ABSTRACT

Tumors often display unrestricted cell cycling attributable to a dysfunctional G1-S checkpoint. One of the mechanisms leading to such a defect is the inactivation of the cyclin-dependent kinase inhibitor p16INK4a. Although inactivation of p16INK4a is observed in a wide range of tumors, including cutaneous melanoma, genetic alteration of p16INK4a is reportedly uncommon in uveal melanoma. Here we show that the p16INK4a promoter is hypermethylated in 6 of 12 uveal melanoma cell lines and in 7 of 22 primary uveal melanomas analyzed. Five of seven patients with a methylated primary tumor died of metastatic disease compared with 2 of 15 patients with a nonmethylated primary tumor. We also show that all uveal melanoma cell lines with a hypermethylated p16INK4a promoter have lost p16INK4a expression but have maintained the expression of p14ARF. Treatment of uveal melanoma cell lines with 5-aza-2'-deoxycytidine results in demethylation of p16INK4a and in reexpression of p16INK4a mRNA, which is maintained upon withdrawal of the 5-aza-2'-deoxycytidine. In conclusion, p16INK4a promoter methylation appears to be a common event in uveal melanoma and is accompanied by the loss of p16INK4a expression.

INTRODUCTION

Progression of cells through the G1 phase of the cell cycle is stimulated by the association of D-type cyclins with CDKs that phosphorylate Rb (1). Upon Rb phosphorylation, E2F is activated and promotes S-phase-specific gene expression. p16INK4a has been identified as an inhibitor of the cyclin D/CDK complex (2). The inhibitory activity of p16INK4a is restricted to the cyclin D-CDK4 and cyclin D-CDK6 kinases and results in cell cycle control at the G1-S restriction point. Exons 2 and 3 of p16INK4a are used by two genes in alternative reading frames. Because of their unique first exons, exon 1a and exon 1b, two transcripts with distinct protein-coding potentials are expressed (3-5). The transcript derived from exon 1a encodes p16INK4a, whereas the transcript derived from exon 1b encodes p14ARF (6). It has been established that p14ARF plays a role in cell cycle control as well, and that it is the actual link between the p16INK4a/Rb pathway and the p53/Rb pathway (Fig. 2A). Upon induction by E2F, p14ARF sequesters MDM2 and thereby prevents degradation and nuclear export of p53 (7, 8).

p16INK4a is commonly inactivated in a wide range of malignancies (9), but p16INK4a/germ-line mutations are uniquely associated with familial cutaneous melanoma (10–13). The occurrence of uveal melanoma as a secondary tumor within some melanoma families indicates that both forms of melanoma share predisposing factors (14, 15). Despite shared risk factors, these tumors present differences in incidence, age at onset, and mortality. Whereas uveal melanoma is a rare tumor with a high age at onset and a high mortality, cutaneous melanoma is much more common, occurs at a younger age, and is associated with a relatively low mortality (16–19). Whereas p16INK4a is the main target of inactivation in cutaneous melanoma, mutation screening and deletion mapping did not reveal such a role for p16INK4a in uveal melanoma (20). An alternative mechanism for tumor suppressor gene inactivation is de novo methylation of CpG islands, which generally represses transcription. De novo methylation of the p16INK4a promoter region occurs in a wide range of malignancies (21–23) and releases the cell from a potent cell cycle inhibitor.

In this study, we show that in both primary uveal melanoma and uveal melanoma cell lines, p16INK4a is frequently inactivated by hypermethylation. Hypermethylation of p16INK4a is accompanied by a down-regulated expression of p16INK4a. Loss of p16INK4a expression attributable to CpG methylation could be reversed when the cell lines were treated with the demethylating agent 5-aza-2'-deoxycytidine. Interestingly, metastatic disease tended to be more common in uveal melanoma patients who had a tumor with a methylated p16INK4a promoter. As illustrated, aberrant methylation can be modulated and, hence, offers a target for treatment of uveal melanoma.

