Epigenetic Silencing of Novel Tumor Suppressors in Malignant Melanoma

Viswanathan Muthusamy,1 Sekhar Duraisamy,2 C. Matthew Bradbury,1 Cara Hobbs,1 David P. Curley,1 Betsy Nelson,1 and Marcus Bosenberg1

1Department of Pathology, University of Vermont College of Medicine, Burlington, Vermont and 2Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts

Abstract

Malignant melanoma is a common and frequently lethal disease. Current therapeutic interventions have little effect on survival, emphasizing the need for a better understanding of the genetic, epigenetic, and phenotypic changes in melanoma formation and progression. We identified 17 genes that were not previously known to be silenced by methylation in melanoma using a microarray-based screen following treatment of melanoma cell lines with the DNA methylation inhibitor 5-Aza-2’-deoxycytidine. Eight of these genes have not been previously shown to undergo DNA methylation in any form of cancer. Three of the genes, QPCT, CYP1B1, and LXX, are densely methylated in >95% of uncultured melanoma tumor samples. Reexpression of either of two of the silenced genes, HOXB13 and SYK, resulted in reduced colony formation in vitro and diminished tumor formation in vivo, indicating that these genes function as tumor suppressors in melanoma. (Cancer Res 2006; 66(23): 11187-93)

Introduction

Melanoma is the most lethal form of skin cancer with an expected 62,190 new cases and 7,910 deaths in the United States in 2006.3 Several genetic changes associated with melanoma have been identified (1). The CDKN2A locus at 9p21 that encodes the p16INK4A and p14ARF tumor suppressor genes is mutated in ~20% of familial melanoma and is inactivated in the majority of spontaneous melanomas (2, 3). Activation of mitogen-activated protein kinase signaling also seems to occur in the vast majority of melanomas, either by activating mutations in the BRAF serine/threonine kinase (~70% of melanomas) or by mutation of NRAS (~20% of melanomas; refs. 4, 5). BRAF is also mutated in ~70% of benign melanocytic nevi, suggesting that additional genetic or epigenetic hits are needed for progression to malignancy (6). Widespread chromosomal instability is another characteristic feature of melanoma, with frequent losses of chromosomal regions 6q, 8p, 9p, 10, 13, and 21q and gains of 6p, 7, 8q, 11q13, 17q, and 20q (7).

Epigenetic changes may also result in inactivation of tumor suppressors and progression to malignancy (8, 9). DNA methylation is one form of epigenetic change and involves the covalent addition of a methyl group to cytosine residues in CpG dinucleotides by DNA methyltransferases. CpG-rich sequences (CpG islands) are infrequent in the genome but are associated with the promoter regions of nearly half of all known human genes (10). Examination of familial cancer genes has revealed that tumor-specific hypermethylation may act as a second hit by preferentially targeting the wild-type allele of tumor suppressors, whereas the promoter region of the mutant allele is not affected by methylation (11). Inhibition of de novo methylation by Dnmt1 down-regulation has been shown to reduce tumor formation in several settings (12-14). Decreased Dnmt1 function completely suppressed polypl formation in Apcmin+ mice, showing that aberrant hypermethylation events are necessary to form and maintain tumors (12, 13, 15). However, in other cases, genomic hypomethylation induced by down-regulation of Dnmt1 increased the rate of specific malignancies, including lymphoma (16).

Epigenetic inactivation of individual tumor suppressors by DNA methylation has also been shown in malignant melanoma (17-22). A high incidence of methylation in uncultured melanoma tissue samples has been reported for RARB (70%), RASSFIA (55%), PYCARD (50%), MGMT (34%), DAPK (19%), and APC (19%), which may play tumor suppressor roles in several tumor settings (summarized in Supplementary Table S1; refs. 19-22). Other tumor suppressor genes confirmed to be methylated in melanoma, albeit at a lower incidence, are the familial melanoma gene CDKN2a (10%) and in CDKN1B (9%) and PRDX2 (8%; refs. 17, 18, 23).

A useful attribute of epigenetic silencing is that it is amenable to reversal by methylation inhibitors. We have exploited this property in a microarray-based assay to screen for genes that are reexpressed following treatment of melanoma cells with 5-Aza-2’-deoxycytidine (5AzadC). We show DNA methylation and silencing of 17 genes in melanoma cell lines and in uncultured melanoma tumor samples. DNA methylation has not been previously shown in eight of these genes in any form of cancer. In addition, we show tumor suppressor properties in melanoma for two of the genes, HOXB13 and SYK.

