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MGMT expression levels predict disease stabilisation, progression-free and overall survival in patients with advanced melanomas treated with DTIC

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ARTICLE INFO

Article history:
Received 18 December 2009
Received in revised form 23 April 2010
Accepted 28 April 2010
Available online 10 June 2010

Keywords:
Melanoma
MGMT
$p16^{INK4a}$
DTIC
Antineoplastic drug resistance
Drug response
Biomarkers
Prognosis

ABSTRACT

Metastatic melanoma responds poorly to systemic treatment. We report the results of a prospective single institution study evaluating O^6-methylguanine-DNA methyltransferase (*MGMT*) status as a potential predictive and/or prognostic marker among patients treated with dacarbazine (DTIC) 800–1000 mg/m^2 monotherapy administered as a 3-weekly schedule for advanced malignant melanomas. The study was approved by the Regional Ethical Committee. Surgical biopsies from metastatic or loco-regional deposits obtained prior to DTIC treatment were snap-frozen immediately upon removal and stored in liquid nitrogen up to processing. Median time from enrolment to end of follow-up was 67 months. *MGMT* expression levels evaluated by qRT-PCR correlated significantly to DTIC benefit (CR/PR/SD; $p = 0.005$), time to progression (TTP) ($p = 0.005$) and overall survival (OS) ($p = 0.003$). *MGMT* expression also correlated to Breslow thickness in the primary tumour ($p = 0.014$). While *MGMT* promoter hypermethylation correlated to *MGMT* expression, *MGMT* promoter hypermethylation did not correlate to treatment benefit, TTP or OS, suggesting that other factors may be critical in determining *MGMT* expression levels in melanomas. In a Cox proportional regression analysis, serum lactate dehydrogenase (LDH, $p < 0.001$), *MGMT* expression ($p = 0.022$) and $p16^{INK4a}$ expression ($p = 0.037$) independently predicted OS, while TTP correlated to DTIC benefit after 6 weeks only ($p = 0.001$). Our data reveal *MGMT* expression levels to be associated with disease stabilisation and prognosis in patients receiving DTIC monotherapy for advanced melanoma. The role of *MGMT* expression as a predictor to DTIC sensitivity versus a general prognostic factor in advanced melanomas warrants further evaluation.

1. Introduction

Although 80–90% of all patients with primary malignant melanoma are cured by surgical treatment, 10–20% of patients develop metastatic disease. For those developing systemic disease, response rates to medical therapy remain poor. Despite multiple approaches being evaluated,[1] dacarbazine (DTIC) remains standard first-line chemotherapy with an

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doi:10.1016/j.ejca.2010.04.023

objective response rate of 10–15% only.[2] Dacarbazine is a prodrug, which is activated in the liver to give MTIC (5-(3-methyltriazen-1-yl)imidazole-4-carboximide). MTIC methylates guanine at the O^6 position,[3] a lesion requiring the O^6-methylguanine-DNA-methyltransferase (*MGMT*) gene product for repair.[4] Defects in the MGMT system lead to increased sensitivity to alkylating agents and apoptosis in experimental systems.[5–8]

Dacarbazine and MTIC are unable to cross the blood–brain barrier. In contrast, temozolomide (TMZ), acting through a similar mechanism, and alkylating agents, such as lomustine (CCNU), may cross the blood–brain barrier and have been used to treat neurological malignancies, including glioblastomas.[9] Notably, reduced MGMT enzyme expression due to promoter methylation has been shown to enhance the response to TMZ in glioblastomas.[10,11] In contrast, studies in patients suffering from malignant melanomas have confirmed neither a correlation between *MGMT* promoter status and prognosis nor a correlation between *MGMT* promoter status and treatment response to TMZ or fotemustine.[12,13] Interestingly, a recent study reported low *MGMT* expression levels to be associated with sensitivity to TMZ in melanoma cells *in vitro*.[14]

The aim of the present study was to explore *MGMT* promoter methylation status and gene expression levels as potential predictors to response, progression-free as well as overall survival (OS) in patients treated with a standard DTIC regimen as first-line therapy in metastatic malignant melanomas. To explore potential effects of *MGMT* expression levels and promoter methylation status on long-term outcome in multivariate analysis, we also determined expression of *BMI-1* and $p16^{INK4a}$, known prognostic factors in melanomas,[15–17] in addition to serum lactate dehydrogenase (LDH), a marker of melanoma 'tumour load' and prognosis.[18] As the *CDKN2A* gene ($p16^{INK4a}$) is known to harbour alterations like mutations, deletions and promoter hypermethylation in melanoma,[19] these alterations were analysed as potential prognostic parameters for inclusion in multivariate models.

