

# Predictive value of the *MGMT* promoter methylation status in metastatic melanoma patients receiving first-line temozolomide plus bevacizumab in the trial SAKK 50/07

PETER SCHRAML<sup>1</sup>, ADRIANA VON TEICHMAN<sup>1</sup>, DANIELA MIHIC-PROBST<sup>1</sup>,  
MATHEW SIMCOCK<sup>2</sup>, ADRIAN OCHSENBEIN<sup>3</sup>, REINHARD DUMMER<sup>4</sup>, OLIVIER MICHIELIN<sup>5</sup>,  
BETTINA SEIFERT<sup>6</sup>, MARC SCHLÄPPI<sup>7</sup>, HOLGER MOCH<sup>1\*</sup> and ROGER VON MOOS<sup>8\*</sup>

<sup>1</sup>Institute of Surgical Pathology, University Hospital Zurich, Zurich; <sup>2</sup>Swiss Group for Clinical Cancer Research (SAKK) Coordinating Center; <sup>3</sup>Inselspital, Bern; <sup>4</sup>Clinic for Dermatology, University Hospital Zurich, Zurich; <sup>5</sup>CHUV, Lausanne; <sup>6</sup>University of Basel, Basel; <sup>7</sup>Canton's Hospital St. Gallen, St. Gallen; <sup>8</sup>Canton's Hospital, Chur, Switzerland

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**Abstract.** The O<sup>6</sup>-methylguanine-DNA-methyltransferase (*MGMT*) promoter methylation status is a predictive parameter for the response of malignant gliomas to alkylating agents such as temozolomide. First clinical trials with temozolomide plus bevacizumab therapy in metastatic melanoma patients are ongoing, although the predictive value of the *MGMT* promoter methylation status in this setting remains unclear. We assessed *MGMT* promoter methylation in formalin-fixed, primary tumor tissue of metastatic melanoma patients treated with first-line temozolomide and bevacizumab from the trial SAKK 50/07 by methylation-specific polymerase chain reaction. In addition, the *MGMT* expression levels were also analyzed by *MGMT* immunohistochemistry. Eleven of 42 primary melanomas (26%) revealed a methylated *MGMT* promoter. Promoter methylation was significantly associated with response rates CR + PR versus SD + PD according to RECIST (response evaluation criteria in solid tumors) ( $p < 0.05$ ) with a trend to prolonged median progression-free survival (8.1 versus 3.4 months,  $p > 0.05$ ). Immunohistochemically different protein expression patterns with heterogeneous and homogeneous nuclear *MGMT* expression were identified. Negative *MGMT* expression levels were associated with overall disease stabilization CR + PR + SD versus PD ( $p = 0.05$ ). There was only a poor correlation between *MGMT* methylation and lack of *MGMT* expression. A significant proportion of melanomas have a methylated *MGMT* promoter. The *MGMT* promoter

methylation status may be a promising predictive marker for temozolomide therapy in metastatic melanoma patients. Larger sample sizes may help to validate significant differences in survival type endpoints.

## Introduction

The incidence of malignant melanoma is increasing worldwide. Stage IV melanoma is still a devastating disease with a median survival time of 6-9 months depending on the bulk and localization of disease. Despite first successful immunotherapies as with ipilimumab, a CTLA4 antibody in first and second line treatment of metastatic melanoma (1,2) and targeted therapy for patients with *BRAF* mutations with PLX4032 (3,4), there is still a need to better define patients responding to alkylating substances such as temozolomide especially in *BRAF* wild-type patients. Single agent dacarbazine has been the standard of care for many years. Response rates (RRs) of 5.5-13% (5) have been reported in recent large phase III trials with a further 15-28% of patients having stable disease (SD), however, few responses are long-lasting (6).

Temozolomide is an oral alkylating agent with a mechanism of action similar to dacarbazine (7), that is spontaneously converted to its active metabolites (8). This oral drug has been used successfully in the treatment of primary brain tumors. It was shown that the gene promoter methylation status of the O<sup>6</sup>-methylguanine-DNA-methyltransferase gene (*MGMT*) is of critical importance for survival in glioblastoma patients treated with temozolomide (9). *MGMT* silencing by promoter methylation impairs the ability of the *MGMT* protein to remove alkyl groups from the O<sup>6</sup>-position of guanine, thereby increasing the mutation rate in rapidly dividing tumor cells and improving patient outcome. Recently, we reported that about one-third of brain metastases from various origins including malignant melanoma revealed a methylated *MGMT* promoter (10). Moreover, homogeneous *MGMT* immunoreactivity correlates with an unmethylated *MGMT* promoter status suggesting that brain metastases may be a potential target for therapy with alkylating substances.

