Silencing of *Peroxiredoxin 2* and Aberrant Methylation of 33 CpG Islands in Putative Promoter Regions in Human Malignant Melanomas

Junichi Furuta,[1,2] Yoshimasa Nobeyama,[1] Yoshihiro Umebayashi,[2] Fujio Otsuka,[2] Kanako Kikuchi,[3] and Toshikazu Ushijima[1]

[1]Carcinogenesis Division, National Cancer Center Research Institute, Tsukiji, Chuo-ku, Tokyo, Japan; [2]Department of Dermatology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ten-noudai, Tsukuba, Ibaraki, Japan; and [3]Department of Dermatology, Faculty of Medicine, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan

Abstract

Aberrant methylation of promoter CpG islands (CGI) is involved in silencing of tumor suppressor genes and is also a potential cancer biomarker. Here, to identify CGIs aberrantly methylated in human melanomas, we did a genome-wide search using methylation-sensitive representational difference analysis. CGIs in putative promoter regions of 34 genes (*ABHD9, BARHL1, CLIC5, CNNM1, COL2A1, CPT1C, DDIT4L, DERL3, DHRS3, DPYS, EFEMP2, FAM62C, FAM78A, FLJ33790, GBX2, GPR10, GPRASP1, HOXA9, HOXD11, HOXD12, HOXD13, p14ARF, PAX6, PRDX2, PTPRG, RASD1, RAX, REC8L1, SLC27A3, TGFB2, TLX2, TMEM22, TMEM30B*, and *UNC5C*) were found to be methylated in at least 1 of 13 melanoma cell lines but not in two cultured normal melanocytes. Among these genes, *Peroxiredoxin 2* (*PRDX2*) was expressed in normal melanocytes, and its expression was lost in melanomas with methylation. The loss of expression was restored by treatment of melanomas with a demethylating agent 5-aza-2′-deoxycytidine. In surgical melanoma specimens, methylation of *PRDX2* was detected in 3 of 36 (8%). Furthermore, immunohistochemical analysis of PRDX2 showed that disappearance of immunoreactivity tends to associate with its methylation. PRDX2 was recently reported to be a negative regulator of platelet-derived growth factor signaling, and its silencing was suggested to be involved in melanomas. On the other hand, 12 CGIs were methylated in ≥9 of the 13 melanoma cell lines and are considered as candidate melanoma biomarkers. (Cancer Res 2006; 66(12): 6080-6)

Introduction

Methylation of promoter CpG islands (CGI) leads to transcriptional silencing of their downstream genes (1, 2). In cancers, tumor suppressor genes are inactivated by methylation of their promoter CGIs, along with mutations and loss of heterozygosity. In addition, genes other than tumor suppressor genes are methylated in cancers (3). If the methylation is specific to cancer cells, it can be used as a biomarker to detect cancer cells or cancer-derived DNA, taking advantage of the high sensitivity of methods to detect aberrant methylation (4, 5). This strategy is reported to be successful in various bodily fluids, biopsy materials, lymph nodes obtained at surgery, and serum.

Malignant melanoma is one of the major causes of cancer deaths, and its incidence is increasing especially in Western countries (6). In melanomagenesis, it was initially expected that aberrant DNA methylation would be rarely involved because UV irradiation is deeply involved in melanomas and causes mutations. However, unexpectedly, silencing of various tumor suppressor genes, such as *RARB, RASSF1A*, and *APC*, has been thus far observed in melanomas (7–9), and involvement of gene silencing in melanomagenesis was suggested. Because analysis of methylation on known genes has limitations, a genome-wide screening for CGIs methylated in melanomas is awaited. CGIs identified to be methylated by genome-wide screenings are considered to offer a source for novel tumor suppressor genes and biomarkers.

In this study, we made a genome-wide screening for CGIs aberrantly methylated in melanomas using methylation-sensitive representational difference analysis (MS-RDA; refs. 10–12). This method prepares a library of unmethylated, CpG-rich regions of the genome, which covers unique CGIs and eliminates repetitive sequences. By subtractive hybridization of two libraries, methylated CGIs, which are missing in one library but not in the other, can be identified.