Materials and Methods

Tissue specimens, primary cells, and cell lines. Melanoma tissue samples were collected in accord with Institutional Review Board–approved protocols at the Memorial Sloan-Kettering Cancer Center (New York, NY) and Dana-Farber Cancer Institute (Boston, MA). All the cell lines used in the study (MelJuSo, UACC 903, C8161, Neo6/C8161, WM1205 Lu, WM35, Roth, Carney, and WM455) were propagated in DMEM–F12 (Invitrogen) supplemented with 5% fetal bovine serum and nonessential amino acids (Invitrogen). Primary cultured human foreskin melanocytes were grown in Medium 254 supplemented with human melanocyte growth serum (Cascade Biologics). Stable MelJuSo cell lines expressing HOXB13 and SYK were generated by reverse transcription-PCR (RT-PCR) amplification of...
full-length coding sequence from primary melanocytes and subcloning into the pTRE expression vector (Clontech). The inserts were sequenced to ensure an absence of introduced mutations. Stable transfectants were produced by transfection with LipofectAMINE (Invitrogen) and selected by growth in medium containing G418 (800 μg/mL; Invitrogen). Colonies were ring cloned, expanded, and analyzed for transgene expression using quantitative RT-PCR.

**5AzadC treatment and microarray analysis.** Cells were grown to 50% confluence in 100-mm culture plates and treated with 5 μmol/L 5AzadC (Sigma) dissolved in growth medium. Fresh 5AzadC medium was added every 24 hours until the end of the assay (96 hours). RNA and DNA were isolated from a batch of 5AzadC-treated cells every 24 hours starting with 0-hour controls. RNA was isolated following 0 and 48 hours of 5AzadC treatment of six melanoma cell lines (Me1Su0, UACC 903, C8161, Neo6, C8161, WM1205, and WM35) and used for the reexpression microarray analysis. Total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. Preparation of double-stranded cDNA and *in vitro* transcription was as per manufacturer’s recommendations. Biotinylated cRNA obtained from *in vitro* transcription was fragmented and hybridized on Human Genome U133A (HG-U133A) array as per manufacturer’s recommendations (Affymetrix). Microarray Suite 5.0 (MAS 5.0; Affymetrix) software was used to analyze the HG-U133A arrays and to determine the statistical significance of a particular probe set. Core set signal calls with Ps >0.01 were not further evaluated. All the arrays were normalized to a target signal intensity value of 500. Baseline analyses were done using 0-hour treatment array signals as a baseline for the respective 48-hour treatment. The data were further processed using customized programs written for conditional formatting analyses to select for genes whose expression on 5AzadC treatment was significantly altered in more than one cell line. These genes were examined for presence of CpG islands in their promoter regions using the National Center for Biotechnology Information MapViewer and the European Molecular Biology Laboratory CpGPlot program.4 Criteria used to identify prospective methylated genes included (a) up-regulation of expression (>4-fold) on 5AzadC treatment in at least one melanoma cell line, (b) significant expression (MAS 5.0 score of present) in cultured melanocytes but no significant changes in expression (<2-fold) of the gene on 5AzadC treatment (in melanocytes), (c) down-regulation of expression of the gene in untreated melanoma cell lines compared with primary melanocytes (>4-fold down-regulation in at least two melanoma cell lines), and (d) presence of a CpG island in the promoter region.

**Bisulfite sequencing and quantitative PCR.** DNA was isolated from cells, and genes using standard phenol-chloroform extraction. Bisulfite modification was done as described previously (24) on DNA from melanocytes, melanoma cell lines before and after 48 hours of treatment with 5AzadC, and DNA from melanoma tissues. PCRs were carried out using 50 ng of bisulfite-modified DNA in a 30 μL volume with 0.2 μmol/L primer concentration (primer sequences and detailed PCR conditions are provided in the Supplementary Table S2). PCR products were purified using the QIAquick Gel Extraction kit (Qiagen) and directly sequenced on the ABI 3100-Avant Automated DNA Sequencer. Particular CpG sites were scored as positive if the C:T peak ratio exceeded 1.5 (at least 25% methylation). Real-time quantitative PCR was done for validation of microarray expression data of selected candidate genes by using 2.5 μL of 100-fold diluted cDNA template and 0.2 μmol/L gene-specific primers in a 25 μL PCR using JumpStart SYBR Green kit (Sigma) according to the manufacturer's instructions in an ABI 7700. The reactions were done in duplicate. Ct values obtained were normalized to glyceraldehyde-3-phosphate dehydrogenase levels, and quantification was done using the comparative Ct method.