*Correspondence to:* Dr Roger von Moos, Medical Oncology, Canton's Hospital Graubünden, Loestrasse 170, CH-7000 Chur, Switzerland  
E-mail: roger.vonmoos@ksgr.ch

\*Shared senior authorship

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Temozolomide demonstrated clinical activity in a phase I trial with 4 out of 23 patients responding to treatment (11). This was supported by a phase II trial where 21% of the patients responded to treatment. A 9 months longer survival was seen in responders (12). Two large phase III randomized controlled trials of intravenous dacarbazine versus oral temozolomide every 4 weeks or dose dense every second week as first-line treatment in patients with melanoma demonstrated toxicity and response rate (13,14) with a trend for superiority in progression-free survival (PFS) and some quality of life domains in one trial in favour of temozolomide (13). A quite recently published phase III trial indicates that extended schedule escalated dose temozolomide has an acceptable safety profile, but does not improve overall survival (OS) and PFS in stage IV melanoma when compared to standard dose dacarbazine (14).

Blocking the vascular endothelial cell growth factor (VEGF) by bevacizumab showed significant survival advantage when combined with chemotherapy in advanced colorectal (15) and non-small cell lung cancer (16). The combination of bevacizumab and temozolomide was chosen in this trial because of the lack of overlapping toxicities. Furthermore, the combination of VEGF antibodies and chemotherapy improved survival in colon and lung cancer (15).

The optimal use of temozolomide in the treatment of metastatic melanoma is under discussion. The combined therapy of temozolomide and bevacizumab used in this phase II trial was an attempt to improve the response rate (17). A disease stabilization rate confirmed by independent external review of 52% at 12 weeks of the trial treatment is considered promising for further investigations. The response rate (16.1%) as well as the PFS (4.2 months) obtained in our trial was slightly higher than reported for single agent temozolomide (9.8-12%) and 2.1 months, respectively, despite having included also patients at higher risk (LDH >2 UNL; ECOG PS 2). Overall survival (9.3 months) in our trial was comparable to single agent temozolomide (12,13,18).

The investigation of the methylation status of the *MGMT* promoter was one of the prospectively defined translational research projects in this SAKK trial. Therefore, we tested *MGMT* promoter methylation and *MGMT* protein expression in our patient set of SAKK 50/07 with metastatic melanoma to elucidate the molecular basis of tumor response and outcome in patients treated with temozolomide and bevacizumab.

## Material and methods

**Patients and tumors.** Formalin-fixed, paraffin-embedded tissue samples of 62 patients previously untreated for metastatic melanoma with ECOG performance status  $\leq 2$  were selected for *MGMT* promoter methylation analysis and immunohistochemical analysis. The patients were treated with temozolomide at 150 mg/m<sup>2</sup> days 1-7 orally and bevacizumab at 10 mg/kg body weight day 1 intravenous every 2 weeks until disease progression or unacceptable toxicity. Response was assessed every 6 weeks by computed tomography according to the RECIST (response evaluation criteria in solid tumors) criteria.

The protocol was approved by local ethics review boards and all patients gave written informed consent. The trial was registered at the National Institute of Health ([www.clinicaltrials.gov](http://www.clinicaltrials.gov); identifier number: NCT00568048).

**DNA extraction and methylation-specific PCR.** All tumors were histologically reviewed by one pathologist (H.M.) on the basis of hematoxylin and eosin-stained tissue sections. One to three biopsy cylinders (0.6-mm diameter) were punched from tumor areas that contained at least 70% tumor cells. Genomic DNA extraction, sodium bisulfite modification of isolated DNA and the analysis of the methylation status of *MGMT* was done using a one-step PCR approach with 40 cycles and the nested primer pair described by Palmisano *et al* (19). DNA of normal lymphocytes and human colorectal adenocarcinoma cell-line SW620 was used as a negative and positive control for methylated alleles of *MGMT*, respectively.

**Immunohistochemistry.** Immunohistochemistry of melanoma sections was performed as described (10). Sections were incubated with clone MT3.1 (dilution 1:160; NeoMarkers, Newmarket, UK). *MGMT*-immunopositive cells revealed a strong nuclear staining. Lymphocytes and endothelial cells served as an internal positive control. The immunoreactivity was scored semi-quantitatively as follows: 0: <5% positive tumor cells, 1+: 5-75% positive tumor cells, 2+: >75-95% positive tumor cells, 3+: >95% positive tumor cells.