Materials and Methods

Cell lines, surgical specimens, and DNA/RNA extraction. Two cultured neonatal normal epidermal melanocytes (HEM1 and HEM2) were purchased from Cascade Biologics (Portland, OR). MeWo, VMRC-MELG, A2058, C32TG, and GAK were obtained from the Health Science Research Resources Bank (Sennan, Japan); G361, SK-MEL-28, and HMV-I were obtained from the Cell Resource Center for Biomedical Research, Institute of Development (Sendai, Japan); COLO 679 and MMAc were obtained from RIKEN BioResource Center (Tsukuba, Japan); and WM-266-4 and WM-115 were obtained from the American Type Culture Collection (Rockville, MD). TK-Mel-1 was established as reported (7).

Thirty-nine surgical melanoma specimens, 21 from primary sites and 18 from metastatic sites, were obtained from 38 patients in stage III or IV by American Joint Committee on Cancer undergoing tumor resections at Tsukuba University Hospital and The University of Tokyo Hospital with informed consents. Specimens 2 and 28 were obtained from a primary site and a metastatic lymph node, respectively, of the same patient. Eight specimens were fresh frozen, and 31 were fixed in formalin and embedded in paraffin. Five lymph nodes specimens were obtained from five nonmelanoma skin cancer cases (three Paget's disease, one basal cell carcinoma, and one squamous cell carcinoma). These specimens were pathologically negative for tumor cells.

From cell lines and fresh-frozen specimens, DNA was extracted by the standard phenol/chloroform procedure, and total RNA was isolated using

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

J. Furuta and Y. Nobeyama are recipients of Research Resident Fellowships from the Foundation for Promotion of Cancer Research.

Requests for reprints: Toshikazu Ushijima, Carcinogenesis Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo, Japan. Phone: 81-3-3542-2511; E-mail: tushijim@ncc.go.jp.

doi:10.1158/0008-5472.CAN-06-0157

ISOGEN (Nippon Gene, Tokyo, Japan). From paraffin-embedded specimens, melanoma tissue was dissected from 50-μm-thick tissue sections by a fine needle, deparaffinized, and incubated in lysis buffer [50 mmol/L Tris-HCl (pH 8.5), 1 mmol/L EDTA, 0.5% Tween 20, 200 mg/mL of proteinase K] at 55°C for 3 days with fresh proteinase K every 24 hours. DNA was purified by phenol/chloroform procedures. Excessive melanin was cleaned up by the cetyltrimethylammonium bromide-urea method (13). Total RNA of the brain and testes was purchased from Ambion (Austin, TX).

MS-RDA. For MS-RDA, an R adaptor was ligated to 1 μg of genomic DNA digested with *Hpa*II, *Sac*II, or *Nar*I (New England Biolabs, Beverly, MA), and the ligation product was amplified by 25 cycles of PCR with R oligonucleotide in the presence of 1 mol/L betaine (Sigma, St. Louis, MO). PCR products (amplicon) of both tester and driver were restricted with the enzyme initially used. A J adaptor was ligated only to the tester amplicon, and 200 ng of it was mixed with 40 μg of the driver amplicon. The DNA mixture underwent heat denaturation and reannealing (competitive hybridization), and double-stranded DNA with the J adaptor on both ends was selectively amplified with a J oligonucleotide (selective amplification). To perform the second cycle of competitive hybridization and selective amplification, the J adaptor was switched to a new N adaptor, and 40 ng of the ligation product was mixed with 40 μg of the driver amplicon. The product was cloned into the pGEM-T Easy Vector (Promega, Madison, WI), and 96 clones were sequenced. Chromosomal positions and relative locations to CGIs that met the Takai and Jones criteria (14) were analyzed at the National Center for Biotechnology Information web site. When at least one end of a clone was derived from a CGI, the clone was regarded as "flanked by a CGI."

Methylation analysis. Sodium bisulfite modification was done as reported (15). Genomic DNA (500 ng) restricted with *Bam*HI (New England Biolabs) was denatured in 0.3 mol/L NaOH. In 3.1 mol/L $NaHSO_3$ (pH 5) and 0.6 mmol/L hydroquinone, DNA underwent 15 cycles of denaturation at 95°C for 30 seconds and incubation at 50°C for 15 minutes. The product was desalted with the Wizard DNA cleanup system (Promega), and desulfonated in 0.6 N NaOH. The sample was ethanol precipitated and dissolved in 20 μL of TE buffer.

