Promoter Hypermethylation of the O^6-Methylguanine DNA Methyltransferase Gene and Microsatellite Instability in Metastatic Melanoma

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Tumor spread to distant organs is the most serious consequence of melanoma, as only 10–20% of stage IV patients respond to current chemotherapies. Tumor sensitivity to alkylating agents is affected by the activity of cellular DNA repair proteins, such as O^6-methylguanine DNA methyltransferase (MGMT) and the DNA mismatch repair proteins. Chemosensitivity may be enhanced by reduced MGMT activity, but the frequency of *MGMT* promoter silencing through hypermethylation is unknown in distant melanoma metastases. The frequency and significance of microsatellite instability (MSI) in metastatic melanoma is also unclear, and it has been suggested that MSI frequency increases during the metastatic process. We undertook an analysis of 84 melanoma metastases from 47 patients. *MGMT* methylation was detected using methylation-specific PCR in 26 of the 84 metastases (31%), but there was discordance between individual metastases from the same patient. Therefore, as a result of this variation, *MGMT* methylation may have only limited value as a predictor of chemosensitivity. High MSI involving mononucleotide repeat markers was not found. Low MSI was detected in five of 50 metastases (10%) and only one of the five metastases also had *MGMT* methylation. These results demonstrate that in contrast to some previous reports, these tumors have a low frequency of MSI.

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INTRODUCTION

Metastatic melanoma is an important cause of cancer mortality, particularly in Caucasian people living in areas of high sunlight exposure. Treatment of metastatic melanoma is based on chemotherapy with the alkylating agents dacarbazine or temozolamide (Eggermont and Kirkwood, 2004). Surgical resection of distant metastatic disease sites is more frequently performed in melanoma than in other tumor types because long-term survival occasionally occurs following resection of all apparent disease. Resection also relieves local complications such as bleeding or bowel obstruction due to metastases in the small intestine (Thompson and Morton, 2004).

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Abbreviations: bp, base pairs; MGMT, O^6-methylguanine DNA methyltransferase; MSI-L, low microsatellite instability

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The efficacy of alkylating agents in the treatment of melanoma is based on widespread DNA damage, which signals cell death through apoptosis. To some extent, cytotoxic drug sensitivity is determined by the activity of cellular DNA repair proteins, such as O^6-methylguanine DNA methyltransferase (MGMT), and the DNA mismatch repair proteins. Functional mismatch repair mediates the cytotoxic effect of these drugs, whereas active MGMT can counteract them by removing alkylating adducts from the O^6-position of guanine. It has long been recognized that inhibiting the activity of the MGMT enzyme may enhance the efficacy of alkylating chemotherapy (Dolan *et al.*, 1990). Inhibitors of MGMT such as O^6-benzylguanine and O^6-4-bromothenyl-guanine are being investigated in clinical trials (Friedman *et al.*, 2000). The *MGMT* gene can be silenced by promoter methylation in many cancers, but its frequency in primary melanoma is thought to be low, at 11% (Esteller *et al.*, 1999). However, the frequency of *MGMT* methylation in distant melanoma metastases has not yet been established.

Defective mismatch repair in cancer leads to widespread DNA replication errors, which are particularly evident in repetitive DNA sequences, such as microsatellites, and can be detected using a standardized test from tumor DNA (Dietmaier *et al.*, 1997; Boland *et al.*, 1998). In sporadic colorectal cancers, the high microsatellite instability (MSI) phenotype is present in about 10% of patients, predominantly as a result of the *MLH1* mismatch repair gene silencing

through promoter hypermethylation (Kane *et al.*, 1997). The biological defect causing the low MSI (MSI-L) phenotype, on the other hand, is not so well understood. Early reports established that the frequency of MSI in primary melanoma ranges from 2 to 20% (Peris *et al.*, 1995; Quinn *et al.*, 1995; Tomlinson *et al.*, 1996). Subsequent studies have suggested that the frequency of MSI in melanoma increases during the metastatic process, up to 38% in a group of regional and distant metastases (Palmieri *et al.*, 2000) and 77% in lymph node metastases (Richetta *et al.*, 2001). The reinterpretation of the literature indicates that most MSI in melanoma involves instability of the dinucleotide repeats, which therefore is closer to the definition of MSI-L. However, there are a limited number of studies addressing distant organ metastases. Furthermore, no previous studies have addressed multiple distant organ metastases from the same patient.

