Formation of DNA Adducts and Induction of lacI Mutations in Big Blue Rat-2 Cells Treated with Temozolomide: Implications for the Treatment of Low-Grade Adult and Pediatric Brain Tumors

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Abstract
Temozolomide (TMZ) is a chemotherapeutic agent used in the treatment of high-grade brain tumors. Treatment of patients with alkylating chemotherapeutic agents has been established to increase their risk for acute myelogenous leukemia. The formation of DNA adducts and induction of mutations are likely to play a role in the etiology of therapy-related acute myeloid leukemia. To evaluate this issue for TMZ, we have measured the formation of DNA adducts and induction of lacI mutations in Big Blue Rat-2 cells treated with TMZ. Treatment of Big Blue Rat-2 cells with either 0, 0.5, or 1 mM TMZ resulted in lacI mutant frequencies of $9.1 \pm 2.9 \times 10^{-5}$, $48.9 \pm 12 \times 10^{-5}$, and $89.7 \pm 40.3 \times 10^{-5}$, respectively. Comparison of the mutant frequencies demonstrated that 0.5 and 1 mM TMZ treatments increased the mutant frequencies by 5.3- and 9.8-fold and that this increase was significant ($P < 0.001$). Sequence analysis of the lacI mutants from the TMZ treatment group demonstrated that they were GC $\rightarrow$ AT transitions at non-CpG sites, which is significantly different from the mutation spectrum observed in the control treatment group. Treatment of Big Blue Rat-2 cells with various concentrations of TMZ produced a linear increase in the levels of N7-methylguanine and O6-methylguanine. The lacI mutation spectrum induced by TMZ treatment is consistent with these mutations being produced by O6-MeG. This study establishes TMZ has significant mutagenic potential and suggests that careful consideration in the use of TMZ for the treatment of low-grade adult and pediatric brain tumors should be given.

Introduction
Each year, $\sim$14,000 patients are diagnosed with primary brain tumors. Approximately 85% of these tumors are classified as GBM. Despite aggressive therapy, the survival of patients with GBM tumors remains 12–18 months. As a consequence of this poor survival, there is a continuous search for new agents and combinations of agents to improve the therapeutic response of GBM tumors. Recently, a new agent, TMZ, developed by Malcolm Stevens has been approved for treatment of anaplastic gliomas (1, 2). In clinical trials, p.o. administration of TMZ has demonstrated therapeutic activity in the treatment of GBM tumors, anaplastic astrocytomas, and oligodendrogliomas (3–8). Because of this activity, TMZ is being evaluated at a number of centers both as a single agent and in combination with other agents for the treatment of adult and pediatric brain tumors (9–11).

Although TMZ is a new agent, it falls within a group of well-characterized methylating chemotherapeutic agents, which include PCB, DTIC, and STZ (Fig. 1). These agents after either P450 oxidation or chemical hydrolysis generate a methylating agent that reacts with genomic DNA to form adducts (12–19). Although these DNA adducts are not directly cytotoxic, their presence in nuclear DNA initiates a cascade of events which ultimately results in cellular death (20, 21). In addition to their therapeutic properties, these agents have been demonstrated to be mutagenic (22–25) and carcinogenic (26–28).

It has been recognized that treatment of patients with alkylating chemotherapeutic agents results in a significant increase in risk for therapy-related leukemias and preleukemias, including t-AML and t-MDS (29, 30). Although the observation of t-AML and t-MDS in adult brain tumor patients treated with alkylating chemotherapeutic agents is rare (31–33), we believe that this is because of the short life span of patients with GBM tumors who make up the majority of patients rather than lack of the process. The survival of patients with GBM tumors is from 12 to 18 months with $\leq 5\%$ of the patients surviving 5 years (34), whereas the appearance of t-AML or t-MDS usually occurs 3–5 years after treatment (29–33). These facts indicate that most patients with GBM tumors have died before either t-AML or t-MDS could be manifested. However, in cases of lower grade astrocytomas and oligodendrogliomas in adults and astrocytomas and medulloblastomas in children, $\approx 80\%$ of the patients may survive $\geq 5$ years (35–37) and, therefore, may be at risk for development of either t-MDS or t-AML.

