SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 4: 551-555, 2012

The promoter methylation and expression of the O6-methylguanine-DNA methyltransferase gene in uterine sarcoma and carcinosarcoma

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Received March 5, 2012; Accepted June 8, 2012

DOI: 10.3892/ol.2012.771

Abstract. O6-methylguanine-DNA methyltransferase (*MGMT*) gene promoter hypermethylation is observed in a number of solid tumors and is correlated with the silencing of *MGMT* expression. In glioblastoma patients treated with the alkylating agent temozolomide, *MGMT* gene methylation status was shown to have predictive value in terms of prolonged overall survival. Recently, temozolomide has demonstrated promising activity in the treatment of soft tissue sarcomas, including those of the uterus. The tissue specimens involving tumor samples and normal uterine fragments were obtained from nine patients with smooth muscle uterine sarcoma, 11 with stromal uterine sarcoma and 17 with mixed uterine tumors. *MGMT* gene promoter methylation was analyzed by combined bisulfite restriction analysis (COBRA) while its expression levels were assessed using the real-time reverse transcription polymerase chain reaction (qRT-PCR). *MGMT* promoter methylation was observed in 27% of all tumor samples analyzed. When stratified by the disease type, 55.5% (5/9) of smooth muscle sarcomas, 23.5% (4/17) of mixed uterine tumor tissues and 9% (1/11) of stromal sarcomas showed *MGMT* methylation. The *MGMT* promoter methylation was associated with lower levels of gene expression in tumors when compared with those with an unmethylated promoter (P=0.0232) or normal tissues (P=0.0141). To conclude, *MGMT* promoter methylation and downregulation of gene expression is observed in a fraction of carcinosarcomas and non-epithelial malignant tumors of *corpus uteri*. The assessment of *MGMT* promoter methylation status may potentially identify patients who would benefit from temozolomide treatment.

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Key words: uterine sarcoma, carcinosarcoma, O6-methylguanine-DNA methyltransferase, gene expression, gene methylation

Introduction

Uterine sarcomas are rare tumors, accounting for 3 to 8% of neoplasms of the uterine corpus and 1% of all tumors of the female genital tract (1). Due to the low incidence of these tumors their molecular biology, including the role of epigenetic events, is poorly understood. Uterine sarcomas are classified into smooth muscle sarcomas, stromal sarcomas and mixed uterine tumors, i.e. carcinosarcomas. Although the latter histological type is reclassified as a dedifferentiated or metaplastic form of endometrial carcinoma, it is still included in most of the studies on uterine sarcomas (2). The standard treatment of the uterine sarcomas comprises surgery and chemotherapy. Uterine sarcomas are among the most lethal uterine malignancies with poorer prognosis compared with other gynecological malignancies; 5-year survival rates remain below 50% for early stages and do not exceed 30% in the remaining stages (3,4).

Recently, several adjuvant chemotherapy regimens have been reported in the treatment of soft tissue sarcomas, including sarcomas of the uterus. Some of the trials were based on the alkylating agents, including carmustine and temozolomide (TMZ). The regiment combining carmustine with O^6-benzylguanine did not result in objective treatment response in 12 enrolled patients with soft tissue sarcoma (5). The initial results concerning TMZ-based therapy as a second-line treatment in 31 patients with advanced soft tissue sarcoma did not show activity of the drug (6). The trial involving patients with soft tissue sarcomas not subjected to standard chemotherapy revealed only minimal efficacy of TMZ (7). Another trial on TMZ demonstrated a modest activity against previously treated unresectable or metastatic soft tissue sarcomas (8). Notably, all responding patients had leiomyosarcoma (of uterine or non-uterine origin) (8).

Better results were obtained in 2005 by the Spanish Group for Research on Sarcomas with the prolonged course of TMZ which had activity in patients with pretreated soft tissue sarcomas (9). Notably, a response was observed in 5 of 11 patients who had gynecological leiomyosarcoma and in one

of two patients with mixed mullerian tumors. The results of the study on the uterine leiomyosarcoma were published in the same year, revealing therapeutic benefit of TMZ in patients with metastatic unresectable disease (10). Of 19 patients pretreated with doxorubicin who underwent TMZ-based therapy, two patients achieved almost complete response and eight showed stabilization of the disease. In a recent study of Ferriss *et al* (11) a clinical benefit of TMZ was achieved in five out of six patients with advanced and recurrent uterine leiomyosarcoma. This therapeutic benefit was associated with silencing of the O6-methylguanine-DNA methyltransferase (MGMT) expression as determined by immunohistochemistry. All the above mentioned studies revealed good tolerance to TMZ (6-11).