2. Material and methods

2.1. Patients

This study was conducted as a single institution prospective study at the Department of Oncology, Haukeland University Hospital, Bergen. The primary aim was to explore potential predictive factors to DTIC monotherapy in melanoma. Thus, patients suffering from locoregional relapses or distant metastases from malignant melanoma, considered candidates for chemotherapy, were offered inclusion. The protocol was approved by the Regional Ethical Committee, and each patient provided written informed consent.

Patient characteristics are listed in Table 1 and Suppl. Table S1. The first patient was included in October 1999 and the last in November 2007. Follow-up was terminated in May 2009. Thus, follow-up time varied from 18 to 105 months, with a median of 67 months. At inclusion, median age was 63 years (range 25–86).

Eighty-five patients with distant metastases or regional relapses ($n = 3$) from malignant melanoma were analysed. Of these 85 patients, 75 patients were subjected to treatment with DTIC monotherapy according to protocol[20] with subsequent response evaluation. The remaining 10 patients were not evaluable for response to therapy for reasons outlined in Table S2. Three of these 10 patients had their metastatic deposit removed by surgery alone, and tissue from these patients was sampled for molecular analysis, as they were considered candidates for DTIC therapy according to protocol if new relapses appeared.

Tumour biopsies were collected from distant metastases or locoregional relapses (Table S1). Tissue samples were collected by incisional or Tru-cut (liver) biopsies and snap-frozen in liquid nitrogen in the theatre.

Because this study was implemented in 1999, our protocol specified the UICC,[21] and not the more recently implemented RECIST criteria,[22] for response evaluation. Accordingly, responses were classified as progressive disease (PD), stable disease (SD), partial response (PR) and complete response (CR) according to these criteria. Clinical evaluations (staging) were conducted by the treating clinicians and recorded in the patient treatment records. Responses were evaluated 6 weeks

Table 1 – Patient demographics.

	n	Mean ± SD
Age at primary diagnosis		57 ± 14
Age at inclusion/referral for metastatic disease		61 ± 13
Male	48	
Female	37	
Localisation of primary melanoma		
Cutaneous	57[a]	
Breslow thickness ⩽ 1.00	6	
1.01–2.00	15	
2.01–4.00	11	
⩾4.01	17	
Breslow thickness not available	8	
Acral	4	
Eye	5[a]	
Mucosal	5	
No primary	15	
Localisation of relapses at inclusion		
1. Soft tissue metastases[b]	20	
2. Visceral organ metastasis	26	
3. Brain metastases	3	
4. Skeletal	0	
Comb. met. loc.		
1 + 2	22	
1 + 4	4	
2 + 4	3	
1 + 3	3	
2 + 3	3	
1 + 2 + 3	1	
Clinical stage at inclusion		
Stage III	3	
Stage IV	82	
Complete follow-up	85	

[a] One of the patients (MM50) had two primary melanomas one uveal melanoma and one skin melanoma.

[b] Soft tissue metastases were defined as subcutaneous, cutaneous or lymph node metastases.

after commencing therapy (=after 2 cycles of DTIC), at 3 months (after 4 cycles of DTIC) and thereafter at 3 monthly intervals until time of progression. OS was defined as time from diagnosis of the first metastasis to death, and time to progression (TTP) as time from treatment initiation to progression. OS was chosen, and not cancer-specific survival, because of the expected survival of only 6–7 months in this metastatic situation. Death was classified as melanoma related for all deaths in patients with distant metastases unless obvious due to other reasons in accordance with international practice. None of the 3 patients with locoregional disease only died during the observation time. Thus, overall survival and cancer-specific survival were equal. None of the patients were lost to follow-up (cut-off date 31st May 2009).