36–t-AML and t-MDS developed in patients treated with alkylating chemotherapeutic agents have been characterized as

$3^3$ The abbreviations used are: GBM, glioblastoma multiforme; PCB, procarbazine; HPLC, high-performance liquid chromatography; DTIC, dacarbazine, STZ, streptozotocin; AML, acute myeloid leukemia; t-AML, therapy-related acute myeloid leukemia; t-MDS, therapy-related myelodysplastic syndrome; TMZ, temozolomide; N7-MeG, N7-methylguanine; O6-MeG, O6-methylguanine; AGT, O6-alkylguanine-DNA-alkyltransferase; Hprt, hypoxanthine guanine phosphoribosyltransferase.
having deletions in the long arm of chromosomes 5 and/or 7 (29, 38–40). In addition, a 4-fold increase in the frequency of p53 mutations in t-AML compared with AML or MDS has been observed (41). In this context, p53 mutations in human malignancies are frequently associated with exposure to chemical carcinogens (42). Recent studies have demonstrated that the risk of t-AML in treated patients is modified by genetic polymorphisms, which alter levels of DNA damage and repair (43–46). Taken together, these results suggest that the formation of genomic damage and induction of mutations may play an important role in the etiology of t-AML in patients treated with alkylating chemotherapeutic agents.

Studies by Shering-Plough (47) have demonstrated that treatment of male and female rats with TMZ results in the induction of tumors in multiple tissues after administration of TMZ. The doses of TMZ used in those studies correspond to 12.5–100% of the dose given to patients (47). Analysis of the reported organ specificity for carcinogenicity by TMZ administration shows that it is very similar to that of PCB (26, 27). These observations suggest that analysis of the mutagenic potential of TMZ is warranted.

The Big Blue Rat-2 cells were derived from a rat embryo cell line and carry stably integrated copies of the lacI gene, it can be easily sequenced, and the lacI shuttle vector. This cell line has been used to investigate the mutagenic potential of several alkylating agents (48–50). This model system has a number of advantages, including ease of determining mutant frequency. In addition, because of the relatively small size of the lacI gene, it can be easily sequenced, and the mutation spectrum can be determined (49, 50).

In these studies, we have investigated the formation of DNA adducts and induction of lacI mutations in Big Blue Rat-2 cells treated with TMZ. These studies demonstrate that treatment of Big Blue Rat-2 cells with TMZ results in a dose-dependent increase in DNA adduct formation and lacI mutant frequency. We interpret these results to suggest that treatment of patients with low-grade brain tumors with this agent may result in an increased risk for t-AML.

Materials and Methods

Materials

TMZ was provided by Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD). The Big Blue Rat-2 cell line was purchased from Stratagene (La Jolla, CA). Supplies for mutation analysis were purchased from Stratagene. N7-MeG and O6-MeG were purchased from Chemsyn Science Laboratories (Lenexa, KS). O6-MeG was prepared previously (51).

Cell Culture and Treatment

Big Blue Rat-2 cells were grown in 175 cm² flasks containing 25 ml of Eagle’s minimum essential medium supplemented with 10% FCS and 50 µg/ml gentamycin. The cells were maintained and treated at 37°C in a humidified 5% CO₂, 95% air atmosphere. For the treatments, TMZ was freshly dissolved in DMSO. TMZ was added directly to the culture medium to achieve the final treatment concentrations. After a 3-h treatment, the cells were washed with HBSS and either collected or fresh medium was added. For control treatments, the cells were treated with DMSO. For measurement of DNA adduct levels, the cells were collected after the treatment. For mutation analysis, the cells were collected 24 h after treatment. The cells were trypsinized and collected by centrifugation. The cellular pellets were quick frozen and stored at −80°C until DNA isolation.

Reaction of TMZ with DNA

Individual reactions, containing 500 µg of purified calf-thymus DNA in 100 mM triethanolamine buffer (pH 7.0) and 500 µM TMZ, were incubated at 37°C for various lengths of time. The individual reactions were stopped by precipitating the DNA with ethanol. The DNA was collected by centrifugation, and the pellet was dissolved in 15 mM sodium chloride and 1.5 mM sodium citrate pH 7.0 (0.1 × SSC).