Table 1. List of genes whose promoter CpG islands were methylated in melanoma cell lines

No.	Gene			Chromosomal location	Accession no.	Map start position	Methylation in cell lines
	Symbol	Description	Accession no.				
1	*ABHD9*	Abhydrolase domain containing 9	NM_024794	19p13.12	AC004257	42,000*	13/13
2	*BARHL1*	BarH-like 1	NM_020064	9q34	AL354735	154,000	2/13
3	*CLIC5*	Chloride intracellular channel 5	NM_016929	6p21.1	AL050336	58,000*	3/13
4	*CNNM1*	Cyclin M1	NM_020348	10q24.2	AL391684	15,000	10/13
5	*COL2A1*	Collagen, type II, α 1	NM_001844	12q13.11	AC004801	134,000	12/13
6	*CPT1C*	Carnitine palmitoyltransferase 1C	NM_152359	19q13.33	AC011495	115,000	12/13
7	*DDIT4L*	DNA damage-inducible transcript 4-like	NM_145244	4q24	AP001961	19,000	10/13
8	*DERL3*	Der1-like domain family, member 3	NM_198440	22q11.23	AP000350	35,000*	3/13
9	*DHRS3*	Dehydrogenase/reductase member 3	NM_004753	1p36.1	AL513016	5,000*	8/13
10	*DPYS*	Dihydropyrimidinase	NM_001385	8q22	AP002847	113,000*	6/13
11	*EFEMP2*	Epidermal growth factor–containing fibulin-like extracellular matrix protein 2	NM_016938	11q13	AC009470	82,000*	4/13
12	*FAM62C*	Family with sequence similarity 62, member C	NM_031913	3q22.3	AC022497	87,000	3/13
13	*FAM78A*	Family with sequence similarity 78, member A	NM_033387	9q34	AL157938	188,000*	1/13
14	*FLJ33790*	Hypothetical protein FLJ33790	NM_173583	11q13.4	AP000744	122,000*	8/13
15	*GBX2*	Gastrulation brain homeobox 2	NM_001485	2q37	AC079135	38,000*	2/13
16	*GPR10*	G-protein–coupled receptor 10	NM_004248	10q26.13	AL356865	81,000*	6/13
17	*GPRASP1*	G-protein–coupled receptor-associated sorting protein 1	NM_014710	Xq22.1	AL035427	89,000	2/13
18	*HOXA9*	Homeobox A9	NM_152739	7p15	AC004080	40,000*	11/13
19	*HOXD11*	Homeobox D11	NM_021192	2q31.1	AC009336	90,000*	3/13
20	*HOXD12*	Homeobox D12	NM_021193	2q31.1	AC009336	81,000	12/13
21	*HOXD13*	Homeobox D13	NM_000523	2q31.1	AC009336	74,000	4/13
22	*p14ARF*	Cyclin-dependent kinase inhibitor 2A, variant 4	NM_058195	9p21	AL449423	87,000*	2/13
23	*PAX6*	Paired box gene 6	NM_000280	11p13	Z95332	11,000	12/13
24	*PRDX2*	Peroxiredoxin 2	NM_005809	19p13.2	AC020934	39,000*	4/13
25	*PTPRG*	Protein tyrosine phosphatase, receptor type, G	NM_002841	3p14	AC103921	63,500	1/13
26	*RASD1*	RAS, dexamethasone-induced 1	NM_016084	17p11.2	AC073621	1,000	1/13
27	*RAX*	Retina and anterior neural fold homeobox	NM_013435	18q21.32	AC067859	141,000*	9/13
28	*REC8L1*	REC8-like 1	NM_005132	14q12	AL136295	40,000*	12/13
29	*SLC27A3*	Solute carrier family 27	NM_024330	1q21.3	AL513523	58,000	6/13
30	*TGFB2*	Transforming growth factor, β 2	NM_003238	1q41	AC096638	52,000	6/13
31	*TLX2*	T-cell leukemia, homeobox 2	NM_016170	2p13.1	AC005041	23,000*	2/13
32	*TMEM22*	Transmembrane protein 22	NM_025246	3q22.3	AC096992	21,500*	9/13
33	*TMEM30B*	Transmembrane protein 30B	XM_090844	14q23.1	AL359220	157,000*	11/13
34	*UNC5C*	Unc-5 homologue C	NM_003728	4q23	AC106881	96,000*	3/13

NOTE: CGIs in putative promoter regions of 34 genes were aberrantly methylated in melanoma cell lines. Gene numbers 1 to 34 correspond to panel numbers in Figure 1.
*positions in the reverse strand.

