Published in final edited form as:

J Clin Oncol. 2007 November 01; 25(31): 4922–4928. doi:10.1200/JCO.2007.12.0667.

A Phase 1, Pharmacokinetic and Pharmacodynamic Study of Temozolomide in Pediatric Patients with Refractory/Recurrent Leukemia: A Children's Oncology Group Study

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Abstract

Purpose: To determine the tolerability, pharmacokinetics and mechanisms of temozolomide resistance in children with relapsed or refractory leukemia.

Patients and Methods: Cohorts of 3–6 patients received 200 mg/m²/day or 260 mg/m²/day of temozolomide by mouth daily for 5 days every 28 days. Toxicities, clinical response, and pharmacokinetics (PK) were evaluated. Pretreatment leukemia cell O⁶-methylguanine-DNA-methyltransferase (MGMT) activity, tumor and plasma MGMT promoter methylation, and microsatellite instability (MSI) were examined in 14/16 study patients and in tissue bank samples from children with ALL not treated with temozolomide (MGMT:n=67; MSI:n=65).

Results: Sixteen patients (9 female, 7 male) (ALL=8, AML=8), median age 11 years (range 1–19 years), received either 200 mg/m²/day (9 enrolled, 3 evaluable for toxicity) or 260 mg/m²/day (7 enrolled, 3 evaluable for toxicity) of temozolomide. No dose limiting toxicities occurred. The mean clearance of temozolomide was 107 mL/min/m² with a volume of distribution of 20 L/m²; and half-life of 109 min. MGMT activity in leukemia cells was quite variable and was highest in patients with relapsed ALL. Only one patient had MSI. Two patients had a PR; neither had detectable MGMT activity with methylated MGMT promoters and both were MSI-stable.

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Disclaimers: none.

Conclusion: Temozolomide was well tolerated at doses as high as 260 mg/m²/day for five days in children with relapsed/refractory leukemia. Increased MGMT activity may account for the temozolomide resistance in children with relapsed leukemia. Leukemia cell MGMT activity was higher in pediatric ALL than AML (p<.0001).

Keywords

methyl guanine methyltransferase; MGMT; alkyl guanine transferase; AGT; promoter methylation; microsatellite instability; acute lymphoblastic leukemia; acute myelogenous leukemia

INTRODUCTION

Although cure rates for children with newly diagnosed leukemia approach 85–90%, the prognosis for children with relapsed leukemia remains poor and novel treatment approaches are needed. Temozolomide, an imidazole tetrazinone approved for the treatment of high-grade glioma,^{1,2} inhibits cell growth in leukemia cell lines and leukemia xenografts.^{3,4} In a phase 1 study of temozolomide in adults with leukemia, two patients achieved a complete response (CR) and two achieved a CR without platelet recovery (CRp) with durable remissions (8–14 months). The dose-limiting toxicity (DLT) was prolonged bone marrow aplasia and the maximum tolerated dose (MTD) was 200 mg/m²/day for 7 days with courses repeated every 28 days.⁵

Temozolomide resistance correlates with activity of the DNA repair enzyme MGMT.^{6–8} Increased MGMT activity is associated with inferior outcomes in adult and pediatric malignant gliomas.^{9,10} Several *in vitro* studies have shown that increased MGMT activity can contribute to temozolomide resistance in leukemia;^{11,12} however, correlations between patient leukemia cell MGMT activity and temozolomide resistance have not been directly examined.

Hypermethylation of CpG islands near gene promoter regions results in transcriptional inactivation of many genes.¹³ MGMT promoter methylation is associated with both decreased MGMT activity¹⁴ and increased response to temozolomide in both CNS tumors^{15,16} and diffuse large-B cell lymphomas.¹⁷ However some patients with leukemia have evidence of MGMT promoter hypermethylation;^{18,19} and it is not known whether MGMT methylation correlates with temozolomide response.

Although increased MGMT activity is associated with temozolomide resistance in AML patients and leukemia cell lines,^{3,20} some leukemia cells remain resistant to temozolomide despite MGMT inhibition.³ Temozolomide resistance in these tumor cells results from mismatch repair mutations,^{21–24} which are frequently associated with microsatellite instability (MSI).²⁴ MSI is uncommon in adults with *de novo* AML;²⁵ however, the frequency of MSI and its contribution to temozolomide resistance in pediatric leukemia has not previously been examined.

