Low Frequency of p16/CDKN2A Methylation in Sporadic Melanoma: Comparative Approaches for Methylation Analysis of Primary Tumors

Mark L. Gonzalgo, Christina M. Bender, Edward H. You, J. Michael Glendening, José F. Flores, Graeme J. Walker, Nicholas K. Hayward, Peter A. Jones, and Jane W. Fountain

ABSTRACT

Methylation of the 5' CpG island of the p16 tumor suppressor gene represents one possible mechanism for inactivation of this cell cycle regulatory gene that is also a melanoma predisposition locus. We have investigated the potential contribution of somatic silencing of the p16 gene by DNA methylation in 30 cases of sporadic cutaneous melanoma. The methylation status of the 5' CpG island of p16 was initially determined by Southern analysis and then reevaluated (in a blinded manner) using methylation-specific PCR, methylation-sensitive single nucleotide primer extension, and bisulfite genomic sequencing. All methodologies yielded concordant results, and significant levels of methylation were observed in 3 of the 30 (10%) melanoma DNAs analyzed. Of the three tumors found to be methylated, two were also positive for LOH on 9p21 (where the p16 gene resides), implying that both p16 alleles were inactivated, one via deletion and the other via methylation-associated transcriptional silencing. The association between methylation and transcriptional silencing of p16 was also further supported by inducing p16 expression with a DNA demethylating agent (5-aza-2'-deoxycytidine) in a melanoma cell line known to harbor a methylated p16 allele. Although methylation-associated gene silencing does not represent a common mechanism for p16 inactivation in sporadic melanoma, our findings provide support that PCR-based techniques, such as methylation-specific PCR and methylation-sensitive single nucleotide primer extension, can be reliably used for the accurate detection and quantitation of aberrant levels of DNA methylation in tumor specimens.

INTRODUCTION

Sporadic cutaneous melanoma accounts for over 80% of melanoma cases worldwide, whereas the remainder arise due to a genetic predisposition and/or originate within the pigmented (chordoid and iris) portions of the eye (1, 2). The sequential inactivation of tumor suppressor genes during the development of cutaneous melanoma is supported both by cytogenetic and molecular genetic studies, where regions of chromosomes 1, 6, 9, 10, and 11 have been found to be consistently deleted or rearranged in metastatic tumors (3–5). Losses of chromosome 9, and specifically the 9p21 region, have also been detected in early-stage melanomas, as well as in precursor lesions (i.e., atypical or dysplastic nevi), implying that mutation of the gene(s) located on this chromosome may be a critical or rate-limiting event in the development of sporadic melanoma (6, 7). Localization of the cyclin-dependent kinase inhibitor gene, p16, to 9p21 (8, 9), along with the identification of frequent homozygous deletions of this gene in melanoma cell lines and tumors (8–11) and the discovery of germ-line p16 mutations in familial melanoma patients (12–16), all suggest this gene is a primary target on 9p21 in both familial and sporadic melanoma. Genetic alterations that specifically disrupt both copies of p16, including the intragenic mutation of at least one allele, however, are uncommon in uncultured tumors (10, 11, 17) and have left some doubt as to whether this gene is the only critical “melanoma” gene on 9p21. In fact, previous molecular studies performed by our group, as well as those of others, suggest that over two-thirds of metastatic melanomas retain at least one wild-type copy of p16 (10, 11, 17). Because most of these tumors have undergone loss of heterozygosity on 9p21 and do not harbor intragenic mutations within the p16 locus, a dominant-negative mechanism for inactivation of the p16 gene product cannot be proposed. These findings have led us to investigate the role that an epigenetic mechanism of gene inactivation, i.e., DNA methylation, may play at the p16 locus during the development of sporadic melanoma.

Methylation of the CpG island that constitutes the 5' end and promoter region of the p16 gene has been described previously in other tumor types, including bladder cancer, non-small cell lung cancer, gliomas, head and neck squamous cell carcinoma, breast cancer, colon cancer, prostate cancer, renal cancer, and nasopharyngeal carcinoma (18–23). Analyses performed on primary bladder, breast, and colon tumors have been most revealing, where 31–67% of these tumors have been found to be methylated at the p16 locus (20, 21). Evidence from tumor cell lines supports the association between methylation and transcriptional silencing; cell lines with methylated p16 alleles do not express RNA from this locus, although the gene can be reactivated after treatment with demethylating agents such as Aza-CdR (19, 23, 24). To our knowledge, no extensive study has yet been performed to determine the frequency of p16 methylation in sporadic melanoma. This is of obvious interest because the p16 gene is a melanoma predisposition locus and may also be the “gatekeeper” in the development of sporadic melanoma (12–16, 25). The relevance of this information to the treatment of melanoma could also be important because therapeutic strategies with demethylating agents could potentially be used on melanoma patients to increase p16 expression in their tumor cells and potentially inhibit the growth of metastatic lesions. Experiments involving the introduction of a wild-type p16 gene in tumor cells in vitro support this notion and demonstrate the growth-inhibitory effects of an active p16 (26–30). Unfortunately, the limited success of adjuvant therapies for metastatic melanoma places these patients in urgent need of new therapeutic options (31).