Measurement of DNA Adducts

Isolation of DNA. The DNA was isolated from the cellular pellets using a chloroform-isomylalcohol extraction procedure. After initial isolation, the samples were incubated with a combination of pancreatic RNase A and T1 and T2 RNase followed by proteinase k. The DNA-containing solutions were subsequently extracted with chloroform-isomylalcohol (24:1). The DNA samples were precipitated using sodium acetate and ethanol and collected by centrifugation. The DNA samples were dissolved in 0.1 × SSC buffer, and their concentration was determined from their absorbance at 260 nm.

DNA Hydrolysis Methods. For quantification of O6-MeG, 100 µg of DNA from each sample were digested for 2 h at 37°C in 10 mM Tris (pH 7.5) and 1 mM MgCl₂ with a mixture of pancreatic DNase I (15 units), snake venom phosphodiesterase (1.5 units), and alkaline phosphatase (0.65 units). After incubation, the DNA was precipitated and ethanol and collected by centrifugation. The DNA samples were dissolved in 0.1 × SSC buffer, and their concentration was determined from their absorbance at 260 nm.

For quantification of N7-MeG, 100 µg of DNA in 100 µl of 0.1 × SSC buffer were heated at 100°C for 30 min (52). The samples were cooled in an ice bath, and 100 µl of cold 1 M HCl were added. The samples were centrifuged at 1300 × g, 0°C for 10 min to collect the DNA. The pellet was washed once with 100 µl of cold 1 M HCl and centrifuged as described above. Both supernatants were combined and adjusted to pH 6 with the

![Fig. 1. Structures of the chemotherapeutic agents TMZ, DTIC, PCB, and STZ.](Image)
addition of 4 mM sodium acetate (pH 6.4) and concentrated NH₄OH. The hydrolysates were filtered before prepurification. **Prepurification of N7-MeG and O⁶-MedG.** An HPLC-UV system consisting of a model 250 Perkin-Elmer solvent delivery system with a Perkin-Elmer LC 235 diode array detector was used (Cupertino, CA). A 5-µm C-18 reversed phase analytical column with column dimensions of 250 × 4.6 mm (Econosphere; Alltech Associates, Deerfield, IL) was used for the prepurification separations. Column temperature was set at 28°C with a Perkin-Elmer column oven. For prepurification of N7-MeG, the column was eluted with an isocratic mobile phase of 10 mM sodium acetate (pH 5.3) with 5% methanol. For prepurification of O⁶-MedG, the column was eluted with 10 mM sodium acetate (pH 5.3) containing 18% methanol. Fractions corresponding to the elution of N7-MeG and O⁶-MedG were collected and reduced in volume. The fractions containing O⁶-MedG were pooled and reduced to 90 µl using a speed-vac concentrator. Ten µl of 1 N HCl were added, and the solutions were heated at 62°C for 30 min to convert O⁶-MedG to O⁶-MeG.

**HPLC-EC Detection.** The HPLC-EC system consisting of a model 580 ESA solvent delivery system with an ESA Coulochem II electrochemical detector with an ESA 5010 analytical cell was used (Chelmsford, MA). A 5-µm Econosphere column was used for the analytical separations. Column temperature was set at 30°C. The column was eluted with an isocratic mobile phase of 50 mM sodium acetate (pH 5.3) with 10% methanol for quantification of N7-MeG.

Electrode 1 was set at an oxidation potential of +600 mV. Electrode 2 was set at oxidation potentials of +850 mV for N7-MeG. Electrochemical response was digitized with a Nelson interface and analyzed using TurboChrom 4 software (Perkin-Elmer). Standard curve(s) was generated by measuring EC response after injection of standard solutions of N7-MeG over the concentration range of 0.05–1 pmol. Levels of adducts in the individual samples were determined by comparison with the standard curve (52).