The *MGMT* gene has been shown to be epigenetically downregulated in several solid tumors. Aberrant promoter hypermethylation of the *MGMT* has been associated with the lack of its mRNA expression, the loss of MGMT protein (12) and loss of enzymatic activity (13). *MGMT* encodes DNA repair protein, an enzyme responsible for the direct removal of alkylating adducts from guanines. The silencing of the gene contributes to the reduction of the genome stability and sensitizes tumor cells to alkylating agents, including dacarbazine, carmustine and TMZ. The main therapeutic target of these drugs are the nitrogen bases of DNA and the most important cytotoxic derivate of their action on the nitrogen bases is O6-methyl-guanine. Alkylation of the guanine leads to an accumulation of the replication errors during the S-phase of the cell cycle and, as a consequence, to the cell cycle arrest and/or apoptosis. The high degree of removal of alkyl adducts causes the resistance to treatment.

As has been shown in tumor cell line-based studies, MGMT prevents TMZ-induced cell death by removing alkyl adducts from the O^6 position of guanine (14). Thus, tumor cells expressing MGMT are resistant to alkylating agents, while those that lack the enzyme appear to be chemosensitive. The predictive value of *MGMT* epigenetic silencing is well documented for glioblastoma treatment with TMZ. The methylation of the promoter of the gene was shown to correlate with improved prognosis in several independent trials and is being considered as a potential stratification marker of the response to TMZ-based therapy (15). However, to date no formal recommendation has been proposed as to the use of this marker in the clinical setting. In melanomas treated with TMZ, the *MGMT* methylation was associated with improved tolerance to treatment, however not with survival (16).

This study aimed to evaluate the frequency of *MGMT* promoter methylation, as well as to assess its possible correlation with the expression levels of the gene, in carcinosarcomas and non-epithelial malignant tumors of *corpus uteri*.

Materials and methods

Patients. A total of nine patients treated for smooth muscle uterine sarcoma, 11 for stromal uterine sarcoma and 17 for mixed uterine tumors in the Maria Sklodowska-Curie Memorial Cancer Centre and Institute of Oncology in Warsaw between January 2009 and December 2010 were enrolled in the present study. The selected patients' characteristics are presented in Table I. The study was approved by the Independent Ethics Committee of the Maria Sklodowska-Curie Memorial Cancer Centre and Institute of Oncology in Warsaw and all patients provided informed consent. Tissue specimens were divided into two parts: one part was examined histologically, the other was frozen in liquid nitrogen and stored at -70˚C until nucleic acid isolation. In addition to 37 tumor tissue samples, 19 samples of normal uterine tissue were also obtained from patients enrolled in the study.

DNA methylation analysis. *MGMT* promoter methylation analysis was performed using the combined bisulfite restriction analysis (COBRA).

DNA was isolated from ~50 mg of pulverized (with the Microdismembrator II, B Braun Biotech International, Melsungen, Germany) tumor samples using NucloSpin Tissue kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. DNA quantity was measured using NanoDrop 2000 (ThermoScientific, Waltham, MA, USA). DNA (1 μg) was bisulfite converted using EpiTect kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Converted DNA was eluted with 40 μl of water. *MGMT* promoter region [chr10:131155461-131155570 genome location as determined by UCSC Genome Browser Database (http://genome.ucsc.edu/) Human March 2006 (hg18) assembly] region was amplified using previously reported PCR primers (17). The reaction volume of 15 μl contained 1X PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.2 mM each primer, 0.5 U of FastStart DNA Polymerase (Roche Applied Science, Mannheim, Germany) and 1 μl of bisulfite-treated DNA as a template. The cycling conditions were as follows: initial denaturation at 94˚C for 3 min; followed by 38 cycles of 30 sec at 94˚C, 40 sec annealing at 58˚C and 50 sec at 72˚C; then final elongation for 7 min at 72˚C. Subsequently, 8 μl of PCR products were digested overnight with *HpyCH4*IV (*Tai*I) restriction enzyme (New England Biolabs, Ipswich, MA, USA) which cleaves sequences containing CpG dinucleotides. Restriction fragments were electrophoresed in 10% polyacrylamide gel (acryl/bis 19:1) and visualized with ethidium bromide. The presence of 61- and 39-bp DNA fragments on the gel (the shortest 15-bp DNA fragment was not visible in certain samples due to the low band intensity) indicates the occurrence of methylated *MGMT* variant. The unmethylated DNA variants, as wells as native DNA (not subjected to bisulfite conversion), have no restriction sites for the chosen enzyme in the analyzed region which excludes the occurrence of false positive results.