A second goal of these studies was to compare and contrast the currently available methods for the detection of DNA methylation. Because we had access to tumor DNAs that were not limited, we chose to test the general applicability of two novel PCR-based techniques, specifically MSP3 (32) and Ms-SNuPE (33), in comparison to the more labor-intensive and reagent-costly techniques of Southern blotting and bisulfite genomic sequencing. If reliable, PCR-based approaches for methylation analysis of primary tumors could potentially be used on melanoma patients to increase p16 expression in their tumor cells and potentially inhibit the growth of metastatic lesions. Experiments involving the introduction of a wild-type p16 gene in tumor cells in vitro support this notion and demonstrate the growth-inhibitory effects of an active p16 (26–30). Unfortunately, the limited success of adjuvant therapies for metastatic melanoma places these patients in urgent need of new therapeutic options (31).

Received 8/1/97; accepted 10/3/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by Grant R01CA66002 (to J. W. F.) and USPHS Grant R35CA49758 (to P. A. J.) from the National Cancer Institute.

2 To whom requests for reprints should be addressed, at Department of Biochemistry and Molecular Biology, Institute for Genetic Medicine, University of Southern California, 2250 Alcazar Street, IGM240, Los Angeles, CA 90033.

The abbreviations used are: MSP, methylation-specific PCR; Ms-SNuPE, methylation-sensitive single nucleotide primer extension; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LOH, loss of heterozygosity; 5-aza-CdR, 5-aza-2'-deoxycytidine; IFNβ1, β-IFN gene; RT-PCR, reversed transcription-PCR.
assays like MSP and Ms-SNuPE could be used routinely to detect methylation changes in tumors where only a small amount of DNA is available (as from paraffin-embedded tissue sections). The ability and sensitivity with which these four different assays could detect methylation at the p16 locus was, therefore, also determined.

Overall, we examined 30 sporadic melanomas, using four different methylation detection assays, to accurately assess the frequency of p16 methylation in cutaneous melanoma. Melanomas known to harbor homozygous deletions of the p16 gene were purposefully excluded from these analyses (10), whereas three congenital nevi were included as normal tissue controls. Parallel assays were also performed on 12 melanoma cell lines (all known to retain at least one p16 allele; Ref. 30) of the melanoma tumors analyzed exhibited significant (≥10%) levels of methylation at the p16 locus, all four methylation-based assays were successful in detecting methylation that ranged from 12% to ≥38% in these three samples.

MATERIALS AND METHODS

Tissue Specimens and Cell Lines. Eight metastatic melanomas (MGH-MEL-02, -04, -06, -12, -13, -14, -15, and -16A) and three congenital nevi (MGH-CN-01, -02, and -03) were obtained from the Massachusetts General Hospital (Boston, MA). Twenty-one additional metastatic melanomas and one primary melanoma were obtained from the Queensland Institute of Medical Research (Brisbane, Australia). Twelve melanoma cell lines were also analyzed and obtained either from the Memorial Sloan Kettering Cancer Center (SK-MEL- designations) or directly from Dr. Jeffrey Weber (cell lines A375, MEL-02, -04, -06, -12, -13, -14, -15, and -16A) and three congenital nevi.

Southern Analyses. Melanoma, congenital nevus, and normal DNAs were digested with appropriate enzymes, electrophoresed on 1.0—1.2% agarose gels, and hybridized with radiolabeled probes as described previously (34). In the case of the methylation-sensitive restriction digestions performed with the enzymes EcoRI, SacII, and Smal, a 6- to 11-fold molar excess of each enzyme (40—80 units) was used to digest DNA samples (7 μg) overnight, followed by an additional "spike" of enzyme (12—20 units) the following morning for ≥2 h additional at 37°C. The probe used to hybridize these blots was a 920-bp EcoRI/Eagl fragment containing the promoter region of p16 (provided by Dr. Toshiro Hayashida); the probes used in the RFLP analyses, D9S33, IFNBJ, and D9S126, have been described previously (32, 35, 36). Sodium bisulfite converts unmethylated cytosines to uracils, and therefore the DNA sequences are completely or partially digested. MSP reactions were performed on bisulfite-converted DNA using the following 5’ primers to discriminate between top-strand unmethylated (M) and methylated (U) CpGs: primer M, 5’-GGG TTG TTT TCG GTT GTT TTTC-3’; and primer U, 5’-GGGG GTG GTT TTGT TTT TCG GTT TTTC-3’. The 3’ anchor (A1) primer (5’-TCT AAT AAC CAA CCA ACC CCT CC-3’) was used in each of the PCR reactions and annealed to a region that did not contain CpG sites. PCR reactions were performed under the following conditions in a total volume of 25 μl: —50 μg bisulfite-converted DNA template, 1X PCR buffer (Boehringer Mannheim, Indianapolis, IN), 0.5 μM of each primer (M + A1 or U + A1), 100 μM deoxynucleotide triphosphates, 1 unit of Taq polymerase. All reactions were hot-started and cycle parameters were: initial denaturation at 94°C for 3 min followed by 32 cycles (M + A1 primers) or 30 cycles (U + A1 primers) at 94°C for 30 s, 68°C for 30 s, and 72°C for 30 s. PCR products were electrophoresed and visualized on ethidium bromide-stained 3% agarose gels.