**HPLC-FL Detection.** An HPLC-FL system consisting of a model 250 Perkin-Elmer solvent delivery system with a Perkin-Elmer LC-240 fluorescence detector was used. Excitation and emission wavelengths were set at 286 and 358 nm, respectively. A 10-µm strong cation exchange column with dimensions of 250 × 4.6 mm (Particle: Alltech) was used for the HPLC-FL analysis. The column was eluted with an isocratic mobile phase of 110 mM ammonium phosphate (pH 2.0) for quantification of O⁶-MeG. Fluorescence response was digitized with a Nelson interface and analyzed using TurboChrom 4 software (Perkin-Elmer). Standard curve(s) was generated by measuring fluorescence response after injection of standard solutions of O⁶-MeG over the concentration range of 0.05–1 pmol. Levels of O⁶-MeG in the individual samples were determined by comparison with the standard curve.

**Analysis of lacI Mutations**

**Genomic DNA Isolation.** Genomic DNA was isolated from the Big Blue Rat-2 cells using the RecoverEase genomic DNA isolation kit according to the manufacturer’s recommendation (Stratagene).

**Packaging and Plating.** The λ bacteriophage shuttle vector was recovered from the genomic DNA by in vitro packaging using Transpack packaging extracts (Stratagene). *Escherichia coli* cells at a density of 0.5 A₆₀₀ nm (SCS-8; Stratagene) were infected with the packaged DNA. The infected bacteria were mixed with top agarose containing 1.5 mg/ml X-gal and plated on 20 × 20 cm agar plaque trays. The total number of plaques was determined by counting duplicate plates of diluted aliquots of the infected bacteria. The total number of plaques per plate was extrapolated from the titr plates.

**Sequencing.** Pure stocks of each mutant clone were made by reinfecing bacteria cells with isolated mutant bacteriophage at a low density. This procedure allowed for the isolation of a single lacI plaque from each original mutant plaque. The isolated mutant lacI DNA was amplified directly by PCR using plaque-forming unit Turbo DNA polymerase (Stratagene) and a Perkin-Elmer 2400 thermocycler. The following conditions were used for the amplification: 95°C for 5 min, followed by 30 cycles of 90°C for 20 s, 59°C for 30 s, 72°C for 40 s, and followed by a 7-min incubation at 72°C after the last cycle. The PCR products were purified with Wizard PCR Prep (Promega, Madison WI) and sequenced with a Big Dye Terminator cycle sequencing kit according to the manufacturer’s suggested procedure (Applied Biosystems, Foster City, CA) using a Perkin-Elmer 2400 thermocycler. Initially, a primer homologous to a 5'-untranslated region of the lacI gene from +53 to +37 (5'-CCCCGACACCATCGAATG-3') was used to identify mutations in the first half of the gene where most of the mutations are found. When no mutations were found in this region of the gene, a second primer homologous to the lacI gene from 1208 to 1191 (5'-TCCGGTCAATTCACAAC-3') in the 3'-untranslated region was used to sequence the later half of the gene (43). Sequence reactions were run on an ABI Prism 377a or 3700 automated DNA sequencer at the Biomolecular Resource Center, University of California at San Francisco. Sequence data were analyzed with EditView (Applied Biosystems) and Blast-2 comparison analysis (National Center for Biotechnology Information).

**Statistical Analysis.** Statistical analysis was performed using Sigma Stat v 2.03 (SPSS, Inc., Chicago, IL). Comparison of mutant frequencies was performed with a z test analysis. P < 0.05 were considered to be significant. Correlation of DNA adduct levels and TMZ treatment concentrations was performed using linear regression analysis. The association of O⁶-MedG levels with lacI mutant frequencies was evaluated with a Spearman rank correlation test. Comparison of the proportion of mutation types in the control and TMZ treatment groups was performed using a χ² analysis with α = 0.05. Comparison of individual mutation types in the control and TMZ treatment groups was performed using a Fisher exact test.

**Results**

To establish the length of treatment of Rat-2 cells with TMZ, the levels of N7-MeG and O⁶-MeG produced by incubation of calf thymus DNA for various lengths of time with 500 µM TMZ were quantified. As shown in Fig. 2, the levels of both of these adducts increased linearly with time ≤~120 min. Between 120 and 180 min of incubation, the level of DNA alkylation plateaued. Comparison of the levels of O⁶-MeG/N7-MeG gives a alkylation product ratio of ~0.1. On the basis of these results with DNA alkylation, we chose to treat Big Blue Rat-2 cells with TMZ for 3 h.