In this study we examined the efficacy, toxicities and pharmacokinetics of oral temozolomide given daily for 5 days at two dose levels, 200 mg/m²/day and 260 mg/m²/day.

In addition, we evaluated MGMT enzymatic activity, MGMT promoter methylation, and MSI in leukemic blasts from patients enrolled on this trial and banked leukemia samples.

PATIENTS AND METHODS

Patient Eligibility:

Patients between the ages of 1 and 21 years (inclusive) with refractory acute lymphoblastic leukemia (ALL) or acute myelogenous leukemia (AML) were eligible for this trial (> 25% blasts on bone marrow aspirate). Other eligibility criteria included: 1) a Karnofsky or Lansky performance score ≥ 50 , 2) recovery from the acute toxic effects of prior chemotherapy, radiotherapy or immunotherapy with a minimum elapsed period of at least 7 days since the last dose of corticosteroids or hematopoietic growth factors, 3) at least 3 months since the last stem cell transplant, and 4) at least 3 months since prior craniospinal radiation, pelvic radiation or TBI, at least 2 weeks since local palliative radiation, and at least 6 weeks since other substantial radiation. Patients could have no evidence of active graft-versus-host disease. Patients were required to have adequate renal function (serum creatinine below the upper limits of normal for age OR a GFR ≥ 70 ml/min/1.73m²); adequate liver function (serum bilirubin ≤ 1.5 mg/dL, ALT ≤ 5 x the institutional upper limit of normal for age, and albumin ≥ 2 g/dL); a platelet count $\geq 20,000/\mu\text{L}$, and a hemoglobin ≥ 8 g/dL. Transfusion support was allowed if necessary to meet hematologic parameters. Study exclusion criteria included: CNS leukemia, pregnancy or lactation in women of child-bearing age, uncontrolled infection, hyperleukocytosis (WBC $> 100,000$ cells/ μL), receipt of concomitant enzyme-inducing anticonvulsants, or concomitant use of other experimental agents. Hydroxyurea was permitted up to 24 hours before the start of therapy if needed for cytoreduction. Institutional Review Board approval and informed consent from the patient or their parent(s) and assent, as appropriate, were obtained in accordance with federal and institutional policies.

Dosage and drug administration:

Temozolomide capsules (Schering-Plough Research Institute, Kenilworth, NJ) were administered orally one hour (h) before or 2 h after meals. Capsules could be opened and the contents resuspended in apple juice or applesauce for children unable to swallow capsules.

Trial design:

The starting dose of temozolomide was 200 mg/m²/dose for 5 days, 70% of the adult MTD.⁵ Subsequent planned dose escalations were in increments of 30%. Courses were repeated every 28 days if there was no evidence of progressive disease, the platelet count was $\geq 20,000/\mu\text{L}$, the hemoglobin ≥ 8 g/dL (with transfusion support) and any other treatment-related adverse events were $\leq$ grade 1. Doses were not held or omitted for hematologic toxicity. A minimum of three patients assessable for DLT were entered at each dose level. Dose levels were expanded to six patients evaluable for toxicity if one patient experienced a DLT during the first course of therapy.

Toxicity assessment:

Adverse events were graded according to the NCI Common Terminology Criteria for Adverse Events (CTCAE v. 3.0). Non-hematologic DLT was defined as any grade 3 or 4 adverse event attributable to temozolomide with the specific exclusion of grade 3 nausea or vomiting, grade 3 hepatic transaminase (AST or/ or ALT) elevation returning to grade 1 before the next treatment course, and grade 3 fever or infection.

Response assessment:

Complete response (CR) was defined as the attainment of an M1 bone marrow (< 5% blasts and adequate marrow cellularity) with no evidence of circulating blasts or extramedullary disease. Partial response (PR) was defined as the complete disappearance of circulating blasts and achievement of M2 marrow status (5% to < 25% blast cells with adequate cellularity). Progressive disease (PD) was defined as an increase of at least 25%, or an absolute increase of at least 5,000 cells/mm³ (whichever was greater) in the number of circulating leukemia cells, development of extramedullary disease, or other laboratory or clinical evidence of PD. Stable disease (SD) was defined as failing to fulfill the criteria for a CR, PR or PD.