M-SNuPE. Bisulfite-converted genomic DNA that was used as a template for MSP analysis was also used as a template for PCR amplification for quantitation of DNA methylation using Ms-SNuPE (33). Primers used to generate top strand-specific PCR product for Ms-SNuPE analysis were: primer B, 5’-GTA GGT GGS GAG GAG TTG TAT T3’; and primer A1, 5’-TCT AAT AAC CAA CCA ACC CCT CC-3’. Primer A1 was the same (anchor) primer used in the MSP analysis. PCR reaction mixtures were performed in a total of 25 μl as described previously (33). Amplification conditions were: initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 30 s. Methylation analysis was performed as described previously using a combination of three primers in the Ms-SNuPE reactions, which monitor the methylation status of three CpG sites in the amplified region (33). The sequences of the primers used in this study are as follows: 21-mer, 5’-TTT TTT TTT TGG AAA AGA TAT T3’; 18-mer, 5’-TTT TAG GGG TGG TAT ATT T3’; and 15-mer, 5’-TTT GAG GGA TAG GGT G3’. Ms-SNuPE reactions were carried out in a total volume of 25 μl containing —25 ng of DNA template, 1X PCR reaction buffer (Boehringer Mannheim), 1 μCi [32P]dCTP or [32P]dGTP, 1 μM of each Ms-SNuPE primer, and 1 unit of Taq polymerase. Conditions for the primer extension were: 95°C for 1 min, 50°C for 2 min, and 72°C for 1 min. Primer extension products were electrophoresed on 15% polyacrylamide gels (7 μl urea) under denaturing conditions, dried, and exposed to a PhosphorImager screen (Molecular Dynamics) for quantitative analysis of methylation levels.

Bisulfite Genomic Sequencing. The method of Frommer et al. (35) was used for the bisulfite genomic sequencing of melanoma DNAs. PCR amplification of bisulfite-converted genomic DNA was performed under the exact conditions used to generate PCR products for Ms-SNuPE analysis (primers B + A1, 35 cycles). PCR products were electrophoresed and isolated from 2% agarose gels. Isolated PCR products were then ligated into the pCR II cloning vector and transferred into the INVivo/Excherichia coli strain (Invitrogen, San Diego, CA). Bacterial colonies containing PCR inserts were picked according to blue/white selection, plasmid DNAs were isolated, and individual clones were sequenced using the Sequenase version 2.0 kit (Amersham, Cleveland, OH) according to manufacturer’s instructions.

RT-PCR Analysis of p16 and GAPDH Expression. Total RNA (~2.5 μg) was isolated from cells and reverse transcribed in a 25-μl reaction volume using random hexamers, deoxynucleotide triphosphates (Boehringer Mannheim), and Superscript II reverse transcriptase (Life Technologies, Inc., Palo Alto, CA). Amplification of CDNA was performed using primers specific for p16 or GAPDH. PCR reactions were performed as previously described (21) in 25-μl volumes at 94°C for 3 min, 24 cycles at 94°C for 1 min, 56°C for 30 s, 72°C for 40 s (p16 amplification) and at 94°C for 1 min, 20 cycles at 94°C for 1 min, 58°C for 30 s, and 72°C for 45 s (GAPDH amplification). Primer sequences for p16 and GAPDH cDNA amplification were as described previously: 5’-AGG CTT CCG CGG CTT GCT GTC G3’ (p16 sense); 5’-CCG GCC ATC ATG ACC TGG A3’ (p16 antisense); 5’-CAG CCG ACC CAC ATG G3’ (GAPDH sense); 5’-TGA GCC TGT TGT CAT ACT TCT C3’ (GAPDH antisense; Ref. 21). PCR products were resolved on 2% agarose gels, transferred to Zetaprobe membrane (Bio-Rad, Richmond, CA), and probed with a digoxigenin-labeled internal oligonucleotide.

RESULTS

Experimental Approaches for Detection and Quantitation of p16 5’ Cpg Island Methylation in Melanoma Cell Lines and Primary Tumors. We studied the frequency of p16 5’ Cpg island methylation in sporadic cutaneous melanoma to determine the potential contribution of methylation-associated gene silencing in this cancer type. The efficacy of screening tumors for p16 methylation was
evaluated by using several techniques for methylation analysis including Southern blotting, MSP, Ms-SNuPE, and genomic bisulfite sequencing. Fig. 1 shows the upstream region of p16 that was assayed for methylation in our tumor samples. The location of restriction enzyme sites and the probe used in genomic Southern blot hybridization is indicated. The EcoRI restriction enzyme was used to generate a 4.3-kb fragment followed by digestion with either SacII or SmaI to determine the methylation status at each of these sites. Several CpG sites in the region of putative transcription initiation (Ref. 37; Fig. 1, upward-pointing arrows) were also assayed by MSP and Ms-SNuPE analysis.

Southern Analysis of Melanoma Tumors and Cell Lines Reveals a Low Frequency of p16 Methylation. A total of 30 sporadic melanomas (29 metastases and 1 primary) and 3 congenital nevi were initially screened for p16 methylation by hybridizing Southern blots containing genomic DNAs digested (in parallel) with EcoRI, EcoRI+SacII, and EcoRI+SmaI with a probe specific for the 5' end and promoter region of the p16 gene (Fig. 1). Fig. 2A shows results from this analysis for five representative (and unmethylated) melanoma DNAs (MGH-MEL-02, -04, -13, -14, and QIMR-901) in comparison to three melanoma DNAs (MGH-MEL-06, -12, and -16A) that yielded partial digestions, presumably indicative of methylated p16 alleles. In all cases, digestion with EcoRI alone produced the expected 4.3-kb band, whereas double digestions with either SacII or SmaI resulted in smaller fragments. All but one DNA (MGH-MEL-06) digested with EcoRI+SacII showed a major band at 0.70 kb, indicative of a complete digestion of the EcoRI fragment with SacII. A smaller complete digestion fragment of 0.22 kb was also consistently detected, although this band was more diffuse and of lower intensity (due to its relative size and overlap with the probe; data not shown). In most instances (including digestions of the three congenital nevi DNAs), a minor partial digestion fragment of 0.92 kb was also observed, which presumably indicated a small amount of methylation at the SacII site located at position -163 (relative to the ATG of the first codon) present in the p16 promoter (Fig. 1 and 2A). The relative intensity of the 0.92-kb fragment, however, appeared to vary consistently with DNA loading and was, therefore, assumed to be of no significance in relationship to inhibiting endogenous p16 expression.