2.2. *Molecular markers and analytical methods*

Tissue samples were stored in liquid nitrogen until nucleic acid extraction and purification. In order to characterise aberrations in *MGMT* and $p16^{INK4a}$ on gene level, several methods like methylation-specific PCR, qRT-PCR, PCR, multiplex ligation-dependent probe amplification (MLPA) were used. A detailed description of each analytical method is given in the Supplementary information.

2.3. *Statistical analysis*

Categorical data were analysed using the Fischer's exact test while the Mann–Whitney test was applied to continuous data. To evaluate the impact of different parameters on treatment response, we initially compared gene expression levels, incidence of promoter hypermethylation or gene mutations between patients responding to treatment (CR or PR) versus patients revealing SD or PD at defined time intervals. Due to the low number of patients obtaining an objective response ($n = 4$), we also compared patients with clinical benefit (CR + PR + SD) versus those having a PD.

Survival data were analysed with the log rank test drawing Kaplan–Meyer plots. A Cox regression (multivariate) analysis was performed to calculate each variable's influence on OS and TTP. Only variables revealing a p-value of less than 0.10 in the univariate analysis were included in the regression model, for which reason variables like age and Clark's level of invasion were not included. Statistical significance was consistently reported as two-tailed, considering a $p < 0.05$ as significant. All data were analysed with PASW Statistics 17.0 software package (Chicago, Illinois, USA).

3. Results

The material contained a total of 85 tumours for tissue analysis and 75 patients for whom results could be related to outcome on DTIC treatment. Individual response to therapy is presented in Table S1. Of the 75 patients evaluable for response to DTIC, only one (1.2%) obtained a CR and three (3.5%) obtained a PR to treatment according to the UICC criteria. Due to the low number of patients obtaining an objective response to therapy, the potential predictive factors comparing patients with PD to those having a 'clinical benefit' (CR + PR + SD) were evaluated. As most patients had a short-term stabilisation only, we compared biological parameters between patients with a 'clinical benefit' versus a PD at 6 weeks and after 3 months of therapy.

Nine of 85 patients (10.6%) expressed hypermethylation of the *MGMT* promoter. Among the 75 patients with evaluable response, the corresponding figure was 7 (9.3%). *MGMT* promoter hypermethylation did not predict benefit to DTIC treatment at 6 weeks or 3 months of therapy ($p = 0.201$ and $p = 0.081$, respectively) and was not associated with TTP ($p = 0.285$) or OS ($p = 0.091$).

MGMT promoter hypermethylation correlated significantly to *MGMT* expression level ($p = 0.020$, Fig. 1). Median *MGMT* expression level in tumours harbouring *MGMT* methylation was 0.54% of the reference standard value versus 6.32% in the unmethylated tumours. Expression levels of *MGMT* varied substantially across individual tumours (Fig. 2). While no statistical difference in *MGMT* expression levels between tumours revealing an objective response ($n = 4$) and non-responding tumours was recorded ($p = 0.914$), clinical benefit to DTIC treatment (response or an SD) was associated with low levels of *MGMT* expression. Thus, mean levels of *MGMT* expression were significantly lower in tumours benefiting from DTIC therapy versus those having no benefit at 6 weeks (mean level of 0.68, 95% CI 0.17–2.6, versus mean level of 8.0, 95% CI 3.5–19, $p = 0.002$, Mann–Whitney) as well as after 3 months (mean level of 0.82, 95% CI 0.14–4.8, versus mean level of 5.2, 95% CI 2.3–12, $p = 0.039$, Mann–Whitney) of therapy, respectively (Fig. 2). Notably, *MGMT* expression correlated strongly to both TTP and OS ($p = 0.005$ and $p = 0.007$, respectively).