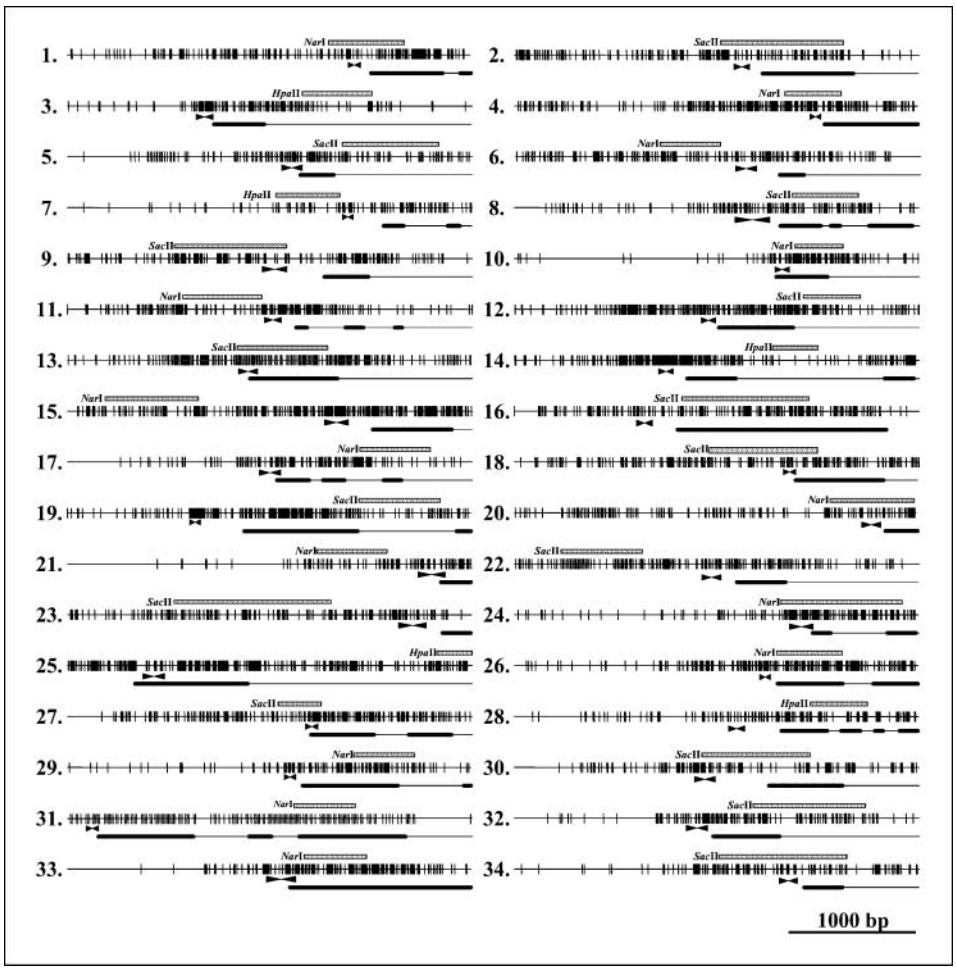

Figure 1. Genomic structures around the 34 CGIs in putative promoter regions and methylated in melanoma cell line(s). Gene names of 1 to 34 are listed in Table 1. *Vertical ticks,* individual CpG sites. *Gray boxes,* DNA fragments isolated by MS-RDA. *Hpa*II, *Nar*I, or *Sac*II, restriction enzyme used for MS-RDA. *Closed boxes,* exons. *Arrowheads,* MSP primers.

