Promoter Hypermethylation: A Common Cause of Reduced p16\textsuperscript{INK4a} Expression in Uveal Melanoma

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ABSTRACT

Tumors often display unrestricted cell cycling attributable to a dysfunctional G\textsubscript{1}-S checkpoint. One of the mechanisms leading to such a defect is the inactivation of the cyclin-dependent kinase inhibitor p16\textsuperscript{INK4a}. Although inactivation of p16\textsuperscript{INK4a} is observed in a wide range of tumors, including cutaneous melanoma, genetic alteration of the p16\textsuperscript{INK4a} promoter is reportedly uncommon in uveal melanoma. Here we show that the p16\textsuperscript{INK4a} 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 p16\textsuperscript{INK4a} promoter have lost p16\textsuperscript{INK4a} expression but have maintained the expression of p14\textsuperscript{ARF}. Treatment of uveal melanoma cell lines with 5-aza-2\textsuperscript{-}deoxycytidine results in demethylation of p16\textsuperscript{INK4a} and in reexpression of p16\textsuperscript{INK4a} mRNA, which is maintained upon withdrawal of 5-aza-2\textsuperscript{-}deoxycytidine. In conclusion, p16\textsuperscript{INK4a} promoter methylation appears to be a common event in uveal melanoma and is accompanied by the loss of p16\textsuperscript{INK4a} expression.

INTRODUCTION

Progression of cells through the G\textsubscript{1} 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. p16\textsuperscript{INK4a} has been identified as an inhibitor of the cyclin D/CDK complex (2). The inhibitory activity of p16\textsuperscript{INK4a} is restricted to the cyclin D-CDK4 and cyclin D-CDK6 kinases and results in cell cycle control at the G\textsubscript{1}-S restriction point. Exons 2 and 3 of p16\textsuperscript{INK4a} are used by two genes in alternative reading frames. Because of their unique first exons, exon 1\textalpha{} and exon 1\beta{}, two transcripts with distinct protein-coding potentials are expressed (3–5). The transcript derived from exon 1\textalpha{} encodes p16\textsuperscript{INK4a}, whereas the transcript derived from exon 1\beta{} encodes p14\textsuperscript{ARF} (6). It has been established that p14\textsuperscript{ARF} plays a role in cell cycle control as well, and that it is the actual link between the p16\textsuperscript{INK4a}/Rb pathway and the p53/Rb pathway (Fig. 2A). Upon induction by E2F, p14\textsuperscript{ARF} sequesters MDM2 and thereby prevents degradation and nuclear export of p53 (7, 8).

p16\textsuperscript{INK4a} is commonly inactivated in a wide range of malignancies (9), but p16\textsuperscript{INK4a} 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 p16\textsuperscript{INK4a} is the main target of inactivation in cutaneous melanoma, mutation screening and deletion mapping did not reveal such a role for p16\textsuperscript{INK4a} 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 p16\textsuperscript{INK4a} 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, p16\textsuperscript{INK4a} is frequently inactivated by hypermethylation. Hypermethylation of p16\textsuperscript{INK4a} is accompanied by a down-regulated expression of p16\textsuperscript{INK4a}. Loss of p16\textsuperscript{INK4a} expression attributable to CpG methylation could be reversed when the cell lines were treated with the demethylating agent 5-aza-2\textsuperscript{-}deoxycytidine. Interestingly, metastatic disease tended to be more common in uveal melanoma patients who had a tumor with a methylated p16\textsuperscript{INK4a} 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\textsuperscript{-}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. G. 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% CO\textsubscript{2} atmosphere. To inhibit methylation of daughter cells, the cell lines were grown in the presence of 1 \mu{}M 5-aza-2\textsuperscript{-}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 oculard pathologist. DNA was isolated by the modified protocol of Isola et al. (25) and Vos et al. (26).
and methylation of a CpG. Starting at position 108 of exon 1, there is a GAGCC*G site. If the second C is methylated, bisulfite will only modify the first C and hence create a $HinfI$ site: GANTC. By performing this restriction analysis, we can ascertain the specificity of the MSP and analyze additional CpGs 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 (Biotechnologies, 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 $p16^{INK4a}$ homologue, $p15^{INK4b}$ (28), was used as the endogenous competitor. Primers used in the PCR reaction were: X2R140, 5′AGCACCACACGCTGTGC 3′; BS.1, 5′TACTAGGAGCAGCACGCTA 3′; AS.1, 5′CAACAAAGCCGA-AGTTAAG 3′; and P15−5′UTF, 5′GGCCGACGGTGTATATCCG 3′. The first primer is chosen in exon 2-conserved sequences and recognizes the gene product from $p16^{INK4a}$, $p14^{ARF}$ and $p15^{INK4b}$; the latter primers are gene-specific and recognize exon 1 sequences from $p14^{ARF}$, $p16^{INK4a}$, and $p15^{INK4b}$, respectively. During competitive RT-PCR, two products are amplified that are separated by size and represent relative expression levels of $p16^{INK4a}$/$p15^{INK4b}$ or $p14^{ARF}$/p15$^{INK4b}$, respectively. Such a quantification is allowed, because after RT-PCR, logarithmic dilutions of cloned $p16^{INK4a}$ with $p13^{INK4b}$ transcript revealed a linear standard curve (data not shown), proving equal amplification efficiencies for both templates and excluding the possibility that the different expression ratios are influenced by sample quality.