Interestingly, *MGMT* expression levels in the metastatic deposits correlated to Breslow thickness in the primary tumours (Mann–Whitney; $p = 0.045$, Fischer's exact test; $p = 0.014$). No correlations between *MGMT* expression and $p16^{INK4a}$ promoter methylation, p16 mutation status or

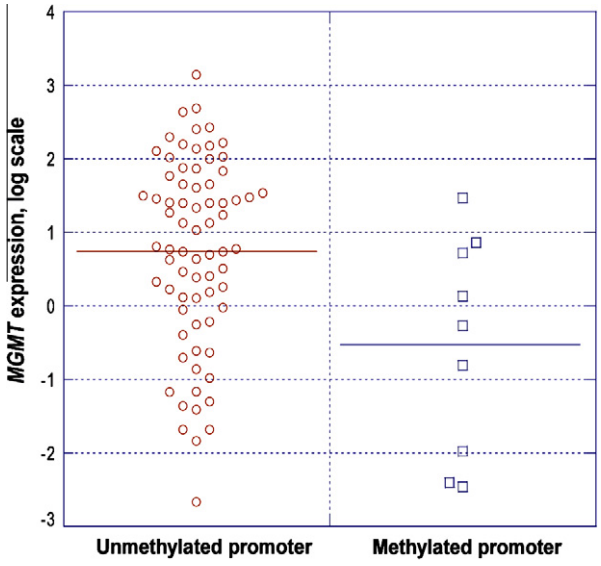

Fig. 1 – *MGMT* expression. Expression values of *MGMT* (log axis) related to promoter methylation status. Mean values are marked with a line.

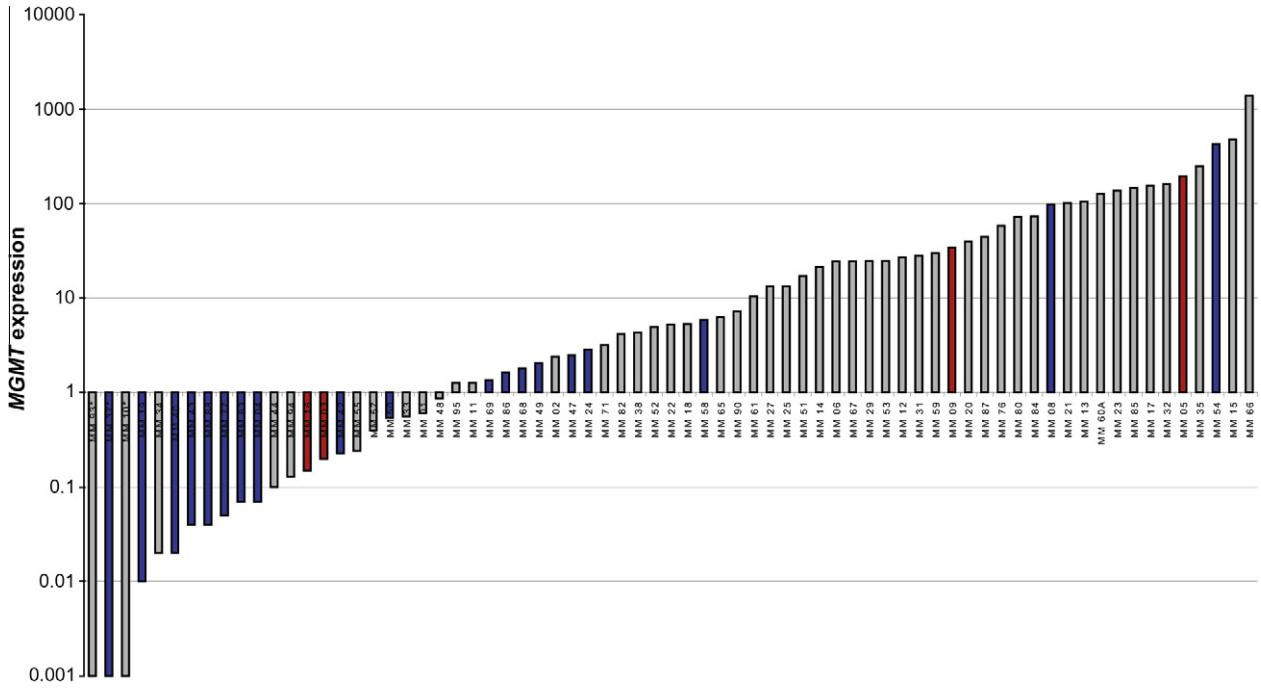

Fig. 2 – *MGMT* expression and response to DTIC treatment in individual patients. Responders to DTIC after 6 weeks are depicted in red, patients with progressive disease are coloured grey and patients with stable disease are marked in blue. Three patients revealed no expression of *MGMT*, for these cases *MGMT* expression level was set as 0001% of the standard.