For methylation-specific PCR (MSP; ref. 16), 1 μL of the sodium bisulfite–treated DNA was amplified with primers specific to methylated or unmethylated sequences. DNA from HEM1 and DNA methylated *in vitro* using *Sss*I methylase (New England Biolabs) were used as a control for unmethylated and methylated DNA, respectively. Minimum cycles to obtain visible bands with these control samples were determined for each primer set, and four cycles were added to analyze test samples. Further four cycles were added for paraffin-embedded samples, which were degraded. For bisulfite sequencing, 1 μL of the sodium bisulfite–treated DNA was amplified with primers common to methylated and unmethylated DNA sequences. The PCR product was cloned into a pGEM-T Easy Vector (Promega), and 10 clones were sequenced using an ABI PRISM 310 sequencer (PE Biosystems, Foster City, CA). Primer sequences and PCR conditions for MSP and bisulfite sequencing are shown in Supplementary Table S1.

Quantitative real-time reverse transcription-PCR. DNase-treated total RNA (3 μg) was reverse transcribed with oligo-dT primer (Promega) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR was done using SYBR Green PCR Core Reagents (PE Biosystems) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The primer sequences and annealing temperatures are shown in Supplementary Table S2. The numbers of target cDNA molecules were normalized to those of *GAPDH* cDNA molecules. Three independent quantitative reverse transcription-PCR (RT-PCR) experiments were done, and the average values are shown.

5-Aza-2′-deoxycytidine treatment. Melanoma cells seeded at a density of 6×10^5 per 10-cm plate on day 0 were exposed to 1 μmol/L 5-aza-2′-deoxycytidine (5-aza-dC; Sigma) for 24 hours on days 1 and 3. The cells were harvested on day 4.

Immunohistochemical analysis. A goat polyclonal antibody raised against a peptide near the NH_2 terminus of human peroxiredoxin II (Prx II), the *PRDX2* gene product, was purchased from Santa Cruz Biotechnology (N-13; Santa Cruz, CA). Formalin-fixed and paraffin-embedded sections were sliced at 5 μm thickness, deparaffinized, and heated in 10 mmol/L citrate buffer (pH 6) for 15 minutes at 121°C. After blocking, the sections were incubated with the antibody at a dilution of 50-fold at 4°C overnight. The binding of the first antibody was detected by a specific second antibody and the Vectastain Elite Avidin-Biotin Complex kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with Mayer's hematoxylin. As a negative control, the absence of staining without the primary antibody was confirmed. As a positive control, staining of epidermal keratinocytes was confirmed. To avoid potential false-positive results due to the presence of melanin granules, regions with little melanin granules were used for immunohistochemical analysis.

Results

Isolation of CGIs aberrantly methylated in melanoma cell lines. MS-RDA was done using HEM1 as the tester and MeWo, WM-266-4, and MMAc as the drivers to obtain DNA fragments methylated in MeWo, WM-266-4, and MMAc, respectively. Three methylation-sensitive restriction enzymes (*Hpa*II, *Nar*I, and *Sac*II) were used, and resultantly, nine series of MS-RDA were done. A total of 864 clones, 96 clones in each series, were sequenced: 321 clones were nonredundant; 273 clones were flanked by CGIs; and 54 clones were flanked by CGIs in putative promoter regions of 55 genes. One CGI was shared by two genes.

MS-RDA isolates clones with differentially methylated restriction sites at their ends, which are not necessarily in a region critical for gene expression (core region). Therefore, methylation statuses of the putative core regions of the 54 CGIs were analyzed by MSP of 13 melanoma cell lines and two cultured normal human epidermal melanocytes (HEM1 and HEM2). CGIs of 34 genes *(ABHD9, BARHL1, CLIC5, CNNM1, COL2A1, CPT1C, DDIT4L, DERL3, DHRS3, DPYS, EFEMP2, FAM62C, FAM78A, FLJ33790, GBX2, GPR10, GPRASP1, HOXA9, HOXD11, HOXD12, HOXD13, p14ARF, PAX6, PRDX2, PTPRG, RASD1, RAX, REC8L1, SLC27A3, TGFB2, TLX2, TMEM22, TMEM30B*, and *UNC5C*) were partially or completely methylated in one or more melanoma cell lines while not in HEMs (Table 1; Fig. 1; representative results in Fig. 2*A*). Complete methylation was observed for 30 of these genes, excluding *FAM62C, HOXD13, p14ARF*, and *RASD1*. Twelve genes (*ABHD9, CNNM1, COL2A1, CPT1C, DDIT4L, HOXA9, HOXD12, PAX6, RAX, REC8L1, TMEM22*, and *TMEM30B*) were methylated in ≥9 of the 13 (>70%) melanoma cell lines.