**RESULTS**

**MSP to Determine $p16^{INK4a}$ Promoter Methylation.** $p16^{INK4a}$ gene mutations and deletions are frequent in many kinds of neoplasias, but neither seems common to uveal melanoma. Because inactivation of $p16^{INK4a}$ 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 melanoma (OCM-1, -3, and -8; MEL-202; Mel-270, -285, and -290; EOM-3; and 92.1) and three cell lines from uveal melanoma metastases (OMM-1, -1.3, and -1.5) were analyzed by MSP after modification with sodium bisulfite. Three of nine primary uveal melanoma cell lines (92.1, Mel-270, and Mel-285) and all three metastases-derived cell lines (OMM-1, -1.3, and -1.5) revealed $p16^{INK4a}$-methylated DNA-specific PCR products; whereas the unmethylated DNA-specific PCR was negative in these samples (Fig. 1). With $HinfI$ digestion, we confirmed the $p16^{INK4a}$ promoter methylation. The three cell lines that were derived from one patient (OMM-1.3 and OMM-1.5 are from two different metastases, and Mel-270 is from the primary tumor) were all methylated, indicating that methylation status was conserved during metastasis and that promoter methylation constitutes a primary event.

**Competitive RT-PCR to Compare $p16^{INK4a}$ mRNA and $p14^{ARF}$ mRNA Expression.** We detected $p16^{INK4a}$ promoter methylation in six of twelve uveal melanoma cell lines. To determine whether promoter methylation is accompanied by reduced $p16^{INK4a}$ mRNA expression, we analyzed $p16^{INK4a}$ gene transcription with competitive RT-PCR (Fig. 2A). Because $p14^{ARF}$ enhances the p53-mediated cell cycle control, and loss of control attributable to reduced $p16^{INK4a}$ expression can theoretically be compensated by an increase in its alternative transcript, we also measured $p14^{ARF}$ expression. Fig. 2B shows expression levels of $p15^{INK4b}$, $p14^{ARF}$ and $p16^{INK4a}$ in the uveal melanoma cell lines. All cell lines that contain a methylated...
Expression of p16 INK4a between promoter methylation and Melanoma Cell Lines.

To test whether there is a causal relationship between p16 INK4a levels of p16 INK4a/p15 INK4b expression and methylation of the p15 INK4b X primers that are used in the competitive RT-PCR. Combination of the loss of p16 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 p16 INK4a locus had no clear effect on p16 INK4a expression. These cells experienced growth arrest for 9 days but then started to form colonies that contained fast-growing clones, we could no longer detect p16 INK4a expression. Recurrent loss of p16 INK4a expression clearly triggered unrestricted cycling and identifies p16 INK4a as a main target of inactivation.

p16 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 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.

Clinicopathological Correlations. Of the 22 primary uveal melanomas tested, seven carried a methylated p16 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 INK4a methylated tumors but did not reach significance (Table 1). Correlation with survival indicated that 5 of 7 patients with a methylated p16 INK4a died of metastatic disease compared with 2 of 15 patients without p16 INK4a methylation. Kaplan-Meier survival analysis showed a tendency to better survival in cases of a nonmethylated p16 INK4a, a result that did not reach significance (P = 0.15).

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 inactivation 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 anticipated previously. In all methylation-positive cell lines, treatment with 5-aza-2’-deoxycytidine resulted in p16INK4a stabilization, which suggests that methylation is a key factor in the high frequency of relapses observed in uveal melanoma. In cutaneous melanoma, the p16INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell, 83: 993–1000, 1995.


ACKNOWLEDGMENTS

We appreciate the help of Dr. A. H. Zwinderman, Department of Medical Statistics, Leiden University Medical Center, Leiden, the Netherlands, for analysis of the patient data. We thank Dr. Gordon Peters and Prof. Dr. Rein Willemze for critical review of the manuscript. Dr. Didé de Wolff-Rouendaal for the histopathological analysis, Prof. Dr. Jan E. E. Keenen and Dr. J. Bleeker for providing patient material, and Wiljo J. F. de Leeuw for tumor DNA isolation.

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