Of the 30 melanoma DNAs analyzed, only 3 (MGH-MEL-06, -12, and -16A) did not digest to completion with EcoRI+SacII; a portion of the DNA in these samples appeared resistant to digestion with SacII, as evidenced by the continued presence of the 4.3-kb EcoRI fragment (Fig. 2A, leftward arrows). This finding suggests that one or both of the p16 alleles present in these tumor DNAs may be methylated at the SacII site within the promoter, as well as the SacII site located within exon 1 (at position +56 relative to ATG), of the p16 gene.

Digestions with SmaI yielded similar results, although there was no indication in any of the samples of consistent minor methylation of the SmaI site located at position -276 in the promoter. Two (MGH-MEL-06 and -12) of the three tumors deemed positive for methylation with SacII also exhibited incomplete digestion with SmaI. However, because additional downstream SmaI sites are present within the 4.3-kb EcoRI fragment, it was more difficult to visualize by eye the partial fragments created in the EcoRI+SmaI digestions (Fig. 2A, rightward arrows). For this reason (that was also supported by the melanoma cell line data discussed below), as well as to conserve on amounts of DNA used in this portion of our analyses, 18 of the melanoma DNAs were assayed using only EcoRI and EcoRI+SacII. All of these 18 melanoma DNAs showed no evidence of partial digestion with SacII (data not shown).

Ten melanoma cell line DNAs were also digested with EcoRI, EcoRI+SacII, and EcoRI+SmaI to screen for methylation at the p16 promoter/5' region. A bladder cancer cell line, T24, was included in these analyses as a positive control. The T24 cell line has already been shown to be methylated at p16 and does not express p16 RNA. Results from the tumor cell lines were analogous to those observed in the uncultured melanomas; methylation was readily detected with both SacII and SmaI, although, again, results were more definitive with SacII (Fig. 2B). Overall, two of the 10 melanoma cell lines (SK-MEL-196 and -241) were determined to be methylated at the p16 locus. The findings for SK-MEL-241 are shown in Fig. 2B, although similar results were also observed for SK-MEL-196. Both SacII sites (Fig. 1) were presumably methylated in these two cell lines because the 4.3-kb EcoRI fragment remained intact and completely undigested. The SmaI digestions, again, were partials, and a portion of the DNA in these samples still digested to completion with SmaI+EcoRI (Fig. 2B and data not shown). Overall, evidence for p16 methylation
in the cell lines with both SacII and SmaI was more striking than that observed in the uncultured melanomas, presumably due to the absence of normal contaminating stromal DNA.

**LOH on 9p21 and Mutational Status at the p16 Locus.** Results from previous p16 mutation and 9p21 LOH analyses were available on the majority of the melanoma tumors and cell lines analyzed in this study (10) and, therefore, provided us the opportunity to assess the relationship between methylation, mutation, and loss of p16. It was of primary interest to determine if complete inactivation of the p16 gene could be proposed in the three uncultured tumors (MGH-MEL-06, -12, and -16A) that were positive for methylation. Because the methylation observed affected at most only 50% of the DNA present in each sample, an explanation was needed to account for the completely digested (unmethylated) fraction. Presumably, it was due to either contaminating normal DNAs or the existence of heterogeneity at the p16 locus within the tumors.

Screens with RFLP markers located on 9p21, which were initially performed to determine LOH status, were also useful in judging the amount of normal contaminating DNA present in each of these three samples. The marker IFNβ proved the most valuable because it was located close to the p16 gene (1 Mb telomeric; Ref. 38) and was informative with BanII in all three tumors. Fig. 3 shows the results obtained with this marker. MGH-MEL-06 was clearly reduced at