MATERIALS AND METHODS

Cell Culture and Treatment with 5-Aza-2'-deoxycytidine. Nine cell lines derived from primary uveal melanomas (92.1; OCM-1, -3, and -8; Mel-270, -285, and -290; MEL-202; and EOM-3) and three cell lines derived from uveal melanoma metastases (OMM-1, -1.3, and -1.5) were used in the experiments. Cell line Mel 270 and OMM-1.3 and -1.5 represent a progression model because they were derived from a primary uveal melanoma and two of its liver metastases. Cell line 92.1 was established in our own laboratory (24). Cell lines EOM-3 and OMM-1 were kindly provided by Dr. Gae P. M. Luyten (Rotterdam University Hospital, Rotterdam, the Netherlands). Cell lines MEL-202, -270, and -285; and OMM-1.3 and -1.5 were a generous gift of Dr. Bruce Ksander (Schepps Eye Research Institute, Harvard Medical School, Boston, MA), and cell lines OCM-1, -3, and -8 were kindly provided by Dr. J. Kan-Mitchell (University of California, San Diego, La Jolla, CA). Cell lines OMM-1 and OCM-1 were cultured in DMEM (Life Technologies, Inc.) containing 25 mM HEPES buffer, 1 mM sodium pyruvate, 1 g/l glucose, and supplemented with 10% heat-inactivated fetal bovine serum and 2% penicillin/streptomycin (Life Technologies, Inc.). The other cell lines were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 3 mM L-glutamine (Life Technologies, Inc.), 2% penicillin/streptomycin, and 10% FBS. Cell cultures were incubated at 37°C in a humidified 5% CO2 atmosphere. To inhibit methylation of daughter cells, the cell lines were grown in the presence of 1μM 5-aza-2'-deoxycytidine for 72 h. Fresh medium was added every 24 h.

Tumor Specimens. We used archival frozen tumor specimens of primary uveal melanomas from 22 patients (Table 1). Patients, of which the median age at presentation was 64 years (range, 44 to 87 years), had not received treatment before enucleation. From one patient, both the primary uveal melanoma (tumor 15) as well as the cell line (92.1) derived from that tumor were studied. Histopathological analysis of all cases was performed by the same ocular pathologist. DNA was isolated by the modified protocol of Isola et al. (25) and Vos et al. (26).

MSP. The MSP method and primer sequences as described by Herman et al. (27) were used. First, genomic DNA was modified with sodium bisulfite. As a result of this modification, cytosine residues are deaminated and changed into uracil residues, but a methylated cytosine is protected and will remain unmethylated upon withdrawal of the 5-aza-2'-deoxycytidine. In conclusion, p16INK4a promoter methylation appears to be a common event in uveal melanoma and is accompanied by the loss of p16INK4a expression.
after RT-PCR, logarithmic dilutions of cloned p16 INK4a cation with sodium bisulfite. Three of nine primary uveal melanoma tases (OMM-1, -1.3, and -1.5) were analyzed by MSP after modifi-

Table 1 Clinical and histopathological data on 22 primary uveal melanoma

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Sex/age (yr)</th>
<th>Location</th>
<th>Cell type</th>
<th>Diameter (mm)</th>
<th>P16 status</th>
<th>Present status</th>
<th>Survival (mo)</th>
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<td>1</td>
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<td>CB</td>
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<td>†, metastases</td>
<td>28</td>
</tr>
<tr>
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<td>Epithelioid</td>
<td>18</td>
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<td>†, metastases</td>
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<tr>
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<td>Mixed</td>
<td>15</td>
<td>N</td>
<td>Alive</td>
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</tbody>
</table>

* N, nonmethylated.
† †, deceased.
† CB, ciliary body.

ified. Hence, sequence differences are introduced that allow for amplification with methylated and unmethylated DNA-specific primers. Amplification with primers specific for methylated DNA is done, then digestion with the Hinf restriction enzyme. Recognition by this enzyme is dependent on both C→T conversion and methylation of a CpG. Starting at position 108 of p16INK4a exon 1, there is a GAGCCG site. If the second C is methylated, bisulfite will only modify the first C and hence create a fI site: GANTC. By performing this restriction analysis, we can ascertain the specificity of the MSP and analyze additional CpG sites apart from the CpGs that determine the primer specificity.