Silencing of PRDX2. As for the 30 genes whose putative promoter CGIs were completely methylated in one or more cell lines, mRNA expression levels were examined by quantitative RT-PCR of the 13 melanoma cell lines, two HEMs, and the normal brain and testes. Brain and testes were included because many genes are highly expressed in these tissues, and these were useful to estimate functional levels of expression of some genes (17, 18). It was found that 18 of the 30 genes were consistently unexpressed in melanomas with complete methylation of the corresponding putative promoter CGIs (representative result in Fig. 3*a*). Six of these 18 genes (*DERL3, FAM78A, PRDX2, PTPRG, SLC27A3*, and *UNC5C*) were expressed in both of the two HEMs, whereas the other 12 genes (*ABHD9, BARHL1, CLIC5, DPYS, GBX2, PAX6, GPR10, HOXD12, RAX, TLX2, TMEM22*, and *TMEM30B*) were expressed in only one of them or in neither of them.

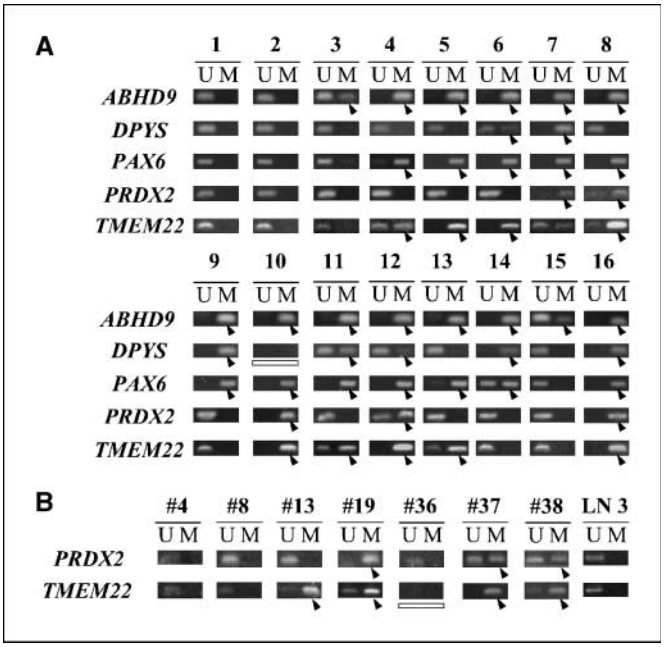

Figure 2. Representative results of MSP. *A,* MSP of representative genes in melanoma cell lines. *Samples 1* and *2,* HEM1 and HEM2 (cultured human epidermal melanocytes 1 and 2). *Samples 3* to *15,* melanoma cell lines MeWo, WM-266-4, WM-115, C32TG, MMAc, VMRC-MELG, COLO 679, GAK, A2058, SK-MEL-28, G361, HMV-I, and TK-Mel-1. *Sample 16,* DNA methylated by *Sss*I methylase (fully methylated DNA). *U* and *M,* primer sets specific to unmethylated and methylated DNA molecules, respectively. *Closed arrows,* bands obtained with the M primer set. *Rectangles,* regions where no PCR products were obtained using the U or M primer sets, suggesting homozygous deletion. *B,* representative results of MSP of *PRDX2* and *TMEM22* in surgical melanoma specimens and normal lymph node specimens. These seven melanoma specimens underwent Prx II immunohistochemical analysis.