**Statistical analyses.** The endpoints of interest defined in the trial protocol and considered for this project include disease stabilization rate at week 12 (DSR12) consisting of either a complete response (CR), partial response (PR) or SD, best overall response, PFS and OS. PFS was defined as the time from trial registration until either a disease progression or death, with patients censored at the time of starting a second line therapy or the last time they were known to be alive without progression. For survival-type endpoints subgroups were compared using the log-rank test. Associations between categorical variables were assessed by either the Chi-squared or Fisher's exact test as well as the Mantel-Haenszel Chi-square test of trend if a variable was ordinal. The data were analyzed in SAS (Statistical Analysis Systems, version 9.2).

## Results

**Patients.** Between January 2008 and April 2009, 62 patients (40 male) were enrolled in the trial SAKK 50/07. None of the patients were ineligible or withdrew participation before treatment start. The median age at enrollment was 59 years (range: 29-82) and the median follow-up time was 20.1 (range: 1.7-32.6) months. All patients were administered at least one cycle of therapy. The independently reviewed DSR12 was 52% including 10 patients with a PR and 22 patients with SD.

***MGMT* promoter methylation and protein expression.** Tumor tissue was not available for 9 of the 62 patients enrolled in the trial. *MGMT* methylation status was reliably determined in 42 of 53 (79%) formalin-fixed and paraffin-embedded samples. Overall, a methylated *MGMT* promoter was detectable in 11 of 42 (26%) of the metastatic melanomas by MS-PCR.

*MGMT* immunoreactivity was assessed in 49 of 53 (92%) tumors. Of 53 tumors, 4 were excluded from further calculations because endothelial cells and/or leukocytes used as internal control for *MGMT* positivity were *MGMT* negative possibly indicating tissue fixation problems. Seventeen (35%) tumor

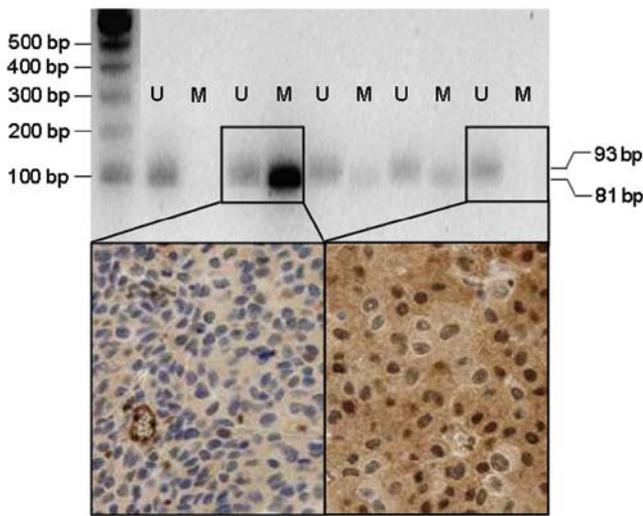


Figure 1. Methylation-specific PCR with 5 metastatic melanomas. U: unmethylated, M: methylated *MGMT* (upper panel). Tumors with methylated *MGMT*/no *MGMT* expression (left) and with unmethylated *MGMT*/strong nuclear *MGMT* expression are highlighted.

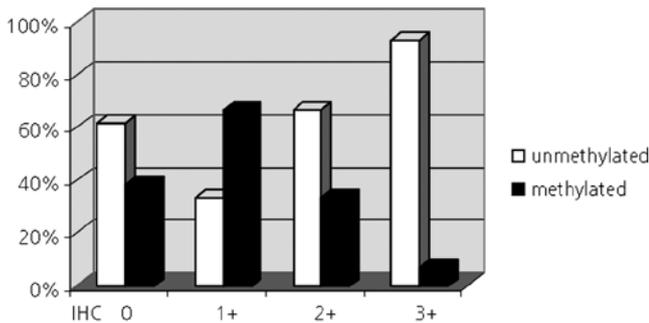


Figure 2. *MGMT* methylation status in the four *MGMT* expression subgroups. IHC 0: <5%, IHC 1+: 5-75%, IHC 2+: 75-95%, IHC 3+: >95% nuclear *MGMT* positive tumor cells.

samples were 3+, 11 (22%) were 2+, whereas 4 (8%) were 1+ and 17 (35%) lacked immunoreactivity for *MGMT*.