Pharmacokinetic studies:

Blood samples (2 mL) were collected in heparinized tubes prior to and at 0.5, 1, 2, 3, 4, 6, and 8 h after the first temozolomide dose. Plasma was separated and frozen at -80°C until analysis. Plasma temozolomide concentrations were measured using a modification of a previously described high-performance liquid chromatography assay.²⁷ In brief, plasma treated with phosphoric acid (20 µg/ml) underwent solid phase extraction using 30 mg/mL StrataX reverse phase columns (Phenomenex, Torrance, CA). Plasma was eluted with acetonitrile and evaporated to dryness under nitrogen at 37°C. Samples were reconstituted in 1% phosphoric acid and injected onto a Luna (2) C18, 3 µ, 4.6 mm x 250 mm column with a C18, 3 µ, 2 mm X 3 mm guard column (Phenomenex, Torrance, CA) and eluted with a mobile phase of 0.5 % acetic acid/methanol (88/12, v/v). Peaks were monitored on a Waters 996 photodiode array detector at 330 nm (Waters Associates, Milford, MA). Recovery of temozolomide was 79 ± 2% and the intra- and inter-assay precisions were 1.7% and 3.9%. The lower limit of quantitation for temozolomide was 0.012 µg/mL.

Temozolomide pharmacokinetic analyses were performed using both compartmental and noncompartmental methods. Time to maximum concentration (T_{max}), peak plasma concentration (C_{max}), and under the concentration-time curve (AUC), apparent volume of distribution (V_{d-ss}/F) were calculated using non-compartmental methods.²⁸ One- and two-compartment models were fit to the concentration-time data using ADAPT II (Biomedical Simulations Resources, University of Southern California, Los Angeles, CA) with maximum likelihood estimation.²⁹ Akaike's Information Criterion (AIC) was used to select the best model fit.³⁰ Individual patient clearance (Cl/F), the absorption half-life, and the elimination-half lives for each phase were estimated from the model.²⁸

Tumor cell isolation:

Pretreatment blood and bone marrow leukemia cells were collected, shipped, and processed as previously described.²⁶ After plasma removal, samples with <90% tumor cells were sorted to >90% purity using a DakoMation cell sorter²⁶. Viability of each patient sample was 90% as determined by trypan blue exclusion. Plasma, tumor cells, and normal lymphocytes (when available) were frozen in RPMI 1640 medium supplemented with 20% bovine growth serum and 10% DMSO.²⁶ An additional 67 previously banked leukemia specimens were obtained from the Texas Children's Cancer Center tissue bank using an IRB approved protocol.

MGMT activity:

MGMT enzyme activity in PBMC or BMA tumor cell lysates was determined by the removal of O^6 -[³H]methylguanine from a [³H] methylated DNA substrate and quantitated as fmol O^6 -[³H]methylguanine / total protein lysate.³¹ All samples contained >90% leukemia cells except patient #5 (44% blasts), #12 (75% blasts) and #15 (50% blasts). Elevated MGMT activity and low MGMT activity were defined as > 2 standard deviation away from the mean MGMT activity from 4 normal volunteers.

MGMT promoter methylation:

MGMT promoter DNA methylation patterns were determined using either tumor cell DNA or free DNA isolated from patient plasma using methylated-specific PCR as described previously.¹⁶

Microsatellite instability assay:

Genomic DNA from pretreatment blasts was extracted and amplified using three MSI multiplex reaction mixtures containing NCI panel markers (BAT-25, BAT-26, D2S123, D17S250, D5S346),³² quasimonomorphic mononucleotide markers (NR-21, NR-22, NR-24, BAT-25, BAT-26),³³ and an alternative panel of markers (D18S35, TP53-DI, TP53-PENTA, D1S2883, and FGA).³⁴ Tumor cell MSI was compared to peripheral blood or non-malignant lymphocytes obtained from the same patient. Each sample was scored according to number of markers positive or negative for MSI. Tumors were categorized into one of three categories; MSI-high (>40% of markers unstable), MSI-low (less than 30% of markers unstable), or MSI-stable using NCI standards.³⁵

Statistics:

The Wilcoxon rank-sum test was used to compare MGMT activity by leukemia subtype (ALL vs. AML) and patient disease status (newly diagnosed vs. relapsed). Three banked specimens had total protein concentrations below the level of quantitation (0.1 mg/mL) and these samples were removed from analysis. The impact of potential confounders, such as age, sex, race and WBC count, on MGMT levels was assessed in stratified analyses using the Wilcoxon rank-sum test. Statistical analyses were applied to patient data and banked samples separately.