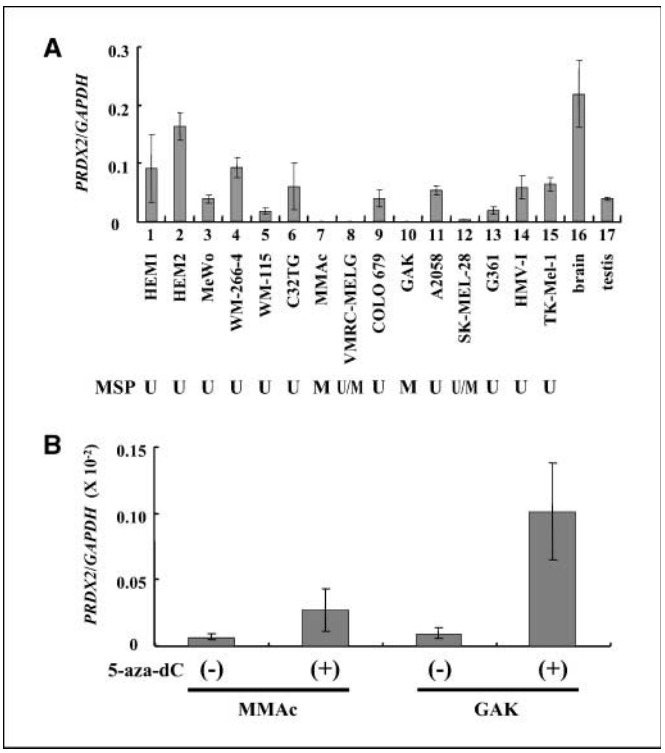

Figure 3. *PRDX2* mRNA expression and their reexpression by 5-aza-dC treatment. *A, PRDX2* expression levels were analyzed by quantitative RT-PCR of two HEMs, the 13 melanoma cell lines, and the normal brain and testes in three independent experiments. Results of MSP are summarized below the sample names by U, M, and U/M, which represent the presence of only unmethylated, only methylated, and both unmethylated and methylated DNA molecules, respectively. Two HEMs (*lanes 1* and *2*), thirteen melanoma cell lines (*lanes 3-15*), and the normal brain and testes (*lanes 16* and *17*) were analyzed. Samples without unmethylated DNA molecules did not express *PRDX2. B,* two cell lines without unmethylated DNA molecules (MMAc and GAK) were treated with a demethylating agent 5-aza-dC, and *PRDX2* expression levels before (−) and after (+) the treatment are shown. *PRDX2* expression was restored in both cell lines by 5-aza-dC treatment.

Because mechanistically important genes were expected to be expressed in both HEMs, the role of methylation of the putative promoter CGIs in the loss of expression was examined for the six genes by treating melanoma cells with a demethylating agent 5-aza-dC. Demethylation of the putative promoter CGI and corresponding reexpression of mRNA were observed for the *PRDX2* gene (Fig. 3*b*). This result supported that methylation of the putative promoter CGI of *PRDX2* caused its silencing. CGIs of the remaining five genes were demethylated, but their expression was not restored. This could be due to missing transcriptional capacity, as observed for many genes silenced by promoter methylation (3). Coincidentally, Prx II, the *PRDX2* product, was recently reported to attenuate the signal transmitted by platelet-derived growth factor (PDGF; ref. 19), and we decided to focus on *PRDX2* as a gene silenced in melanoma cell lines.

Methylation of PRDX2 in surgical melanoma specimens. Dense methylation of the putative promoter CGI of *PRDX2* was confirmed by bisulfite sequencing before analysis of a large number of surgical melanoma specimens (Fig. 4). A 414-bp region in the