DNA isolated from the blood sample of a healthy donor was methylated *in vitro* with *Sss*I DNA methyltransferase (New England Biolabs) and used as a positive (methylated) control. The same DNA sample after whole genome amplification (GenomiPhi, GE Healthcare, Piscataway, NJ, USA) was used as a negative (unmethylated) control.

Expression analysis. *MGMT* mRNA level was evaluated using the real-time reverse transcription polymerase chain reaction (qRT-PCR).

Total RNA from ~50 mg of pulverized (with the Microdismembrator II, B Braun Biotech International) tumor and normal uterine samples was extracted using RNeasy Mini kit with on-column DNase digestion (Qiagen) according to

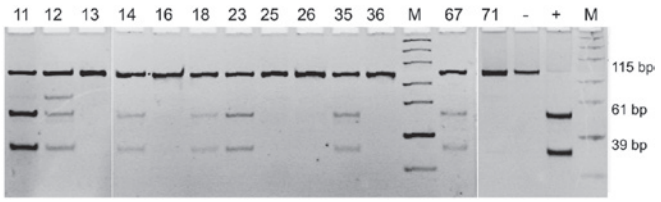

Figure 1. The representative results of *MGMT* promoter methylation analysis. Lanes 12-71, tumor samples; -, negative control (whole genome amplified DNA from the blood sample of a healthy donor); +, positive control (*in vitro* methylated DNA from the blood sample of a healthy donor); M, molecular weight marker; MGMT, O6-methylguanine-DNA methyltransferase.

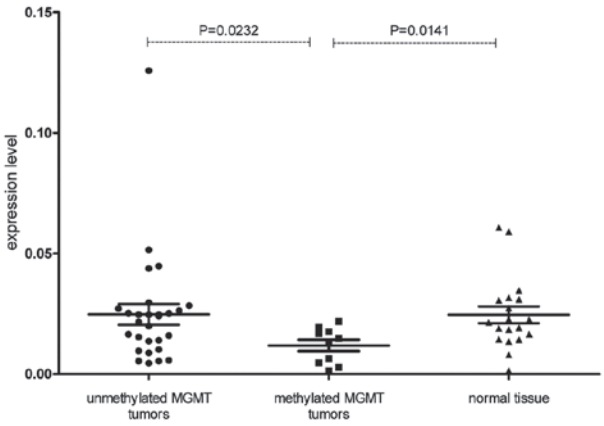

Figure 2. The *MGMT* expression levels in tumors with methylated or unmethylated *MGMT* and normal uterine tissues; MGMT, O6-methylguanine-DNA methyltransferase. Horizontal lines indicate standard error of the mean (SEM).

the manufacturer's instructions. RNA quantity was measured using NanoDrop (ThermoScientific), while the overall RNA quality was assessed by electrophoresis on a denaturing agarose gel (FlashGel, Lonza, Rockland, ME, USA). The RNA samples (1 μg each) were reverse-transcribed using the RT^2 First Strand kit (SA Biosciences, Hilden, Germany) according to the manufacturer's instructions. Quantitative real-time PCR was performed in triplets using ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). The reaction mixture of 25 μl contained 2.5 μl of 15X diluted cDNA template, 1X Power SYBR Green PCR Master Mix (Applied Biosystems) and 0.1 mM of the forward and reverse primers. PCR was performed as follows: precycling hold at 95˚C for 10 min, 45 cycles: 95˚C for 30 sec and 60˚C for 60 sec. To assess the reaction specificity, the amplification products were subjected to melting curve analysis.

UBC was used as a reference gene, as its stable expression in carcinosarcoma tumors and non-epithelial malignant tumors of the *corpus uteri* as well as in normal uterine tissues has been recently demonstrated (18). Primer sequences for the *MGMT* and *UBC* were obtained from the *qPrimerDepot* database (19). These were: *MGMT* forward, CTCCGGACCTCCGAGAAC, and *MGMT* reverse, GTCTGCACGAAATAAAGC, producing 94-bp amplicons; as well as *UBC* forward, TTGCCTTGACATTCTCGATG, and *UBC* reverse, ATCGCTGTGATCGTCACTTG, producing 108-bp amplicons.