$p16^{INK4a}$/*BMI-1* expression levels were observed (data not shown).

To further evaluate the effect of *MGMT* expression levels on outcome, we evaluated the impact of multiple variables (gender, age, Breslow thickness, Clark's level of invasion, $p16^{INK4a}$ hypermethylation, $p16^{INK4a}$ mutation status, hypermethylation, $p16^{INK4a}$ copy number status, $p16^{INK4a}$ expression levels as well as *BMI-1* and serum LDH expression levels) in addition to *MGMT* status on TTP as well as OS. The results are summarised in Table S3, while Kaplan–Meyer plots for *MGMT* expression and $p16^{INK4a}$ expression are depicted in Fig. 3A and B, respectively. As shown, univariate analysis revealed *MGMT* expression levels to be associated with progression-free ($p = 0.005$) as well as overall ($p = 0.007$) survival. In contrast, $p16^{INK4a}$ was associated with OS only ($p = 0.033$).

Multivariate analysis was performed entering different sets of variables into a Cox model. Complete listing of different variables and models applied for multivariate analysis are given in Supplementary information, Table S4. As *BMI-1* expression was not a significant predictor of OS ($p = 0.219$) in the univariate model and, in addition, strongly correlated to $p16^{INK4}$ expression (Pearson's $R = 0.459$), this parameter was excluded from multivariate analysis.

The results obtained with different models in the multivariate analysis are presented in Table S4. Regarding progression-free survival, only serum LDH and DTIC response after 6 weeks proved significant ($p = 0.027$ and $p = 0.001$, respectively) (see Table 2).

Regarding OS, serum LDH and Breslow thickness proved to be independent, significant markers (HR = 0.321 and HR = 0.483, respectively). While both *MGMT* expression and $p16^{INK4a}$ expression and/or mutation predicted survival in models including Breslow thickness (p-values of 0.012–0.021 and 0.016–0.036, respectively), both lost their significant impact when DTIC response was entered into the model.

4. Discussion

The identification of *MGMT* promoter methylation as a predictor of TMZ chemosensitivity in glioblastoma[23] has encouraged researchers to look for similar correlations in malignant melanomas. However, *MGMT* promoter hypermethylation has previously been reported neither to correlate to response to TMZ[12] nor to fotemustine[13] in advanced melanomas.

In this prospective study, we detected no significant correlation between *MGMT*-promoter hypermethylation and response to DTIC treatment or outcome among patients treated with DTIC for advanced melanoma. In contrast, *MGMT* expression level determined by qRT-PCR correlated to stabilisation on DTIC treatment as well as TTP and OS. To the best of our knowledge, our results presented here are the first suggesting *MGMT* expression levels to be of importance as a predictor to DTIC/MTIC sensitivity as well as TTP and OS in humans suffering from metastatic melanoma.

Interestingly, using various melanoma cell lines, Augustine and collaborators recently reported a correlation *in vitro* between *MGMT* expression and TMZ response, but not between *MGMT* promoter hypermethylation and TMZ response.[14] Taken together, these data support the hypothesis that *MGMT* expression is of importance in regulating cytotoxic activity