Both the methylation status of the *MGMT* promoter as well as the *MGMT* immunoprofile were available in 39 patients. Examples of melanomas with unmethylated and methylated *MGMT* promoter as well as with strongly positive and negative *MGMT* protein expression are shown in Fig. 1. No significant association was observed when the *MGMT* expression patterns in the methylated and in the unmethylated patient subgroups were compared. Of 28 (46%) melanomas with an unmethylated *MGMT* promoter, 13 displayed a homogeneous 3+ *MGMT* immunoreactivity, 6 (21%) tumors had a 2+ score, one (4%) had a 1+ score and 8 (29%) were *MGMT* negative. Within the subgroup of melanomas which had a methylated *MGMT* promoter 5 (45%) were also *MGMT* negative, two (18%), three (27%) and one (9%) were scored 1+, 2+ and 3+, respectively (Fig. 2).

When we looked at the methylation status in the four *MGMT* expression subgroups we noted that in the patient subgroup with a 3+ score 13 of 14 (93%) tumors were also

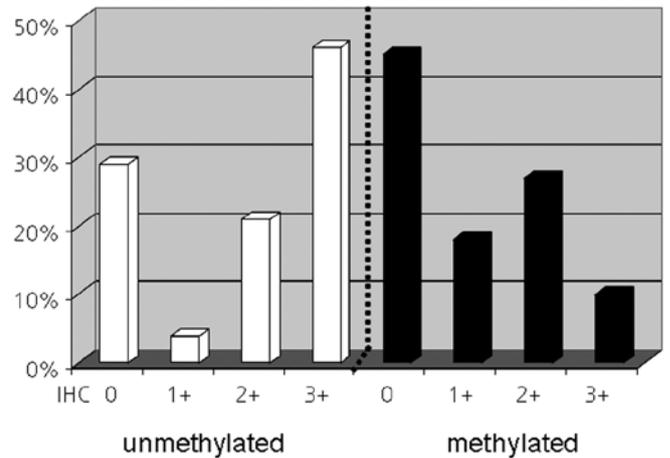


Figure 3. Association between *MGMT* methylation and *MGMT* protein expression in metastatic melanoma. IHC 0: <5%, IHC 1+: 5-75%, IHC 2+: 75-95%, IHC 3+: >95% nuclear *MGMT* positive tumor cells.

Table I. Association between response and *MGMT* promoter methylation status.

Methylation status	Response	Mantel-Haenszel Chi-square test of trend p-value
Methylated vs. Unmethylated	CR, PR, SD, PD	0.0803
	CR + PR + SD, PD	0.5573
	CR + PR, SD + PD	0.0426

unmethylated. In contrast, no association was observed between *MGMT* negativity and promoter methylation. Only 5 of 13 (38%) *MGMT* negative tumors had a methylated promoter (Fig. 3). However, no statistical correlation existed between expression and methylation status.

*MGMT promoter methylation status and patient outcome.* Patients with methylated *MGMT* promoter had higher response rates according to RECIST, when CR + PR versus SD + PD were compared ( $p=0.0426$ , Table I). No significant differences were obtained for other response groupings.

PFS and OS were stratified by *MGMT* methylation status. Median PFS time in the methylated subgroup was 8.1 months [95% confidence interval (CI): 1.7, 8.4] versus 3.4 months (95% CI: 1.4, 5.4) in the unmethylated subgroup. Median OS time in the methylated subgroup was 9.9 months (95% CI: 8.4, 15.9) versus 9.1 months (95% CI: 6.6, 11.9) in the unmethylated subgroup. Although, in the methylated subgroup median PFS was exceeded by more than 4 months compared to the unmethylated subgroup the results did not reach statistical significance.

*MGMT protein expression and patient outcome.* The different groupings for the *MGMT* protein expression (0-5, 5-75, 75-95 and 95-100% nuclear positive tumor cells) were compared in various groupings with regard to best overall response. The best response outcome was obtained from patients that were

Table II. Best overall response by MGMT protein expression and promoter methylation status.

Methylation status	Best response	MGMT expression (% positive tumor cells)				
		Unknown	0-5%	5-75%	75-95%	95-100%
Methylated	Complete response	-	-	-	-	-
	Partial response	-	5	-	-	1
	Stable disease	-	-	1	2	-
	Disease progression	-	-	1	1	-
	Unknown	-	-	-	-	-
Unmethylated	Complete response	-	-	-	-	1
	Partial response	-	1	-	2	1
	Stable disease	2	4	-	3	6
	Disease progression	1	2	1	1	5
	Unknown	-	1	-	-	-
Unknown	Complete response	-	-	-	-	-
	Partial response	2	1	-	-	1
	Stable disease	3	3	-	-	2
	Disease progression	1	1	2	2	2
	Unknown	-	-	-	-	-

MGMT negative (<5%) versus otherwise when CR + PR + SD versus PD were compared ( $p=0.05$ ).