Competitive RT-PCR. Total RNA from the cell lines was isolated as described by the manufacturer (Biotecx Laboratories, Houston, TX). RNA was primed with random primers and reverse transcribed into cDNA in a 20-μl reaction volume containing 200 units of MMV (SuperScript II) reverse transcriptase (Life Technologies, Inc., Breda, the Netherlands). The cDNA was subsequently used in a competitive PCR in which the transcript of the p16INK4a homologue, p15INK4b (28), was used as the endogenous competitor. Primers used in the PCR reaction were: X2R140, 5′AGCACAACCGGTGTGTC 3′; BS, 1,5′ATTACG 3′; AS, 1,5′CAACGAACCGAT- GTTACG 3′; and P15–5′UTF, 5′GGCCAACCGTTGATATCCG 3′. The first primer is chosen in exon 2-conserved sequences and recognizes the gene product from p16INK4a /p14ARF and p15INK4b; the latter primers are gene-

RESULTS

MSP to Determine p16INK4a Promoter Methylation. p16INK4a gene mutations and deletions are frequent in many kinds of neoplasias, but neither seems common to uveal melanoma. Because inactivation of p16INK4a can also occur by promoter methylation, it was of interest to determine whether this applies to uveal melanoma. Genomic DNA of nine cell lines derived from primary uveal melano-

Fig. 1. MSP in 12 uveal melanoma cell lines. Top, amplification with the unmethylated DNA-specific PCR (U-p16); middle, methylated DNA-specific PCR (M-p16); bottom, Hinf digestion of M-p16. Partial digestion is seen in almost all reactions and is an indication of suboptimal bisulfite modification and/or digestion rather than partial methylation, because there is no correlation with U-p16 PCR signal.

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p16\textsuperscript{INK4a} do not express p16\textsuperscript{INK4a} mRNA, whereas all methylation-negative cell lines do express p16\textsuperscript{INK4a} mRNA. In contrast, methylation of the p16\textsuperscript{INK4a} locus had no clear effect on p14\textsuperscript{ARF} expression.

\textbf{p16\textsuperscript{INK4a} Expression in 5-Aza-2'-deoxycytidine-treated Uveal Melanoma Cell Lines.} To test whether there is a causal relationship between promoter methylation and p16\textsuperscript{INK4a} expression, six methylation-positive cell lines were treated with 5-aza-2'-deoxycytidine. 5-Aza-2'-deoxycytidine treatment inhibits methylation of daughter strands during DNA synthesis. A gain of p16\textsuperscript{INK4a} expression is expected if promoter methylation really causes the lack of p16\textsuperscript{INK4a} expression. Competitive RT-PCR performed on treated and untreated cell lines with a methylated p16\textsuperscript{INK4a} promoter showed p16\textsuperscript{INK4a} expression in all treated cell lines but not in any of the untreated cell lines (Fig. 3). Subsequent MSP revealed a loss of methylation in the treated cells and supports the notion that promoter methylation caused the loss of p16\textsuperscript{INK4a} expression (data not shown). Treatment of methylated cell lines always resulted in p16\textsuperscript{INK4a} reexpression but showed strong variation in expression.

After treatment of cell line Mel-270, this cell line expressed p16\textsuperscript{INK4a}. We subsequently cultured the treated cells for 3 months in the absence of 5-aza-2'-deoxycytidine and repeatedly observed p16\textsuperscript{INK4a} expression. These cells experienced growth arrest for ~75 days but then started to form colonies that contained fast-growing cells. When we tested the mRNA expression of these fast-growing cell clones, we could no longer detect p16\textsuperscript{INK4a} expression. Recurrent loss of p16\textsuperscript{INK4a} expression clearly triggered unrestricted cycling and identifies p16\textsuperscript{INK4a} again as a main target of inactivation.

\textbf{p16\textsuperscript{INK4a} Methylation in Primary Uveal Melanoma.} DNA from 22 snap-frozen primary uveal melanomas was modified with bisulfite and applied to MSP. Positive methylation signals were always accompanied by signals in the unmethylated DNA-specific PCR, indicating a mixture of tumor and normal tissue. Or even worse, the methylated DNA-specific signals might represent false-positive signals. But the gain of a Hinfl restriction site revealed its value in the analysis and distinguished tumors with a truly methylated p16\textsuperscript{INK4a} promoter from the methylation-negative tumors. Of a total of 22 primary tumors, 7 (32%) were positive for methylation. From one patient, we analyzed the primary tumor (tumor 15) as well as the cell line derived from this primary uveal melanoma (92.1) and found methylation in both (Fig. 4). This demonstrates that methylation in the cell line originates from the primary tumor, and that methylation in the cell line is not attributable to a tissue culture artifact.