**Western blotting.** Western blotting experiments were done by separation of 15 μg of cell lysate per sample on SDS-PAGE, transfer to Immobilon-P membranes (Millipore), blocking with 0.1 mol/L PBS containing 0.2% Tween 20 and 5% nonfat milk, and incubation with antisera to HOXB13 (F-9), SYK (4D10; both from Santa Cruz Biotechnology, Santa Cruz, CA), or actin (AC-40; Sigma). Following washes and incubation...

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4 http://www.ebi.ac.uk/emboss/cpgplot/.

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Figure 1. Expression changes induced by 5AzadC treatment in melanoma cell lines. A, changes in expression of selected candidate genes on 48-hour 5AzadC treatment validated by quantitative RT-PCR analysis. B, expression of the genes in untreated melanoma cell lines relative to primary melanocytes by quantitative RT-PCR. C, microarray profile data of genes showing significant expression changes on 48-hour 5AzadC treatment. Genes were ordered by median expression change relative to corresponding untreated cells.
with peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences), Enhanced Chemiluminescence Advance Detection System (Amersham Biosciences) was used for chemiluminescent detection.

In vitro proliferation assays and tumor formation in nude mice. For in vitro growth curve assays, three replicates of 10,000 cells each of the vector and HOXB13- or SYK-transfected cells were seeded in six-well cell culture plates for each time point. The cells were trypsinized, diluted, and counted on a flow cytometer (Beckman Coulter) at 24-hour intervals starting at 0 hour and ending at 120 hours. Colony formation assays were done by plating three replicates each of 1,000, 500, or 250 cells of the vector and HOXB13- or SYK-transfected cells in six-well cell culture plates. Colonies were allowed to form for 2 weeks and stained with 0.005% crystal violet. For xenografting experiments, 0.5 × 10^6 cells of the vector or HOXB13- or SYK-transfected cells were injected s.c. into flank skin of nude mice. Four injections were carried out per condition. Tumor dimensions were measured once every week, and the mice were sacrificed at 10 weeks after injection when end point measurements of tumor weights were obtained. Statistical significance of the end point data of the in vitro and in vivo assays was evaluated using the Student’s paired t test.

Results
Identification of candidate genes silenced by DNA methylation in melanoma. To determine DNA methylation-related expression changes, we treated six human melanoma cell lines with 5AzadC and compared expression changes at 48 hours after treatment relative to untreated melanoma cell lines. Expression of 150 genes was increased by >4-fold on 5AzadC treatment in at least one of the six melanoma cell lines. This represents a small fraction (0.8%) of the 18,400 transcripts represented on the microarray. Eighty percent (120 of 150) of the genes with altered expression were associated with promoter region CpG islands, suggesting that these genes might be directly methylated in the melanoma cell lines (Fig. 1). We selected a subset of 25 candidate genes for further validation by bisulfite sequencing using the additional criterion of >4-fold down-regulation of expression of the gene in at least two untreated melanoma cell lines compared with primary melanocytes. We found that 68% (17 of 25) of these genes were densely methylated in the promoter region in at least 1 of 9 melanoma cell lines or 20 melanoma tumor tissue samples (Fig. 2A and B). No DNA methylation was detected in primary human melanocytes at any of the loci. DNA methylation of specific genes occurred at similar frequencies in cell lines and tumor tissues and was present both in melanoma cell lines and melanoma tissue samples for all genes tested (Fig. 2C). Several of the melanoma cell lines (7 of 9) and melanoma tumor specimens (7 of 20) exhibited DNA methylation of >50% of the genes tested. The methylation status of each potential CpG methylation site for all 17 genes in all melanoma cell lines, primary melanocytes, and melanoma tumor samples is depicted in Supplementary Fig. S3.