---

Footnotes:
5 G. J. Walker, J. F. Flores, J. M. Glendening, A. Lin, I. D. C. Markl, and J. W. Fountain. Virtually 100% of melanoma cell lines harbor alterations at the DNA level within CDKN2A, CDKN2B, or one of their downstream targets, manuscript in preparation.
7 The non-deleted DNA present in MGH-MEL-06 and MGH-MEL-12 was judged to be normal contaminating DNA based on the comparison of LOH results obtained on chromosome 9 with those from markers located elsewhere in the genome (data not shown). A relatively consistent level of “background” signal was observed in all instances for deleted alleles, supporting the assumption that the original tumors were grossly contaminated with normal stroma and not composed of different tumor cell populations.
IFNB1, both with BanII (Fig. 3) as well as with MspI (data not shown). The level of normal DNA contamination in this tumor sample, however, was not insignificant; quantitative calculations using PhosphorImager data suggested that about 50% (38–62.5%) of the DNA present in this sample arose from normal contaminating stroma. This level of normal contamination was consistent with the portion of DNA digested to completion with SacII on the methylation blot (Fig. 2A). MGH-MEL-06 was, therefore, assumed to have lost one copy of p16 and retained a second allele that was fully methylated. In our initial assessment of MGH-MEL-12, this tumor was not scored as reduced at IFNB1 or any other locus on 9p21 (10). However, upon reevaluation, we noted a subtle shift in the intensity of the IFNB1 alleles in the tumor relative to the control DNA, suggestive that the higher molecular weight allele had been lost in the tumor (Fig. 3). This same shift in relative allele intensities was also observed with a more telomeric marker, D9S126, but not with a more centromeric marker, D9S533, that resides 3–5 Mb from p16 (data not shown). Assuming this tumor is reduced on 9p21 and grossly contaminated with normal DNA, calculations based on the IFNB1 and D9S533 PhosphorImager results suggested that only 20% (17–23%) of this sample was comprised of DNA from tumor tissue. Again, this amount of normal DNA contamination is consistent with the methylation result observed with SacII (Fig. 2A); MGH-MEL-12 was, therefore, considered to be reduced on 9p21 and fully methylated on its remaining p16 allele. The last tumor, MGH-MEL-16A, showed no evidence of LOH on 9p21 either at the IFNB1 locus (Fig. 3) or any other RFLP marker tested (Ref. 10; data not shown). This tumor DNA could, therefore, be either: (a) more grossly contaminated than MGH-MEL-12; (b) only partially methylated at the p16 locus due to tumor heterogeneity; or (c) incompletely digested with SacII for reasons other than methylation.

Table 1 shows the LOH, mutation, and methylation status for p16 in all of the melanomas, congenital nevi, and melanoma cell lines analyzed in this study. Two additional melanoma cell lines (for a total of 12) that were not assayed by Southern blot are also listed because they were determined to be unmethylated (888) or unmethylated (SK-MEL-37) at the p16 locus by other means (see Table legend). The 888 cell line was also used in further analyses that monitored p16 expression after induction with 5-Aza-CdR (discussed below). A number of tumors and cell lines (n = 10 total) harboring intragenic mutations within the p16 gene were purposefully included in this study to assess the relationship between mutation and methylation at this locus. A priori, we assumed that mutation and methylation would be two exclusive mechanisms for p16 inactivation. This was what we observed in five of the six methylated cases, except the melanoma cell line 888, which retained a methylated p16 allele that had also suffered an 18-bp deletion in exon 1 (E1a). Notably, none of the three methylated tumors carried a mutated p16 allele.

MSP Detects Methylation of the p16 5' CpG Island in Melanoma Tumor Specimens. Nine melanoma tumors and one congenital nevus analyzed previously for methylation of the p16 5' CpG island by Southern blotting (at SacII and Smal sites) were screened in a blinded manner (in an independent laboratory) by MSP. The top strand-specific sites assayed by MSP included a CpG located within the Smal recognition sequence assayed previously in the Southern analyses. Fig. 4 shows results obtained from the MSP analysis on these 10 samples. DNA from the T24 bladder cancer cell line was again used as a positive control for methylation in this assay. This cell line DNA is heavily methylated at the p16 locus as can be deduced by the presence of a band in the methylated-specific primer reaction and absence of a band in the unmethylated-specific primer reaction (Fig. 4). WBC DNA was used as a negative control and was found to be unmethylated by MSP analysis.

Three melanoma tumor DNAs showed detectable levels of partial methylation by MSP (Fig. 4). These same melanoma samples (MGH-MEL-06, -12, and -16A) were those determined previously to be methylated by Southern analysis (Fig. 2). Notably, methylation of the Smal site was more readily detected in this analysis compared to the Southern blot results obtained on the same tumor DNAs. The relative ratio of methylated to unmethylated template, however, remained similar to what had been observed previously; MGH-MEL-16A was the least methylated, followed by MGH-MEL-12, and MGH-MEL-06. The remaining melanomas showed no methylation at the sites examined by MSP as indicated by the absence of bands in the methylated-specific primer lanes and presence of bands only in the unmethylated-specific primer lanes (Fig. 4).

Quantitative Methylation Analysis of Sporadic Melanomas Using Ms-SNuPE. Methylation levels were determined in 18 of the melanoma tumors and 1 of the congenital nevi by Ms-SNuPE (33). This assay allowed for quantitative measurement of the amount of methylation present in each DNA sample at three top-strand CpGs in the promoter region of p16. A multiplex primer extension array was used, and methylation levels at the three monitored CpG sites were averaged to determine the percentage of methylation of the region analyzed. The T24 human bladder cancer cell line DNA remained a positive control and showed an average of 92% methylation, as indicated by the strong intensities of bands in the C (methylated) compared to the T (unmethylated) lanes (Fig. 5). WBC DNA from melanoma patient MGH-MEL-16, as well as congenital nevus DNA from MGH-CN-02, served as negative controls and showed an aver-
This mutation potentially arose due to an unequal crossing-over event between or within a p16 allele(s).
Figure 4. MSP analysis of nine primary melanomas and a congenital nevus. Two CpG sites in the p16 promoter region (including a CpG located within the Smal site at position -276 relative to ATG) were analyzed for methylation. PCR products were generated if CpG sites were methylated (M) or unmethylated (U). T24 DNA was a positive control for methylation and amplified only with the methylation-specific primers. WBC was a negative control for methylation, as indicated by the presence of a product in the U lane but not in the M lane. MGH-MEL-16A, -12, and -06 exhibited partial levels of methylation at the monitored CpG sites as indicated by products in both the M and U lanes. No significant amount of methylation was observed in the remainder of the melanoma or congenital nevus samples, as supported by the presence of robust methylation and amplified only with the methylation-specific primers. WBC was a negative control for methylation, as indicated by the presence of a product in the U lane but not in the M lane. MGH-MEL-16A, -12, and -06 exhibited partial levels of methylation as indicated by the presence of robust products in both the M and U lanes. No significant amount of methylation was observed in the remainder of the melanoma or congenital nevus samples, as supported by the presence of robust products only in the U (unmethylated) lanes.