\textbf{Clinicopathological Correlations.} Of the 22 primary uveal melanomas tested, seven carried a methylated p16\textsuperscript{INK4a} gene. Two bad prognostic factors, i.e., location of the uveal melanoma in the ciliary body (5 of 10) and the presence of epithelioid cells (4 of 5) in the tumor, occurred more frequently in p16\textsuperscript{INK4a} methylated tumors but did not reach significance (Table 1). Correlation with survival indicated that 5 of 7 patients with a methylated p16\textsuperscript{INK4a} died of metastatic disease compared with 2 of 15 patients without p16\textsuperscript{INK4a} methylation. Kaplan-Meier survival analysis showed a tendency to better survival in cases of a nonmethylated p16\textsuperscript{INK4a}, a result that did not reach significance (P = 0.15).

\section*{DISCUSSION}

Uveal melanoma and cutaneous melanoma share a common ancestry; both uveal melanocytes and the skin melanocytes are derived from the
neural crest. Genetic involvement of p16INK4a distinguishes the two tumors. In cutaneous melanoma, p16INK4a is the most important susceptibility gene, whereas p16INK4a involvement in uveal melanoma seems limited. Most commonly, loss of one p16INK4a allele in uveal melanoma is reported without mutational inactivation of the remaining wild-type allele (20, 29). Promoter methylation has also been reported, though in a low frequency (3%; Ref. 20). Our results, however, indicate that p16INK4a inactivation by way of promoter methylation occurs much more frequently in primary uveal melanoma (32%) and uveal melanoma cell lines (50%), indicating that the role of p16INK4a is more important than anticipated previously. In all methylation-positive cell lines, treatment with 5-aza-2′-deoxycytidine resulted in p16INK4a reexpression. We conclude that lack of expression is caused by hypermethylation of the p16INK4a promoter and that, subsequent to inhibition of methylation, p16INK4a expression can be rescued. Because demethylation of the p16INK4a promoter has such a dominant effect on the expression, we suppose that apart from promoter methylation, there is no other major genetic or epigenetic inhibitor of expression present in our cell lines to prevent p16INK4a expression. Cell culturing in the absence of 5-aza-2′-deoxycytidine resulted in a relapse of uncontrolled cell growth after 75 days, coinciding with loss of p16INK4a expression. This repeated loss illustrates that p16INK4a inactivation is a key feature of unrestricted cell growth in our cell lines.

Furthermore, we show that in uveal melanoma, p16INK4a is the only target in the 9p21 region where, besides p16INK4a, the genes that encode the CDK inhibitors p15INK4B and p14ARF are localized. p14ARF might even be slightly up-regulated in methylated cell lines, possibly revealing the cellular response to loss of p16INK4a.

Follow-up of the patients with methylated tumors indicate that p16INK4a is involved in the development of metastases, but additional studies with a larger group of patients and a longer follow-up will be necessary to substantiate this. Functional studies are required to determine the invasive pathways that are stimulated by loss of p16INK4a. In this respect we can expand on the experiments performed on gliomas. Chintala et al. (30) demonstrated that the malignant behavior of glioma cells with regard to invasive capacity in Matrigels could be reversed by infecting cells with an adenovirus carrying the p16INK4a cDNA. Harada et al. (31) added that restoration of wild-type p16INK4a expression into p16INK4a-deleted glioma cells inhibited angiogenesis induced by tumor cells in vivo. Because increased vessel density or complicated vessel networks are important risk factors for metastases, p16INK4a may not only play a role in cell cycle control but also in angiogenesis. Additional studies are necessary to test this hypothesis. In conclusion, p16INK4a promoter methylation in uveal melanoma is a frequent event and seems to be a good marker to study metastases risk, and it offers a target for possible adjuvant treatment of uveal melanoma.

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