Identification of methylation-targeted tumor suppressor candidate genes in melanoma. A very high incidence of promoter methylation (>75%) was observed in CYP1B1, QPCT, LNX, COLIA2, and GDF15, and an incidence of 50% to 60% was observed in PCSK1, BST2, and DNAJC15 in melanoma tumor samples (Fig. 2B and C; Supplementary Table S1). The genes CDKNIC, MAP2P2, SYK, HOXB13, PTGS2, and WDFC1 were methylated in 20% to 35% and CDH8, DAL1, and LRRC1 in 5% to 10% melanoma tumor tissues. Using quantitative RT-PCR, we confirmed that promoter methylation of several of the above candidate genes was accompanied by reduced expression relative to melanocytes and that this loss of expression could be reversed in the majority of cases by 5AzadC treatment (Figs. 1A and C and 3). Demethylation was also induced following 5AzadC treatment (Supplementary Fig. S4). We chose to evaluate two candidate genes SYK and HOXB13 further because of their loss of expression in most melanoma cell lines relative to melanocytes (Fig. 1B).

Tumor suppressor properties of SYK in malignant melanoma. We found that SYK mRNA expression was markedly reduced in all nine melanoma cell lines compared with primary cultured human melanocytes (Fig. 4A), and 5AzadC treatment resulted in >2-fold up-regulation of SYK in a subset (four of eight) of cell lines with SYK methylation (Fig. 3B). Immunoblotting showed complete absence of protein expression in all but one cell line (Fig. 4B). SYK mRNA expression was reduced >4-fold in 77% of the primary melanoma tissue samples (10 of 13; Fig. 4C). Promoter region methylation of SYK was detected in 89% of the melanoma cell lines and 30% of primary tumors (Fig. 24-C). To examine if SYK has a tumor suppressor role in melanoma, we analyzed phenotypic changes resulting from stable reexpression of SYK in a SYK-negative melanoma cell line (MeljuSo). Expression of SYK at comparable levels with those seen in primary melanocytes (Supplementary Fig. S5) resulted in reduced cell proliferation (Fig. 6B) and markedly reduced colony formation in vitro (Fig. 6B). In s.c. xenografting experiments with nude mice, the average tumor...
size was reduced by >8-fold in SYK-expressing clones compared with vector controls (Fig. 6B).

**Tumor suppressor properties of HOXB13 in malignant melanoma.** We found that HOXB13 mRNA expression was markedly reduced (>16-fold) in all the melanoma cell lines (Fig. 5A); however, one of the cell lines had retained protein expression (Fig. 5B), which may indicate increased protein stability in that cell line. In the melanoma tumor samples, we observed >4-fold reduction of HOXB13 mRNA compared with melanocytes in >60% (8 of 13) of the samples (Fig. 5C). Promoter region methylation was detected in 33% of the melanoma cell lines (Fig. 2A) and 20% of the tumor samples (Fig. 2B) tested. Reexpression of HOXB13 occurred on treatment with 5AzadC in two of three cell lines with promoter region methylation (Fig. 3A). We stably expressed HOXB13 in a HOXB13-deficient cell line (MelJuSo) at similar levels to that seen in primary melanocytes (Supplementary Fig. S5) to characterize the possible tumor suppressor function of HOXB13 in melanoma cells. Cell proliferation and colony formation were reduced in HOXB13-transfected clones compared with vector controls (Fig. 6A). HOXB13-transfected MelJuSo lines exhibited >4-fold reduction in tumor size relative to vector controls in xenografting experiments in immunodeficient mice (Fig. 6A and C). These results indicate that HOXB13 is a tumor suppressor in melanoma and is targeted for silencing by promoter hypermethylation.

**Discussion**

Several genes have been analyzed for promoter methylation in melanoma using candidate gene-based approaches (17–22). We have used a microarray-based strategy to identify 17 genes that are not previously known to be silenced by DNA methylation in malignant melanoma. Eight of these genes have not been previously shown to undergo DNA methylation in any form of cancer. Several of these genes are candidate tumor suppressors in melanoma. Reexpression of either of two of the silenced genes, HOXB13 and SYK, resulted in reduced colony formation in vitro and diminished tumor formation in vivo, indicating that these genes function as tumor suppressors in melanoma.