Although no clear biological difference has yet been found between MSI-L and microsatellite stable tumors, we and others have shown that the MSI-L phenotype is associated with an MGMT defect in colorectal cancer (Whitehall *et al.*, 2001; Kohonen-Corish *et al.*, 2005). This raised the intriguing hypothesis that this may also be the case in melanoma, given the potential importance of both types of alterations in the metastatic process. Therefore, we undertook an analysis of *MGMT* promoter methylation and MSI in metastatic melanoma. *MGMT* hypermethylation was determined with methylation-specific PCR and MSI status was analyzed with at least six microsatellite markers, including the standardized panel used in colorectal cancer.

RESULTS

Analysis of *MGMT* promoter hypermethylation

We detected *MGMT* hypermethylation in 31.0% (26/84) of the tumors, using methylation-specific PCR. In five of these patients, methylation was also found in the matched control DNA specimen prepared from the resected tissue outside the tumor margin. The 22 patients who had at least one methylated tumor are listed in Table 1. The organ sites with methylated tumors included the lung (six patients), liver (two), small intestine (two), rectus muscle (one), brain (two), mesentery (one), and pancreas (one). Three methylated specimens were derived from subcutaneous metastases and eight from lymph nodes. For four organ sites, where 10 or more specimens were analyzed, methylation frequency was highest in the lymph node metastases (47.5%) and lowest in the small intestine (12.5%) (Table 2). Seven of the 22 patients had a single metastasis analyzed. Eleven patients had two to four metastases but only one methylated tumor. Four patients had two methylated tumors, out of a total of two to four metastases analyzed. In each of these four cases, the methylated tumors were resected in two separate operations. Four patients had two metastases synchronously resected in the same operation that showed discordant results (Patients 7, 9, 21, and 22).

Analysis of MSI

A subset of 50 metastases were initially available for MSI analysis. We observed MSI in five of the 50 specimens

Table 1. Organ sites of the melanoma metastases showing *MGMT* methylation or MSI

Patient	Site of methylated melanoma metastases	Site of unmethylated melanoma metastases
1	Lung	Lung/pleura
2	Lung	
3	Lung	
4	Lung	
5	Lung, subcutaneous metastasis	
6	Lung	
7	Liver[1]	Skeletal muscle[1]
8	Liver	
9	Small intestine, pelvic lymph node[1]	Inguinal lymph node[1]
10	Small intestine	Small intestine
11	Skeletal muscle	Lung
12	Brain	Subcutaneous metastasis, inguinal lymph nodes
13	Brain	Lung
14	Mesentery[MSI]	Small intestine
15	Pancreas	Retroperitoneal lymph node
16	Subcutaneous metastasis, axillary lymph node	Subcutaneous metastasis, dermal metastasis
17	Subcutaneous metastasis, inguinal lymph node	Lung
18	Axillary lymph node	Axillary lymph node
19	Axillary lymph node	
20	Inguinal lymph node	
21	Hilar lymph node[1]	Hilar lymph node[1], small intestine[2], lymph node[2]
22	Mediastinal lymph node[1]	Lung[1, MSI]
23		Brain[MSI], lymph node
24		Lung[MSI], dermal metastasis
25		Colon[MSI], small intestine

[1] and [2]Metastases resected synchronously.
[MSI]Patients 14, 22, 23, 24, and 25 each had only one metastasis showing MSI with the *D9S161* marker.

Table 2. Frequency of *MGMT* methylation in the main sites of melanoma metastases analyzed

Site	*MGMT* methylated No. (%)	*MGMT* unmethylated No. (%)
Lung	6 (37.5)	10 (62.5)
Small intestine	2 (12.5)	14 (87.5)
Lymph nodes	8 (47.5)	9 (52.5)
Subcutaneous/dermal	3 (30.0)	7(70.0)

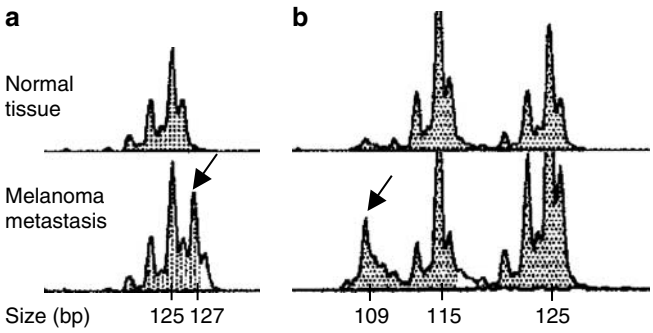

Figure 1. Analysis of the dinucleotide repeat *D9S161* from two patients with metastatic melanoma (lung) and their matching normal lung tissue. MSI is seen as the presence of extra peaks in the tumor tissue as indicated by arrows. (**a**) Expected allele 125 bp and extra allele in tumor 127 bp; (**b**) expected alleles 125 and 115 bp and extra allele 109 bp.