RESULTS

From July 2004 until April 2006, 16 pediatric patients (8 with AML, 8 with ALL) were enrolled (Table 1). Ten patients were not fully assessable for toxicity because they had early progressive disease (PD) and either received non-protocol therapy before completing the first course of temozolomide (n=8) or died from PD during the first course of treatment (n=2). None of these patients experienced unusual or severe temozolomide-related toxicities. Three patients were fully assessable for toxicity at each dose level.

Toxicity:

Temozolomide was well tolerated in children with recurrent or refractory leukemia. There were no DLTs at the two dose levels evaluated. Non-DLTs related to temozolomide included 1 case each of grade 3 nausea, vomiting, fever with neutropenia, pneumonia, and elevated serum transaminases levels.

Response:

One five year-old male with AML (M1 subtype) treated with 200 mg/m² temozolomide had a partial response (PR) with a decrease in bone marrow blasts from 60% to 10%, received four courses before developing PD. A 14-year-old female with AML/MDS (M2 subtype) also had a PR, with a decrease in bone marrow blasts from 56% to 6% after one course of temozolomide. Her parents refused further treatment and she developed PD six weeks after cessation of therapy. Four patients had PD after the completion of course one and the remaining ten patients had early PD.

Pharmacokinetics:

Pharmacokinetic samples were obtained from 10 patients (Table 2). Temozolomide was both rapidly absorbed and eliminated from plasma. Based on the non-compartmental analysis, the time to maximal concentration was 74 ± 50 minutes and the terminal half-life was 109 ± 24 minutes. The V_d was 20 ± 5.2 L/m², and the apparent oral systemic Cl was 107 ± 31 mL/min/m². Both AUC and C_{max} increased in proportion with dose. Compartmental analysis resulted in a mean elimination t_{1/2} of 111 ± 37 minutes. Compartmental and non-compartmental t_{1/2}, Cl and V_d were similar.

Assessment of pre-treatment MGMT enzyme activity:

We assessed MGMT enzyme activity in 12 patients (Table 3). Patients had a wide range of MGMT activity (<100 to >2500 fmol/mg protein) compared to normal volunteers, who had an average MGMT activity of 771 ± 170 fmol/mg protein (Figure 1). MGMT activity appeared to correlate with leukemia subtype; 3/7 patients with AML had MGMT activity below the level of detection (<5%) while no ALL patients had undetectable MGMT activity. Although 4/5 patients with ALL had elevated MGMT enzyme activity (>1100 fmol/mg protein), no patients with AML had elevated MGMT activity (Table 3). There was no correlation between patient MGMT activity and age, sex or race. The two patients with a PR to temozolomide had undetectable MGMT activity.

We further explored MGMT activity in an independent set of 67 banked ALL and AML samples obtained from the Texas Children's Hospital tissue tumor bank using an IRB-approved protocol. Three banked specimens had total protein concentrations below the level of quantitation (0.1 mg/mL) and were excluded from analysis. As shown in Figure 1, MGMT activity correlated with leukemia subtype. ALL lymphoblasts had significantly higher MGMT activity than AML myeloblasts ($p < 0.0001$). The highest MGMT activity was found in specimens from patients with newly-diagnosed ALL. In general, specimens from patients with relapsed ALL had higher MGMT activity than specimens from either newly-diagnosed ALL ($p = 0.02$), or relapsed AML ($p < 0.01$). MGMT activity did not correlate with sex but it appeared to correlate with race (whites: 1316 ± 1227 fmol/mg protein vs. non-whites: 623 ± 761 fmol/mg protein; $p = 0.048$). Differences in MGMT activity between leukemia subtypes, however, remained significant after controlling for this potential confounder.