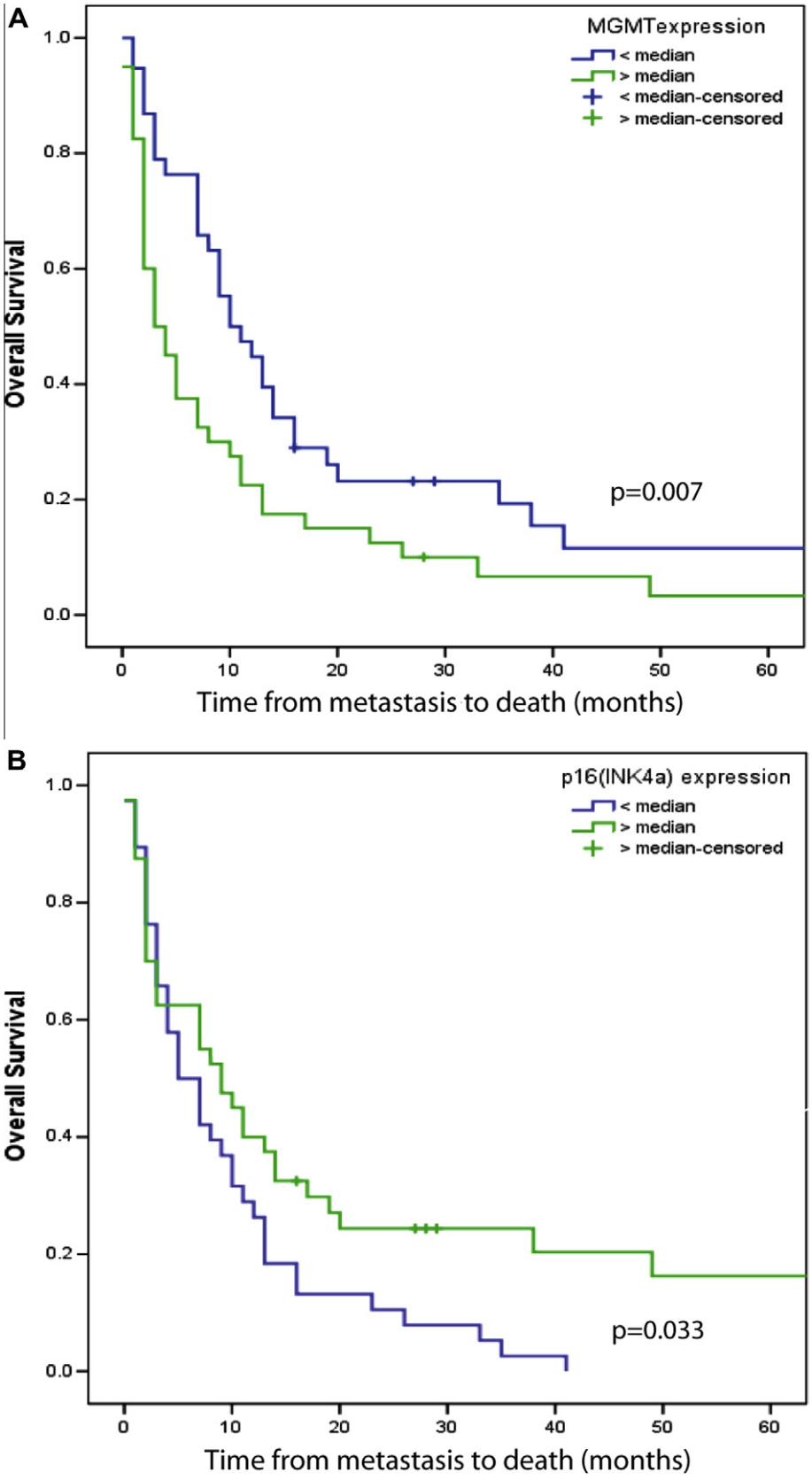

Fig. 3 – Kaplan–Meyer plots on overall survival with respect to (A) *MGMT* and (B) $p16^{INK4a}$ expression levels. In both cases patients were stratified into those expressing *MGMT* or $p16^{INK4a}$ levels above (green) or below (blue) median expression level.

Table 2 – Cox proportional regression analysis regarding overall survival entering the three categorical variables; serum LDH (over normal versus normal), $p16^{INK4a}$ expression (high versus low), and *MGMT* expression (high versus low).

Variables	Categories	Sig.	*n*	HR	95% CI
LDH in serum	High and low	$p < 0.001$	78	0.333	0.193–0.574
MGMT expression	High and low	0.021	81	0.557	0.337–0.919
$p16^{INK4a}$ expression	High and low	0.036	81	1.726	1.034–2.881

of alkylating agents acting through MTIC, but mechanisms other than promoter hypermethylation seem to be of importance in regulating *MGMT* expression levels in melanoma *in vivo*. While evidence has linked CpG methylation of the coding region of *MGMT* to increased protein expression levels and fotemustine resistance in melanoma cell lines,[24,25] further studies are warranted to address patient differences between different tumour forms such as glioblastomas and melanomas with respect to MGMT regulation *in vivo*.