Big Blue Rat-2 cells were treated with various concentrations of TMZ for 3 h. The levels of N7-MeG and O⁶-MeG in DNA produced by these treatments were quantified. As shown in Table 1, treatment of Big Blue Rat-2 cells with TMZ produced a dose-dependent increase in the level of both of these DNA adducts. These adducts were not observed in DNA iso-
are indicated.

lated from the control treatment group. Linear regression analysis of the data showed a significant association between TMZ concentration and the level of both N7-MeG, $R^2 = 0.96$, ($P < 0.001$) and $O^6$-MeG, $R^2 = 0.91$, ($P < 0.001$).

Big Blue Rat-2 cells were treated with either 0, 0.5, or 1 mM TMZ and then collected 24 h after treatment for the analysis of lacI mutations. The results of these measurements are summarized in Table 2. The mean levels of lacI mutants in the control, 0.5 mM, and 1 mM TMZ treatment groups were 9.1 ± 2.9 × 10^-5, 48.9 ± 12 × 10^-5, and 89.7 ± 40.3 × 10^-5, respectively. Comparison of the mutant frequencies demonstrates that the 0.5 and 1 mM TMZ treatments have increased the lacI mutant frequency by 5.3- and 9.8-fold, respectively. This increase in mutant frequency was significant for both the 0.5 mM ($P < 0.001$) and 1 mM TMZ ($P < 0.003$) treatment groups.

Sequence analysis of 21 lacI mutants from the 1 mM TMZ treatment group demonstrated that 19 of the 21 mutants were independent (Table 3). Eighteen of these mutations (95%) were transition mutations at GC bp. Only one of the GC→AT transitions (6%) occurred at a CpG site (Table 3). This lacI mutation spectrum observed in the TMZ treatment group is significantly different from the mutation spectrum observed in the control treatment group, where ∼80% of the GC→AT transitions occur at CpG sites (Table 3; Refs. 49 and 50).

**Discussion**

The purpose of these studies has been to measure the formation of DNA adducts and induction of lacI mutations in Big Blue Rat-2 cells treated with the chemotherapeutic agent TMZ. Treatment of these cells with TMZ produced a dose-dependent increase in the levels of $O^6$-MeG and N7-MeG. The ratio of $O^6$-MeG/N7-MeG alkylation products is ∼0.1 over the entire treatment dose range. This result suggests that significant repair of $O^6$-MeG by AGT did not occur in Big Blue Rat-2 cells over the treatment interval investigated (51).

Big Blue Rat-2 cells were treated with 0.125–2.5 mM TMZ for these studies. Pharmacokinetic analysis of the plasma levels of TMZ after a single treatment gives a area under the curve level of 17 μg h/ml (53). This is equivalent to a TMZ concentration of 0.088 mM. A single course of therapy with TMZ corresponds to five daily treatments which would represent a cumulative exposure dose of 0.44 mM TMZ. Therefore, we believe that the concentrations of TMZ used in this study are representative of the concentrations achieved in patients during one course of multiday therapy.

In a preliminary study, we have measured the levels of $O^6$-MeG formed in the liver, lung, and kidney of athymic rats 24 h after i.p. administration of 128 mg/kg TMZ. The levels of $O^6$-MeG in the liver, lung, and kidney after this treatment are 3.6, 11.9, and 13.3 pmol/μmol DNA, respectively (54). These levels of DNA alkylation produced in vivo by treatment with 128 mg/kg TMZ are similar to the levels of $O^6$-MeG produced by treatment of Big Blue Rat-2 cells with TMZ (Table 1). For treatment of brain tumors, one treatment cycle with TMZ consists of 150–200 mg/m² TMZ taken daily for 5 days (4–6). In rats, this would correspond to a TMZ treatment dose of 28.8–38.4 mg/kg daily (54). Over a 5-day period, the total dose of TMZ administered to rats would be 144–192 mg/kg. On the basis of these comparisons, we believe that the levels of $O^6$-MeG produced by treatment of either Big Blue Rat-2 cells with 0.5–1 mM TMZ or athymic rats with 128 mg/kg TMZ are representative of the DNA adduct levels produced in patients after one treatment cycle with TMZ.