Figure 5. Bands in the C lanes at CpG dinucleotides indicate the presence of unmethylated cytosines, and black circles indicate methylated cytosines. The level of methylation in MGH-MEL-06 estimated by genomic bisulfite sequencing of multiple plasmid clones. Again, in these two instances, alleles were either densely methylated or largely unmethylated, as had been observed for MGH-MEL-06 (data not shown). The bisulfite sequencing data support our previous findings of methylation at the Smal and SacII sites in these three melanomas and demonstrate the utility of PCR-based assays such as MSP and Ms-SNuPE, which appear to have the same sensitivity (in the case of SacII) or are more sensitive (in the case of Smal) at detecting and quantitating levels of methylation compared to genomic Southern blots.

Genomic Bisulfite Sequencing of the p16 5' CpG Island in Melanoma Tumors. Bisulfite genomic sequencing was used to determine the detailed pattern of p16 methylation in the three cases of melanoma (MGH-MEL-16A, -12, and -06) that were found previously to be methylated by Southern hybridization, MSP, and Ms-SNuPE. The methylation status of 15 CpG sites in the p16 5' CpG island was determined, and results for MGH-MEL-06 are shown in Figure 6. Each row of circles represents a single plasmid molecule that was cloned and sequenced from PCR products generated from amplification of bisulfite-treated DNA. Open circles indicate unmethylated cytosines, and black circles indicate methylated cytosines. The autoradiographs shown are actual examples of the genomic sequencing results obtained on MGH-MEL-06 (Fig. 6). The location of CpG sites is indicated by horizontal bars adjacent to the sequencing gels. Bands in the C lanes at CpG dinucleotides indicate the presence of unmethylated cytosines, whereas bands in the T lanes at CpG sites represent unmethylated cytosines. The level of methylation in MGH-MEL-06 estimated by genomic sequencing of multiple plasmid clones at the Smal site was 50% (11 of 22 CpGs), whereas the overall level of methylation throughout this region was 52% (172 of 330 CpGs) in this sample. This value was slightly higher than the quantitated level of methylation determined by Ms-SNuPE analysis (38%) for three sites within the same region and was also consistent with the results generated in the Southern and MSP analyses.

Individual plasmid molecules were generally either fully methylated or unmethylated at 14 of 15 of the CpG sites analyzed with the exception of plasmid clone 10, which showed methylation of only the 3' CpGs in the region (Fig. 6). The SacII site analyzed by Southern analysis is located within this region, whereas the Smal site is further 5'. Interestingly, there was one CpG just downstream of the Smal site that was found to be unmethylated in the majority of the sequenced plasmids. Findings generated on melanoma tumors MGH-MEL-16A and -12 also indicated low levels of methylation by genomic bisulfite sequencing of multiple plasmid clones. Again, in these two instances, alleles were either densely methylated or largely unmethylated, as had been observed for MGH-MEL-06 (data not shown). The bisulfite sequencing data support our previous findings of methylation at the Smal and SacII sites in these three melanomas and demonstrate the utility of PCR-based assays such as MSP and Ms-SNuPE, which appear to have the same sensitivity (in the case of SacII) or are more sensitive (in the case of Smal) at detecting and quantitating levels of methylation compared to genomic Southern blots.

Treatment of a Melanoma Cell Line with 5-Aza-CdR Is Associated with Demethylation of the p16 5' CpG Island and Induction of Gene Expression. Modulation of p16 expression by treatment of cell cultures with 5-Aza-CdR has been demonstrated previously in a number of cell lines including those derived from lung and head and neck tumors (19, 23, 24). The 888 human melanoma cell line was treated with 5-Aza-CdR to determine if p16 transcriptional activity could also be altered in this type of tumor cell line. Fig. 7A shows the results from Ms-SNuPE analysis of p16 in the 888 human melanoma cell line before and after treatment with 5-Aza-CdR. The 888 human melanoma cell line was used as a negative control for methylation and has been shown to be only minimally methylated throughout the region analyzed (6% average methylation) (21). The 888 melanoma cell line was very heavily methylated (82%) prior to drug treatment, but following exposure to 5-Aza-CdR, methylation levels were reduced to an overall average of 38% at the three monitored sites.