Raw data were analyzed using ABI Prism 7000 SDS Software Version 1.1 (Applied Biosystems). Relative expression levels were calculated using the $2^{-\Delta Ct}$ method, where ΔCt was defined as a difference between Ct value for *MGMT* and *UBC* reference gene.

Statistical analysis. The Chi-square test was used to compare *MGMT* methylation frequencies among the three histopathological subtypes of the analyzed tumors. The difference in the *MGMT* expression levels between *MGMT*-methylated and unmethylated tumors and normal uterine tissues was assessed with the use of a two-sided Mann-Whitney U test with a significance threshold level α=0.05. The values of the *MGMT* expression levels were visualized in a plot using GraphPadPrism (La Jolla, CA, USA).

Results

MGMT promoter methylation was observed in 27% (10/37) of tumors obtained from all the patients enrolled into our study. In three cases that showed *MGMT* promoter methylation, an additional band on the gel was observed indicating incomplete digestion and therefore incomplete promoter methylation (Fig. 1, sample 12). When stratified by the disease type, 55.5% (5/9) of smooth muscle sarcomas, 23.5% (4/17) of mixed uterine tumor tissues and 9% (1/11) of stromal sarcomas showed *MGMT* methylation. The difference in frequency of *MGMT* promoter methylation in smooth muscle sarcomas compared with the two other subtypes of the analyzed tumors was statistically significant (P=0.0489).

The *MGMT* expression level was significantly lower in tumor samples with gene promoter methylation when compared with unmethylated tumor tissues (P=0.0232). The *MGMT* expression level was also significantly lower in tumor samples with gene promoter methylation than in the normal uterine samples obtained from the same patients (P=0.0141; Fig. 2). The promoter methylation status and values of *MGMT* expression levels for individual uterine sarcoma and carcinosarcoma tumors are provided in Table I.

No difference between *MGMT*-unmethylated tumors and normal samples was observed.

Discussion

MGMT promoter methylation status has previously been shown to correlate with the downregulation of the gene expression in different types of solid tumors (20). The aim of this study was to evaluate the frequency of *MGMT* promoter methylation and its correlation with the gene expression status in carcinosarcomas and non-epithelial malignant tumors of the *corpus uteri*. We observed the methylated *MGMT* variant in a relatively high fraction of tumors (27%), being the highest in smooth muscle sarcomas where the gene was methylated in over half of the cases. The tumors with *MGMT* promoter methylation showed a significantly lower gene expression level than tumors with an unmethylated promoter as well as normal uterine tissue samples.

A number of cell line-based experiments have revealed the inverse correlation between the *MGMT* expression and the cytotoxic effect of TMZ. These observations have been

Table I. Individual patient data.

Patient	Age (years)	*MGMT* methylation status	*MGMT* expression level	Tumor type	Tumor histology/ histological grade
Mixed uterine tumors					
3	75.5	Negative	0.010	Recurrent	Carcinosarcoma heterologous[a]
4	54.1	Negative	0.025	Primary	Carcinosarcoma heterologous[b]
5	79.5	Negative	0.009	Primary	Carcinosarcoma heterologous
11	61.4	Positive	0.017	Primary	Carcinosarcoma homologous
14	23.2	Positive	0.015	Primary	Adenosarcoma homologous
18	66.4	Positive	0.013	Primary	Carcinosarcoma heterologous
24	66.6	Negative	0.025	Primary	Carcinosarcoma heterologous
25	61.0	Negative	0.020	Recurrent	Carcinosarcoma homologous
30	64.3	Negative	0.025	Primary	Carcinosarcoma heterologous
33	61.6	Negative	0.022	Recurrent	Carcinosarcoma heterologous
39	54.7	Negative	0.044	Primary	Adenosarcoma homologous
44	68.0	Negative	0.030	Primary	Adenosarcoma heterologous
47	65.7	Negative	0.014	Primary	Carcinosarcoma homologous
51	56.4	Negative	0.005	Primary	Mixed endometrial stromal and smooth muscle tumor
66	55.1	Negative	0.045	Primary	Carcinosarcoma homologous
67	59.7	Positive	0.003	Primary	Adenosarcoma homologous
71	55.5	Negative	0.126	Recurrent	Adenosarcoma (dediff)
Smooth muscle sarcomas					
1	36.6	Positive	0.022	Primary	Rhabdomyosarcoma
2	52.6	Positive	0.001	Recurrent	Leiomyosarcoma/G3
12	56.3	Positive	0.005	Primary	Leiomyosarcoma/G3
23	63.2	Positive	0.006	Recurrent	Leiomyosarcoma/G2
26	45.8	Negative	0.025	Recurrent	Leiomyosarcoma/G3
28	51.5	Negative	0.005	Recurrent	Leiomyosarcoma/G2
35	57.5	positive	0.018	Recurrent	Leiomyosarcoma/G2
37	25.5	Negative	0.016	Recurrent	STUMP
63	40.5	Negative	0.005	Recurrent	Leiomyosarcoma/G3
Stromal uterine sarcomas					
6	76.7	Negative	0.024	Recurrent	Endometrial stromal sarcoma, low grade
8	59.5	Positive	0.019	Primary	Undifferentiated endometrial sarcoma
13	60.1	Negative	0.025	Recurrent	Undifferentiated endometrial sarcoma
16	43.3	Negative	0.017	Recurrent	Endometrial stromal sarcoma, low grade
17	74.8	Negative	0.006	Primary	Undifferentiated endometrial sarcoma
31	51.0	Negative	0.014	Primary	Sarcoma stromale, low grade
36	64.6	Negative	0.052	Primary	Undifferentiated endometrial sarcoma
38	44.8	Negative	0.010	Primary	Endometrial stromal sarcoma low grade
52	78.1	Negative	0.027	Primary	Undifferentiated endometrial sarcoma
62	53.4	Negative	0.026	Primary	Undifferentiated endometrial sarcoma
64	68.9	Negative	0.028	Primary	Undifferentiated endometrial sarcoma