Treatment of Big Blue Rat-2 cells with 0.5 and 1 mM TMZ resulted in a 5.3–9.8-fold increase in lacI mutant frequency (Table 2). This increase in mutant frequency is similar to what is observed after treatment of Big Blue Rat-2 cells with 1 mM methyltritosourea (48). There is a significant association between lacI mutant frequency and level of $O^6$-MeG produced by TMZ administration ($r = 0.88$ and $P < 0.001$) as determined by the Spearman rank correlation test. Recent studies by Cai et al. (55) have demonstrated that treatment of Chinese hamster ovary cells with TMZ resulted in a dose-dependent increase in Hprt mutant frequency. The increase in Hprt mutant frequency after TMZ treatment was dependent on the cellular levels of AGT (55). Taken together, these studies demonstrate that TMZ has significant mutagenic potential in the absence of AGT.

Sequence analysis of 21 lacI mutants from the 1 mM TMZ treatment group demonstrated that 94% of the GC→AT transitions occurred at non-CpG sites (Table 3). This lacI mutation spectrum observed in the TMZ treatment group is significantly different from the mutation spectrum observed in a control treatment group analyzed previously where the GC→AT tran-

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**Table 1.** Levels of $O^6$-MeG and N7-MeG in Big Blue Rat-2 cells after treatment with TMZ

<table>
<thead>
<tr>
<th>TMZ (mM)</th>
<th>$O^6$-MeG (pmol/μmol DNA)</th>
<th>N7-MeG (pmol/μmol DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>0.125</td>
<td>1.6</td>
<td>14.3 ± 2.4</td>
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<tr>
<td>0.25</td>
<td>3.4 ± 0.4</td>
<td>38.3 ± 13.7</td>
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<tr>
<td>0.5</td>
<td>8.6 ± 1.1</td>
<td>86.8 ± 27.0</td>
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<td>1.0</td>
<td>19.1 ± 1.2</td>
<td>153.1 ± 33.6</td>
</tr>
<tr>
<td>2.5</td>
<td>43.9 ± 2.3</td>
<td>385.8 ± 17.9</td>
</tr>
</tbody>
</table>

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4 W. J. Bode, N. W. Gaikwad, and T. Otawa. DNA adduct levels in intracerebral human glioma xenografts and normal tissues of athymic rats treated with temozolomide, submitted for publication.
molecular surrogate for this process in bone marrow (61). Furthermore, because treatment with 1 mM TMZ increased the formation of DNA adducts and induction of mutations by TMZ in the treatment of these patients should be given. Since the initial observation by Loveless (56), the miscoding properties of O2-alkylguanine derivatives have been extensively studied (57). The formation of O2-MeG and induction of GC→AT transition mutations by TMZ treatment are consistent with these mutations being derived from O2-MeG. This interpretation is also consistent with the observation by Cai et al. (55) that Hprt mutant frequency induced by TMZ treatment was dependent on the cellular levels of AGT.

TMZ is a group member of methylating chemotherapeutic agents, which include PCB, DTIC, and STZ. Treatment with these agents results in the formation O2-MeG and N7-MeG in both animal models and patients (12–19). Several investigators (22, 24, 25) have demonstrated that treatment with PCB results in a dose-dependent increase in lacZ mutant frequency in the bone marrow. The similarity of PCB and TMZ in the adducts formed and their relative levels suggests that treatment of patients with TMZ will result in the formation of DNA adducts and mutations in their bone marrow. The measurement of DNA adduct levels (15–17) and mutations in their bone marrow. The measurement of DNA adduct levels (15–17) and mutations in their bone marrow.

Table 3 Summary of base substitutions found in spontaneous and TMZ-induced lacI mutations

<table>
<thead>
<tr>
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<th>TMZ</th>
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<tr>
<td></td>
<td></td>
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<tr>
<td>Transitions</td>
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<tr>
<td>G→C−→A</td>
<td>7 (24)</td>
</tr>
<tr>
<td>A→T−→G</td>
<td>5 (17)</td>
</tr>
<tr>
<td></td>
<td>1 (6)</td>
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<tr>
<td>Transversions</td>
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</table>

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