Fig. 7B shows expression levels of p16 in the 888 melanoma cell line assayed by RT-PCR analysis before and after treatment with 5-Aza-CdR. The 888 human melanoma cancer cell line was used here as a positive control for gene expression. The 888 cell line did not show detectable levels of p16 expression prior to treatment with 5-Aza-CdR. Treatment of the 888 cell line with 1 × 10^{-6} M 5-Aza-CdR resulted in the induction of p16 expression as indicated by the presence of a robust product in the (+ Aza) lane. These data, therefore, confirm that demethylation of the p16 5' CpG island by 5-Aza-CdR treatment in melanoma cells is also associated with the induction of p16 expression.
This study represents the first detailed investigation of the frequency of p16 methylation in sporadic cutaneous melanoma. Determining the contribution that methylation-based inactivation of the p16 gene plays in melanoma development is critical because this gene is a melanoma predisposition locus (12—16) and also appears to be a specific target for mutation/deletion in sporadic melanoma (10, 11, 17, 45, 46). However, excluding melanomas with homozygous deletions of the 9p21 region that potentially include other candidate genes in addition to p16, few melanomas (≤5%) have been identified that fulfill Knudson’s “two-hit” hypothesis (47) for dual inactivation (via loss of heterozygosity and intragenic mutation or intragenic mutation only) of both copies of this gene (10, 11, 17). Given that there is no evidence for the dominant-negative mutation of this gene, some other explanation must be used to account for the fact that ≥50% of melanomas with LOH on 9p21 retain a wild-type p16 allele (10, 11, 17). One explanation would be that the second copy of p16 is methylated in these tumors.

The current study was designed to determine the potential role of methylation at the p16 locus in sporadic melanoma and, overall, revealed methylation of the 5′ CpG island in the promoter region of this gene in three uncultured melanomas (MGH-MEL-16A, -12, and -06). The frequency of this event, however, was not high (10%; 3 of
Fig. 6. Genomic bisulfite sequencing results on melanoma MGH-MEL-06. Individual plasmid clones were sequenced following PCR of bisulfite-treated DNA to determine the methylation status of 15 CpG sites in the p16 5′ CpG island. The region analyzed is indicated by the black bar on the gross map provided above the autoradiographs and includes CpG sites monitored by genomic Southern blotting, MSP, and MS-SNuPE. Symbols are the same as those used in Fig. 1. Left, methylation patterns of individual plasmid clones: ○, unmethylated cytosines; ●, methylated cytosines. Examples of sequencing gel results from three plasmid clones are shown. Horizontal bars adjacent to each sequence indicate the location of CpG sites. Bands in the C lanes are indicative of methylated cytosines, whereas bands in the T lanes are indicative of unmethylated cytosines. An asterisk (*) by the sequence generated on clone 22 indicates the presence of a cytosine residue (outside of a CpG site) that was not converted to uracil during the original bisulfite treatment.

30) and could, therefore, not be used to justify the low intragenic mutation rate of p16 observed in sporadic melanoma. In fact, 9–10 of the 30 (≥30%) melanomas analyzed were reduced on 9p21 and retained a wild-type, unmethylated p16 allele. Another 23–30% (7–10/30) were not reduced, not mutated, and not methylated for p16. Of the remaining one-third (10 melanomas), only one-half of these (5 of 30; 17%) showed evidence for inactivation of both copies of p16 either via LOH and mutation (n = 3) or LOH and methylation (n = 2). The others were either: (a) not analyzed for LOH or mutations (n = 3); (b) mutant and not reduced (n = 1); or (c) not reduced and only partially methylated (n = 1). The specific role that methylation plays in inactivating the p16 gene in melanoma is even further reduced (to 8–9%) if one considers that melanomas with homozygous deletions of this gene were purposefully excluded from the current study. These findings contrast dramatically with those from other tumor types (e.g., breast cancer, colon cancer, and bladder cancer), where the potential contribution of methylation-associated gene silencing of p16 has been reported to be between 31 and 67% (20, 21),
and continue to support the model that loss of only one copy (haplo-insufficiency) of p16 may be all that is required during the initial (as well as potentially late) stages of melanoma development (10, 11, 17, 48–50).

The analyses we performed on melanoma cell lines also supported the conclusions drawn from the uncultured tumors. In this instance, only a small number of samples were analyzed because we had determined previously that 33 of our 42 (79%) melanoma cell lines were either homozygous deleted (n = 21) or reduced and mutant for p16 (n = 12; Ref. 10). Of the 12 melanoma cell lines examined, two were found to retain methylated and unmethylated p16 alleles. A third cell line (888) was also identified that retained a methylated, as well as mutant, copy of p16. The frequency of p16 methylation in the melanoma cell lines (2–3 of 42; 5–7%), therefore, compares directly with that observed in the uncultured melanomas and, again, supports that this epigenetic mechanism for inactivation of the p16 gene is uncommon in sporadic melanoma. Notably, in contrast to what we have observed previously for both homozygous deletions and intransic mutations of p16 (10), there appears to be no in vitro selection bias for melanoma cell lines harboring methylated copies of p16. This implies that methylation of p16 arises as an in vivo event during tumor progression and is not an in vitro artifact induced during the process of establishing or propagating a melanoma cell line in culture.

As has been detected in cell lines established from tumors other than melanoma (18–24), we also observed an association between p16 methylation and transcriptional silencing. Specifically, no p16 RNA was detected in the 888 melanoma cell line that retained a methylated p16 allele. Treatment of this cell line with 5-Aza-CdR, a demethylating agent, was also associated with a reduction in the level of p16 methylation and a concordant induction of p16 expression. Methylation of p16 in melanoma cells, therefore, appears to have the same function as that observed in other tumor types in providing an alternative mechanism by which the p16 gene can be inactivated. The assumption that p16 is not expressed in the two uncultured melanoma samples (MGH-MEL-06 and -12) retaining fully methylated copies of this gene appears to be a reasonable one.