studied (10.0%) using the *D9S161* marker (Figure 1), each from a different patient (Table 1). Only one of these tumors in patient no. 14 also showed *MGMT* methylation ($P=0.99$, Fisher's exact test). Each patient had at least one other metastasis with no MSI. Metastases demonstrating MSI were from four sites, lung (two), colon (one), brain (one), and mesentery (one). No cases demonstrated MSI using the mononucleotide markers, and only one of the five tumors also showed mild instability with a dinucleotide marker from the colon cancer microsatellite panel.

DISCUSSION

Hypermethylation of gene promoters is an epigenetic process through which tumor suppressor genes can become dysfunctional and hence cancer promoting. Methylation of *MGMT* is associated with a poor outcome in non-small-cell lung cancer (Brabender *et al.*, 2003). On the other hand, chemosensitivity to alkylating agents may be enhanced by inactivation of MGMT, as shown in glioma and diffuse large B-cell lymphoma (Esteller *et al.*, 2000, 2002). In melanoma, low MGMT expression prior to treatment also tends to correlate with better chemotherapy response (Ma *et al.*, 2003), but until now, the importance of *MGMT* promoter methylation has not been assessed in distant metastases. This study has shown that *MGMT* methylation occurs in about 31% of distant melanoma metastases. This percentage compares very well with the recent findings by Hoon *et al.* (2004), who found 34% *MGMT* methylation in a series of 86 tumors from 44 stage III/IV cutaneous melanoma patients. However, we have also shown that in stage IV patients with multiple distant metastases, methylation was not uniformly present in all tumors. There was no prevalence of methylation in any particular organ site, although it was striking that only two out of 16 metastases occurring in the small intestine were methylated. Therefore, it would be difficult to predict, based on the analysis of one resected tumor, which patients are likely to respond to chemotherapy. In five patients, methylation was also detected in the normal tissue adjacent to the tumor, which could be due to small clusters of tumor cells that were not detected in the histopathology review of slides. Alternatively, it is possible that *MGMT* methylation is an early epigenetic change, as discussed by Brabender *et al.* (2003), who made similar findings in primary lung cancer.

The biological significance of *MGMT* methylation in carcinogenesis is currently under investigation. It has been hypothesized that inactivation of the *MGMT* gene in sporadic colorectal cancer can overload the mismatch repair system due to accumulation of DNA mispairs, which can result in a mild mutator phenotype, and is characterized by MSI-L (Whitehall *et al.*, 2001). Although the definition of MSI-L in sporadic colorectal cancer has been questioned (Tomlinson *et al.*, 2002), there is now emerging evidence that MSI-L tumors form a distinct molecular group when gene expression data from microarray experiments are analyzed using principal components analysis (Mori *et al.*, 2003). We have also recently shown that MSI-L is a distinct phenotype in stage C colon cancer, with an adverse prognosis (Kohonen-Corish *et al.*, 2005). Therefore, it was of interest to determine whether there was any association between these two phenotypes in metastatic melanoma, where the frequency of MSI has been reported to range from 19 to 77% (Tomlinson *et al.*, 1996; Palmieri *et al.*, 2000; Kroiss *et al.*, 2001; Richetta *et al.*, 2001). As there is considerable variation in the microsatellite markers used in analyzing melanoma MSI, we decided to test the same microsatellite panel that was originally used to define the association between *MGMT* methylation and MSI-L in colorectal cancer (Whitehall *et al.*, 2001). This was supplemented by the dinucleotide marker *D9S161*, which has been reported as a sensitive marker for detecting loss of heterozygosity and for defining a subset of melanomas with high metastatic potential (Puig *et al.*, 2000).