Assessment of MGMT promoter methylation:

MGMT promoter methylation correlated with MGMT activity in 12 patients. (Table 3, Figure 2). Patients with MGMT promoter methylation had little or no MGMT activity, whereas patients with an unmethylated MGMT promoter had MGMT activity present. Although the two patients with a PR to temozolomide had methylated MGMT promoters and no MGMT expression, patients with no objective responses had both unmethylated ($n = 10$) and methylated ($n = 2$) MGMT promoters, implying that factors other than MGMT activity can result in temozolomide resistance. MGMT promoter methylation status was concordant in plasma and leukemia blasts in the 9 patients where both were evaluated.

Microsatellite instability:

Thirteen of the 14 patients tested were MSI-stable, whereas only one patient was MSI-high (Table 3). This patient had MSI at multiple MSI loci (Figure 3A). MSI was also examined in 65 banked leukemia samples. As shown in Figure 3B, samples from patients with newly diagnosed ALL or AML were MSI-stable or MSI-low. In contrast, samples from 7/18 patients with relapsed ALL were MSI-high and 2/18 patients were MSI-low. Several patients with relapsed AML (3/7) were also MSI-low. This suggests that MSI is common in pediatric patients with relapsed leukemia.

DISCUSSION

In this study we examined the toxicities, response and pharmacokinetics of temozolomide given as a single agent to pediatric patients with relapsed or refractory leukemia. Temozolomide drug disposition in children with leukemia was similar to that observed in pediatric patients with solid tumors³⁶ as well as in adults.^{28,37-40} Due to the high number of patients with PD during the first treatment course, the MTD of temozolomide in pediatric patients with relapsed leukemia was not determined. However, three evaluable patients tolerated 260 mg/m^2 without evidence of DLTs. Of the sixteen patients enrolled, 2 had objective responses.

Another objective of this trial was to examine the mechanisms of temozolomide resistance. Increased MGMT enzymatic activity has been correlated with temozolomide resistance in both glioma and leukemia cell lines.^{1,3,41} Patients in this study with elevated MGMT activity were also resistant to temozolomide. MGMT expression is decreased by MGMT promoter methylation, which is common in many tumor types, and MGMT methylation may predict temozolomide sensitivity.⁴² However, others have found only a moderate association between MGMT promoter methylation and MGMT enzyme activity^{43–45}. In our study, 12 samples showed concordance between MGMT activity and MGMT promoter methylation.

We also noted a concordance between MGMT methylation in tumor cells and plasma. Since only tumor DNA is abnormally methylated (normal tissue remains unmethylated), plasma MGMT methylation could be used to monitor disease response and detect early relapse in patients whose tumors have methylated MGMT promoters.⁴⁶ Balana et al. reported a correlation between plasma MGMT promoter methylation and chemotherapy response in patients with glioblastoma multiforme,¹⁶ suggesting that further research in this area is warranted.

Some tumors remain resistant to temozolomide despite low MGMT activity due to mismatch repair pathway defects,⁴⁷ which may arise from mutations or gene silencing of mismatch repair enzymes.²³ Mismatch repair defects and the resulting MSI, which are common in leukemia cell lines,^{22,24,48,49} are less common in primary pediatric leukemias (approx. 10% prevalence)^{48,50–52} and in adults with newly diagnosed leukemia.^{53,54} MSI, however, is more prevalent in relapsed leukemia,^{55–57} treatment-related AML,^{25,58,59} and adult T-cell ALL.⁶⁰ In our study, only one patient was MSI-high. Since several patients with PD had stable MSI (Table 3), it did not appear that MSI was a major determinant of temozolomide resistance. However, MSI stability may be necessary for temozolomide sensitivity since previous work has shown that leukemia cells with mismatch repair defects are resistant to temozolomide despite low MGMT activity.

In summary, we determined that in children with relapsed and refractory leukemia, temozolomide could be tolerated at doses of 260 mg/m²/day for five days. In this heavily pre-treated population, however, we did not observe significant clinical activity. As patients with relapsed AML in general had lower MGMT activity than patients with relapsed ALL, further study of temozolomide in this leukemia subtype should be considered.

Acknowledgements:

Thanks to Anu Gannavarapu, Gaye Jenkins, John Hyatt and Alexander Aleksic for technical assistance.

Supported and funded by: NCI grants U01-CA97452 (COG), U01CA63187 (University of Chicago Cancer Research Center), K12CA90433 (TMH), The Lady Tata Memorial Fund (TMH), the Scott Carter NCCF Research Fellowship (TMH).