Based on the findings presented in this study, proposing a new approach for the treatment of metastatic melanoma that involves indiscriminate administration of chemotherapeutic agents aimed at up-regulating p16 expression from methylated p16 alleles is not warranted. The potential use of these agents, however, on patients whose tumors have been prescreened for methylated p16 alleles is still a viable option. Present studies indicate that reactivation of a dormant (methylated) p16 gene or, alternatively, the re-introduction of a wild-type p16 gene into a tumor cell, can dramatically reduce the ability of these cells to proliferate in vitro, as well as form tumors in nude mice in vivo (30). Thus, there is the potential that the administration of agents such as 5-Aza-CdR might lead to the complete or partial regression of metastatic disease in a melanoma patient whose tumors carry methylated p16 alleles.

Our analyses also provided valuable information regarding the usefulness of different methylation detection assays. Although the choice to initially analyze our tumor DNAs by MSP, Ms-SNuPE, and bisulfite genomic sequencing was to provide confirmation of our Southern blotting results, the comparison of findings obtained from these four different assays allowed us to draw important conclusions regarding the relative ability that each of these methods has to detect DNA methylation at the p16 locus. Because the extent of methylation varied in our three methylated melanoma DNA samples, we were also able to compare each assay quantitatively. Results from the MSP, Ms-SNuPE, and bisulfite genomic sequencing analyses all supported our initial Southern results, although MSP and bisulfite sequencing were more sensitive in detecting methylation at the SmaI site in the p16 promoter than the Southern blots indicated. This was probably due to differential hybridization of the probe to a number of partially digested fragments of DNA that were generated by SmaI. Conversely, when analyzing this region by Southern analysis, SacII appears to be the restriction enzyme of choice because only two SacII sites reside within the 5' end of the p16 gene [one within the promoter and one within exon 1 (E1a)], eliminating any interference from other partial digestion fragments. Interestingly, we did not observe preferential methylation of the SmaI over the SacII site within this region. Methylation of the SacII site at position —276 in the p16 promoter (within the region where transcription initiates; Ref. 37) has been proposed to be a critical event in silencing expression of this gene (19). In this particular study, we observed a high degree of methylation throughout the entire region between and immediately adjacent to this SmaI site and two SacII sites (at positions —163 and +56) in our melanoma tumors and cell lines.

The levels of methylation observed in the three methylated melanoma samples were directly comparable using any one of the four methylation assays and, in two of the three cases, suggested that the tumor DNA (versus the normal contaminating DNA) present in these samples was completely methylated. For example, the extent of methylation detected in MGH-MEL-12 and MGH-MEL-06 by Ms-SNuPE was observed to be between 22 and 38%. These values corresponded directly to the amount of actual tumor DNA (17–23% for MGH-MEL-12 and 38–63% for MGH-MEL-06) determined to be present in each of these samples. The sensitivity of each assay was most chal...
lenged by a third melanoma sample, MGH-MEL-16A, which was only partially methylated at the p16 locus. All four assays, however, were still successful in detecting methylation in this sample that was estimated (by Ms-SNuPE) to involve only 12% of the total DNA. Anything under this amount of methylation (from 2–8%) was considered insignificant because it was within the range of methylation detected in normal (nontumorigenic) controls.

Overall, these results suggest that the two novel PCR-based assays, MSP and Ms-SNuPE (32, 33), have similar sensitivities as Southern blots and bisulfite genomic sequencing for detecting methylation at the p16 locus. Both assays were successful in accurately detecting levels of methylation (22%), and the Ms-SNuPE technique was useful for quantitatively distinguishing between a DNA sample that was heavily methylated (≥38%) versus one that showed intermediate levels of methylation (22%) versus one that was minimally methylated (12%). The comparable results from all four assays also indicated that MSP and Ms-SNuPE can be used to detect methylation in tumor samples that are grossly contaminated with (≥77%) normal stroma or in which there is heterogeneity within the tumor. In both instances, bisulfite modification of DNA has made it possible to use these PCR-based methods for the accurate detection of methylated DNA molecules present in tumor samples. The ability to screen for DNA methylation using PCR-based approaches has several obvious advantages, including efficiency, ease, and a requirement for only a small amount of starting material. Ultimately, adaptation of these methods for high-throughput screening of tumor samples may prove to be of global use in establishing the presence or pattern of aberrant DNA methylation at multiple loci in a variety of different cancers.

ACKNOWLEDGMENTS

We thank Dr. Jefferey Weber and Catherine Kunyoshi for supplying the A375, 526, and 888 melanoma cell line DNAs, Dr. Raymond Barnhill for eight of the melanoma tumor DNAs, and Drs. Sally Lynch, Alan Houghton, and Anthony Albino for the remaining melanoma cell line DNAs used in these experiments. We are also grateful to Amy Lin for technical support and Dr. Toshiro Hayashida for the p16 promoter fragment used as a probe in these studies.

REFERENCES


Low Frequency of $p16/CDKN2A$ Methylation in Sporadic Melanoma: Comparative Approaches for Methylation Analysis of Primary Tumors

Mark L. Gonzalgo, Christina M. Bender, Edward H. You, et al.

*Cancer Res* 1997;57:5336-5347.

Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/57/23/5336

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.