Altogether, the five tumors analyzed here displayed clear instability with *D9S161* (Figure 1). Only one of these specimens also had a methylated *MGMT* promoter. The *D9S161* marker is located on chromosome 9 in the region implicated in melanoma carcinogenesis. It is possible that there are cancer-specific differences between the various microsatellites used in detecting MSI. The chromosome 9 markers may be more sensitive in melanoma, although these were not used in at least one previous study that reported the highest frequency (77%) of MSI in melanoma metastases (Richetta *et al.*, 2001). In any case, the overall frequency of MSI-L detected here was only 10%, and therefore our results do not support the hypothesis that MSI is a significant factor during the progression of melanoma to distant organ metastases. However, it cannot be excluded that once the biology of this phenotype is better understood, new diagnostic tests or markers will emerge that will define a wider extent of this subset of metastatic melanoma. On the other hand, our results also suggest that the type of instability involved in melanoma is not likely to be high microsatellite instability, which is characterized by instability in the mononucleotide repeats. Another laboratory has reported a 21% frequency of high MSI in metastatic melanoma, but their definition included all tumors that displayed instability with any two unstable markers, not just with mononucleotide markers (Palmieri *et al.*, 2003).

In conclusion, the results establish the frequency of *MGMT* methylation in almost one-third of distant melanoma metastases. However, we observed discordant results, which is likely to limit the potential value of *MGMT* methylation as a predictor of chemosensitivity. We have further shown that these tumors have a low frequency of MSI, and therefore we were unable to demonstrate an association between *MGMT* methylation and MSI-L in metastatic melanoma with the microsatellite marker panel used here.

MATERIALS AND METHODS

Patients and specimens

The Sydney Melanoma Unit database contains details of surgical resections for all patients attending the Unit. From the database, patients who had undergone resection of distant metastatic melanoma at the Royal Prince Alfred Hospital between 1996 and 2001 were identified. Patients who had surgery at other institutions were excluded. Of the 47 patients for whom suitable tissue specimens could be retrieved, 18 had one metastasis surgically removed, and 22 patients had two metastases and seven patients had three to four metastases resected. The metastatic sites included the lung (16 metastases), small intestine (16), liver (five), spleen (three), muscle (three), brain (four), adrenal gland (two), stomach (one), colon (one), pancreas (one), pelvis (two), and omentum/mesentery (three). Also, 17 lymph node metastases and 10 subcutaneous metastases were included. Of the 29 patients with multiple metastases, 11 had them resected in the same operation, whereas the rest had two (16 patients) or three to four (two patients) operations, most over a 1- to 2-year period. This study had the approval of the Ethics Review Committee of the Central Sydney Area Health Service and was exempted from obtaining informed consent from the patients because only archival tumor specimens were used.

Archival hematoxylin and eosin sections of melanoma metastases were first re-evaluated by a pathologist and the most suitable blocks from each patient were chosen for subsequent analysis. Tumor and normal tissue areas were marked on the slides. Serial sections (4 μm) were manually microdissected for tumor and matched normal tissue. DNA was purified using the Puregene DNA Isolation Kit (Gentra, Minneapolis, MN).

MSI analysis

The international reference panel of microsatellite markers *BAT25/40*, *D2S123*, *D5S346*, *D17S250*, and *MYCL1* (Boland *et al.*, 1998), as well as *D9S161* (Puig *et al.*, 2000), was analyzed. One primer from each primer pair was synthesized with a 5′ fluorescent tag. PCR amplification was performed as described previously (Kohonen-Corish *et al.*, 2005). Fragments were visualized using ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) capillary electrophoresis and the microsatellite patterns of the tumors were compared with normal tissue from the same patient.

Methylation-specific PCR

Aliquots of about 1 μg of the tumor and normal DNA specimens were bisulfite-treated, as described previously (Herman *et al.*, 1996). For *MGMT*, each specimen was amplified, as described previously (Kohonen-Corish *et al.*, 2005), using primers specific for both the methylated and the unmethylated promoter sequences (Esteller *et al.*, 1999; Harden *et al.*, 2003). The predicted fragment size is 122 base pairs (bp) for the methylated PCR and 93 bp for the unmethylated PCR. The *MYOD1* primers, which do not contain any CpG nucleotide sequences, were used for an internal reference PCR to ensure integrity of each bisulfite-treated DNA specimen (Brabender *et al.*, 2003). PCR conditions were 95°C (45 seconds), 57°C (45 seconds), and 72°C (60 seconds) for 40 cycles, with an initial denaturation step at 95°C for 12 minutes and final elongation at 72°C for 7 minutes. All methylation-specific PCR products were visualized on 6% polyacrylamide gels.

CONFLICT OF INTEREST

The author states no conflict of interest.

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