The fact that *MGMT* expression was not significantly suppressed among our 4 patients achieving an objective response to treatment may be due to the small numbers, preventing robust statistical analysis. Thus, we defined 'therapy failures' as tumours progressing at 6 weeks or 3 months of therapy. Comparing different parameters at *MGMT* expression levels between these patient groups, we detected significant differences, suggesting an interaction with treatment efficacy.

MGMT expression was associated with TTP as well as with OS in univariate analysis. Considering multivariate analysis, *MGMT* expression significantly predicted OS but not TTP. These results need to be interpreted carefully. While they may be consistent with *MGMT* predicting benefit from DTIC treatment, alternatively, *MGMT* expression may be a general prognostic factor[26] related to melanoma outcome in general, independent of DTIC treatment. Further studies on the potential prognostic role of *MGMT* expression among patients receiving different therapeutic approaches are warranted to resolve this issue.

Notably, while we detected a significant correlation between *MGMT* expression level and outcome defined as disease stabilisation as well as long-term outcome, no correlation to *MGMT* promoter methylation status was recorded. However, the number of tumours (9/85) harbouring an *MGMT* promoter methylation was low, suggesting other mechanisms to influence on *MGMT* expression level in the majority of tumours. This hypothesis is in accordance with the *in vitro* findings of Augustine et al.[14] and results from other clinical studies.[12,13]

While Breslow thickness has proven to be one of the strongest prognostic[27] markers in primary melanoma indicating vertical growth to predict metastatic propensity, the biological mechanisms of this effect are incompletely understood. Our finding of a correlation between Breslow stage and *MGMT* expression indicates Breslow thickness to be related to biological characteristics of the tumour beyond local invasion potential.

Expression of $p16^{INK4a}$ is recognised as a prognostic marker in primary melanoma.[28] Notably, one study reported a moderate correlation between high $p16^{INK4a}$ expression levels and response to melphalan/actinomycin D high-dose ILI treatment in advanced melanomas.[29] While, to the best of our knowledge, no study has reported any correlation between $p16^{INK4a}$ expression or mutation status and response to either DTIC or TMZ in melanomas, $p16^{INK4a}$ may represent a statistical covariate to MGMT expression, explaining an association to treatment outcome. Our data, however, do not support such a hypothesis.

In conclusion, we show a correlation between *MGMT* expression and benefit to DTIC monotherapy as well as OS in advanced melanomas. The fact that most patients benefiting from treatment obtained SD of short duration only indicates additional mechanisms to be involved in directing resistance of melanoma patients to DTIC therapy. Yet, these findings represent the first successful step aiming at identifying potential predictive factors in this therapy-resistant cancer type. The fact that expression levels of *MGMT* had an influence on drug sensitivity suggests future studies exploring combination regimens including alkylating compounds to stratify patients for *MGMT* expression. Further, identification of additional predictive factors in other gene pathways[30] could lead to identification of novel combined treatment regimens attacking different critical pathways simultaneously.

Conflict of interest statement

None of the authors have any actual or potential conflict of interests including any financial, personal or other relationships with other people or organisations within that could inappropriately influence their work.

Acknowledgements

We are grateful to the patients who participated in the study. We would also like to recognise Dept. of Oncology and Dept. of Surgery, Section of Plastic Surgery, Haukeland University Hospital, in particular, the consultants Stephanie Geisler, Svein Inge Helle and Åse Sivertsen who included many of the patients. We would like to thank Astrid Lund for participating in the statistical analysis, Dagfinn Ekse and Hildegun Helle for data collection and Nhat Kim Duong and Elise de Faveri for technical assistance.

Grant information: This work was supported by the Norwegian Cancer Society, the Norwegian Health Region West (Helse Vest) and the Innovest program of Excellence, Haukeland University Hospital. Christian Busch is a recipient of a fellowship from the Norwegian Cancer Society.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.04.023.

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