Prior presentation of work: The patient data from this study was presented in abstract form at the American Society of Hematology meeting, December, 2005.

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Figure 1: Summary of MGMT enzyme activity in an independent set of leukemia samples: MGMT activity was determined in 67 pediatric patients with newly-diagnosed ALL (nALL) (n=21), relapsed ALL (rALL) (n=19), newly-diagnosed AML (nAML) (n=20), relapsed AML (rAML)(n=7), and PBMC from healthy volunteers (n=4). Median values for each group noted as solid line. Differences that are statistically different are noted (Wilcoxon rank-sum test).

patient 13 (PD) Patient 9 (PR) patient 15 (PD)

plasma tumor plasma tumor plasma tumor

Mar U M M U M U M U M U M U M U M

100bp →
75bp →

NL Co Leu

Figure 3: Microsatellite instability in pediatric leukemia:

(A) Representative fluorescent-based microsatellite alterations from a patient demonstrating microsatellite instability in the leukemia cells (T), but not the normal lymphocytes (B) for the NCI MSI markers BAT-25, BAT-26, and D2S123³² and the quasimonomorphic MSI markers NR-21, NR-22 and NR-24³³. (B) Microsatellite instability was assessed in 65 pediatric leukemia samples categorized by subtype and relapse status. Microsatellite instability was determined at 13 alleles. Samples were classified as stable, having instability

at a single site (MSI-low), or having instability at multiple sites (MSI-high), consistent with a mutator phenotype (RER+).

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Table 1.

Patient Characteristics for Eligible Patients (n=16)

Characteristic	Number
Age (years)	
Median (Range)	11 (1–19)
Sex (Male:Female)	7:9
Race/ethnicity	
White	7
Black or African American	4
Asian	3
Hispanic	1
Other	1
Diagnosis	
ALL	8
AML	8
Prior Therapy	
Chemotherapy Regimens	
Median (Range)	4 (1–7)
Prior Radiation	8
Prior Bone Marrow Transplant	10
Karnofsky/Lansky	
Median (Range)	90 (60–100)

Table 2:

Summary of Day 1 pharmacokinetic parameters from 10 pediatric patients treated with temozolomide.

Parameter ^a	200 mg/m ² (n=6) ^b	260 mg/m ² (n=4) ^b
T _{max} (min)	82 ± 57	61 ± 36
C _{max} (µg/mL)	9.3 ± 3.0	13 ± 2.8
AUC _{0-24h} (µg/m•h)	28 ± 7.3	46 ± 6.4
AUC _{0-∞} (µg/ml•h)	30 ± 7.8	51 ± 8.7
V _{d-ss} /F (L/m ²)	21 ± 5.7	17 ± 3.1
Cl/F (mL/min/m ²)	120 ± 38	90 ± 21
t _{1/2} abs (min)	19 ± 18	8.7 ± 3.4
t _{1/2} el (min)	100 ± 24	129 ± 45

^aNoncompartmental analyses were used to determine T_{max}, C_{max}, AUC and V_d;compartmental analyses were used to determine Cl and t_{1/2}.

^bMean ± standard deviation

Table 3:
Mechanisms of temozolomide resistance:

MGMT activity and microsatellite instability in peripheral blood or bone marrow aspirate samples (n=14)

Pt	Dx	Response	MGMT activity (fmol/mg protein)	MGMT promoter methylation (plasma) ²	MGMT promoter methylation (cells) ²	MSI ³
1	Infant ALL	PD	356	U	U	Stable
3	ALL	PD	ND	U	ND	Stable
5	ALL	PD	1607	U	U	Stable
6	ALL	SD	1756	U	U	Stable
11	ALL	PD	>2500	U	ND	Stable
13	ALL	PD	2393	U	U	Stable
15	AML	PD	390	M	M [#]	Stable
2	AML	PD	Undetectable	M [#]	M [#]	Stable
7	AML	PD	430	U	ND	Stable
8	AML	PD	837	U	U	Stable
9	AML	PR	Undetectable	M[#]	M[#]	Stable
12	AML	PD	835	U	U	Stable
14	AML	PR	Undetectable	M	ND	Stable
16	AML	PD	ND	U	ND	MSI-high

²U=unmethylated, M= methylated, M[#]= both methylated and unmethylated PCR products present

³Microsatellite instability