PFS and OS were stratified by the MGMT expression groups. In general, patients with negative or heterogeneous MGMT expression had longer PFS and OS rates than patients with high and homogeneous MGMT expression. However, these differences did not reach statistical significance (data not shown). Best overall response by MGMT protein level and promoter methylation status is summarized in Table II.

## Discussion

We investigated the epigenetic silencing of *MGMT* by both *MGMT* promoter methylation and the MGMT protein expression status in metastatic melanomas to assess the predictive value for benefit from temozolomide in combination with bevacizumab. In our study, 26% of metastatic melanomas revealed a methylated *MGMT* promoter. This result is in line with previous studies on similar patient cohorts resulting in *MGMT* promoter methylation between 20 and 31% (10,20,21). Of note, a methylated *MGMT* promoter correlated significantly with complete and partial responses. Compared to patients with unmethylated *MGMT* promoter this patient group had also a trend to prolonged median PFS and OS of more than 4 months.

Our combined analysis of *MGMT* promoter methylation status and protein expression is unique and revealed a strong association between homogeneous MGMT expression and unmethylated MGMT promoter. Ninety-three percent of the tumors with >95% MGMT-positive tumor cells had an unmethylated *MGMT* promoter. In contrast, only 44% of the tumors with no or heterogeneous MGMT positivity had a methylated *MGMT* promoter. Similar results were observed

in patients with brain metastases of various solid tumors, as previously published (10).

There are several possible explanations for this discrepancy: i) gene mutations may lead to loss of functional MGMT expression independent of the *MGMT* promoter methylation status; ii) in a certain set of unmethylated and MGMT negative melanomas, *MGMT* may be induced after addition of alkylating, DNA damaging agents (22,23); iii) both discordance between *MGMT* promoter methylation and individual metastases from the same patient (20) and/or intra-tumoral heterogeneity of promoter hypermethylation (21) may significantly weaken *MGMT* methylation as a reliable predictor of chemosensitivity; iv) some tumors may be interpreted unmethylated because the corresponding PCR product is not detectable or not reproducible. In contrast to MS-PCR, pyrosequencing enables to identify promoter regions methylated from 0 to 100%. A previous melanoma study showed that in 50% of the tumors, *MGMT* promoter methylation was 10% or less (18). It is therefore tempting to speculate that tumors with low *MGMT* methylation are considered unmethylated when MS-PCR is used.

In a recently published study methylation of the *MGMT* promoter was analyzed in metastatic melanoma with the combined bisulfite restriction analysis technique (24). In contrast to our findings, those patients with a methylated *MGMT* promoter neither had a survival advantage nor a better response rate but suffered from more severe adverse events compared to those with an unmethylated promoter. One possible explanation for the discrepant outcomes may be the difference in treatment. The lack of MGMT in temozolomide-treated melanoma cells causes an increase of toxicity, which may even be enhanced in the presence of bevacizumab by attenuating vascularization and therefore oxygen and energy supply in the tumor.

We also believe that the use of different methods and the analysis of different CpG regions within the *MGMT* promoter may lead to contradictory results. Consequently, a standardized protocol with a still to be defined threshold that more reliably separates *MGMT* methylated (inactive) from unmethylated (active) melanoma patients would be highly desirable. Here we selected to use a protocol which was previously described for investigating the *MGMT* methylation status in glioma patients (9) and which allowed us to directly compare the results obtained from melanoma and glioma patients.

A reproducible and reliable promoter methylation analysis was only possible in 42 of 53 cases. DNA treated with formalin and bisulfite is highly modified and degraded and thus of limited quality to perform reliable PCR. To minimize the amplification of artificial PCR products we used a one-step PCR approach. The success rate for DNA amplification was much higher compared to that obtained from a recent study in which nested PCR with 80 cycles was used (10). All 53 melanoma DNA samples were amplifiable, the results of 11 (19%) cases, however, were not reproducible. Similar error rates were reported in the literature (10,25,26). The reasons why some of the DNA samples extracted from formalin fixed tissue are not suitable for MS-PCR may be a too long tissue fixation time, the use of unbuffered formalin or insufficient dehydration of the tissue.

Based on our data we suggest that both molecular and immunohistochemical approaches may be helpful to identify responders (methylated) as well as non-responders (unmethylated and homogeneous expression). For routine diagnosis further optimized standard protocols with preferably unfixed, snap-frozen biopsies are necessary to improve specificity and sensitivity.

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