[a]heterologous tumor, representing the malignant counterparts that normally do not occur in the uterus; [b]homologous tumor, representing the malignant counterparts of tissues indigenous to the uterus. MGMT, O6-methylguanine-DNA methyltransferase; STUMP, smooth muscle tumor of uncertain malignant potential.

confirmed in clinical trials. In the study by Ferriss *et al* (11) on the effectiveness of TMZ in uterine leiomyosarcomas treatment, the *MGMT* expression was inversely correlated with treatment response.

Using the *MGMT* promoter methylation status as a predictive biomarker has certain advantages. As cytosine methylation is a stable covalent modification, it may be analyzed in a wide range of tissue samples, including formalin-fixed and

paraffin-embedded (FFPE) tissues. FFPE samples are probably the most accessible clinical tissue material for molecular analysis, although inappropriate for the mRNA expression analysis. Currently available laboratory techniques allow relatively fast and sensitive *MGMT* methylation detection with qualitative or quantitative results. Compared with immunohistochemical expression analysis, the determination of *MGMT* methylation status is not dependent on subjective microscopic evaluation and if a quantitative technique is applied, more precise quantitative results may be achieved.

TMZ-based therapy is the current standard in the treatment of glioblastoma patients and *MGMT* methylation status has already been shown to be strong predictive factor of significantly longer progression free survival and overall survival of patients with methylation of the gene promoter (21). The systematic comparison of the application of immunohistochemical staining with the promoter methylation analysis in the glioblastoma patients treated with TMZ revealed the superiority of methylation analysis as a survival predictive factor (22).

As the group of patients enrolled in our study is relatively small, the findings have value as preliminary results. However, the presence of *MGMT* promoter methylation in a notable proportion of patients and the observation that gene methylation is associated with the downregulation of the gene expression levels indicate that methylation analysis should be included in the clinical trials on the effectiveness of TMZ in patients with uterine sarcoma and carcinosarcoma. The results of the present study advocate the use of TMZ in uterine leiomyosarcoma treatment and also suggest that a smaller percentage of patients with stromal sarcoma and carcinosarcoma may benefit from this type of therapy. The qualitative techniques for *MGMT* promoter methylation detection, including COBRA that was used in our study, potentially allow prediction of the patients' response to TMZ-based treatment and thus their stratification.

To conclude, as TMZ-based chemotherapy showed promising results in recently reported trials on the treatment of soft tissue sarcomas, determination of *MGMT* promoter methylation status may have significant clinical implications in a fraction of patients with carcinosarcoma and non-epithelial malignant tumors of the *corpus uteri*. Such studies should be applied in the clinical practice and ultimately contribute to future therapeutic strategies for these rare gynecological tumors.

Acknowledgements

This study was supported by the research grant No. NN 407 125 937 from the Ministry of Science and Higher Education.

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