**QPCT** was methylated in all melanomas and encodes a glutaminyl cyclase that converts precursor glutaminyl peptides to their bioactive pyroglutaminyl peptide forms (25, 26). **LXN** was methylated in 95% of melanomas and encodes a global inhibitor of mammalian carboxypeptidases and may function to limit prostate tumor aggressiveness by inhibiting carboxypeptidase 4 (27, 28). **COL1A2**, which was found to be methylated in 80% of melanomas, has been found to be frequently hypermethylated in several human malignancies, including breast cancer, hepatocellular carcinomas, and colorectal cancer (29). Expression of COL1A2 in a tumorigenic cell line led to increased adhesion, slower growth, and reduced colony formation in soft agar, features that are suggestive of a tumor-suppressive role for COL1A2 (29).
Interestingly, two genes involved in the metabolic modification of chemotherapeutic agents were identified with the microarray screen: \textit{CYP1B1}, which was universally methylated, and \textit{DNAJC15}, which was methylated in 50% of melanomas. \textit{CYP1B1} is a member of the cytochrome \textit{P}450 family of monooxygenases and has a wide range of substrates, including estrogen, androgens, and chemotherapeutic drugs (30). Methylation of \textit{CYP1B1} is associated with a poor prognosis in breast cancer (31). \textit{DNAJC15} (also known as \textit{DNAJD1} and \textit{MCJ}) is inactivated by promoter hypermethylation in ovarian carcinoma, pediatric brain tumors, and Wilm’s tumor (32–34). Loss of \textit{DNAJC15} confers resistance to various chemotherapeutic agents used in the treatment of ovarian cancers (32). Thus, inactivation of genes involved in metabolic activation of chemotherapeutic drugs may contribute to the drug resistance phenotype commonly observed in malignant melanoma.

The \textit{SYK} cytoplasmic tyrosine kinase plays a role in coupling activated immune receptors to downstream signaling effectors (35). It is expressed in normal breast epithelial tissue and has tumor suppressor properties in breast cancer cells and may affect mitotic progression (36, 37). Promoter hypermethylation of \textit{SYK} occurs frequently in breast tumors and cell lines and is associated with loss of expression that could be restored on treatment with 5AzadC (38). Similar findings confirming the role of \textit{SYK} as a tumor suppressor in melanoma have recently been described (39). We now show that promoter hypermethylation is one of the mechanisms that results in loss of \textit{SYK} expression in melanoma.

\textit{HOXB13} is a member of the highly conserved HOX transcription factors that regulate differentiation and pattern formation during embryogenesis (40). \textit{HOXB13} has been found to negatively regulate wound healing possibly by down-regulating hyaluronic acid

![Figure 4. SYK expression in melanoma cell lines and melanoma tumor samples. A, quantitative RT-PCR analysis of SYK mRNA expression in melanoma cell lines compared with primary human melanocytes. B, Western blot of SYK expression in melanoma cell lines. C, quantitative RT-PCR analysis of SYK mRNA expression in tumor samples compared with melanocytes.](image)

![Figure 5. HOXB13 expression in melanoma cell lines and melanoma tumor samples. A, quantitative RT-PCR analysis of HOXB13 mRNA expression in melanoma cell lines compared with primary human melanocytes. B, Western blot of HOXB13 expression in melanoma cell lines. C, quantitative RT-PCR analysis of HOXB13 mRNA expression in tumor samples compared with melanocytes.](image)
HOXB13 was found to be down-regulated in prostate and colorectal cancer cells, where it has an antiproliferative role (41, 42). More recently, HOXB13 was found to be epigenetically inactivated in a subset of renal cell carcinomas and had growth-inhibitory effects in vitro (45). We have shown that HOXB13 has tumor-suppressive properties in melanoma and is frequently inactivated by promoter region hypermethylation.

The high rates of methylation of CYP1B1 (100%), QPCT (100%), and LXN (95%) in uncultured metastatic melanoma tumor samples suggest that these loci would be ideal markers for a variety of melanoma clinical trials. In particular, QPCT could be used as a marker for successful induction of demethylation (as per Supplementary Fig. S4) in melanoma patients being treated with demethylating agents. Alternatively, methylation-specific PCR assays for CYP1B1, QPCT, and LXN on blood or serum of melanoma patients could be used as possible staging markers as has recently been described using the lower frequency markers RARB (70%) and RASSF1A (55%; ref. 46).

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