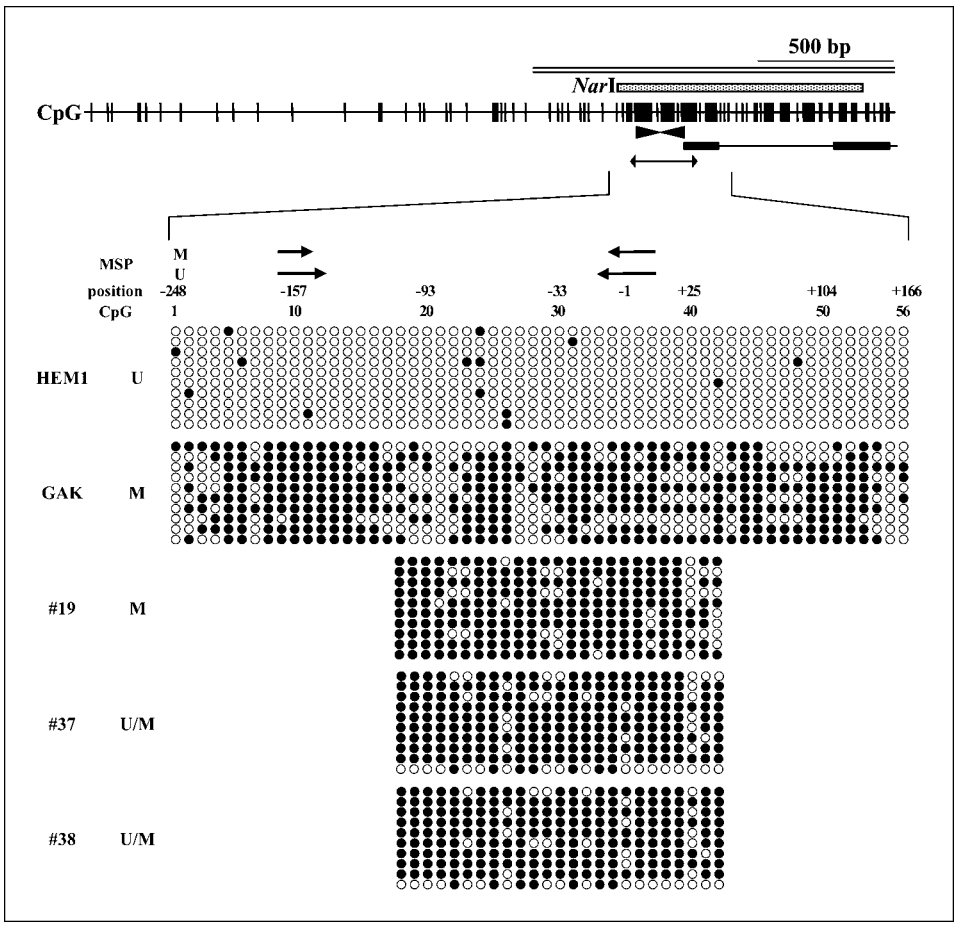

Figure 4. Dense methylation of the putative promoter CGI of *PRDX2. Vertical ticks,* individual CpG sites; *double line,* a region that met the CpG island criteria by Takai and Jones. *Gray box,* the DNA fragments isolated by MS-RDA using *Nar*I; *closed boxes,* exons; *arrowheads,* MSP primers; *arrow,* putative promoter region predicted by WWW Promoter Scan (http://bimas.cit.nih.gov/molbio/proscan/). Methylation status of the *PRDX2* promoter CpG island was analyzed by bisulfite genomic sequencing of 10 clones for each sample. *Open* and *closed circles,* unmethylated and methylated CpG sites, respectively. *Arrows,* the positions of CpG sites recognized by MSP primers specific to unmethylated (*U*) or methylated (*M*) DNA molecules. The transcription start site was defined as +1. Whereas 56 CpGs were analyzed in HEM1 and GAK, a smaller region with 25 CpGs was analyzed in surgical specimens due to DNA degradation. Dense methylation of the promoter CpG island was observed for GAK and specimens 19, 37, and 38, whereas it was unmethylated in HEM1.

5′ flanking region of *PRDX2* was densely methylated in the GAK melanoma cell line, which showed complete methylation by MSP but not in HEM1, which showed no methylation by MSP.

Confirming the specificity of the MSP primers, we screened methylation of the putative promoter CGI of *PRDX2* by MSP in 36 surgical melanoma specimens (19 primary and 17 metastatic sites), excluding three of 39 specimens (Supplementary Table S3). *PRDX2* methylation, which was present in 4 of 13 (30%) in cell lines, was observed in 3 of 36 (8%) in specimens (representative results in Fig. 2*b*). The presence of dense methylation in the specimens with methylation (specimens 19, 37, and 38) was confirmed by bisulfite sequencing (Fig. 4). The presence of methylated DNA in surgical specimens indicated that *PRDX2* was silenced also in surgical melanoma specimens. Although the number of specimens with methylation was limited, no significant association between *PRDX2* methylation and origin of tumors (primary or metastatic) or sample type (fresh or paraffin embedded) was observed.

Immunohistochemical analysis of Prx II in surgical melanoma specimens. Immunohistochemical analysis of Prx II was done using four surgical melanoma specimens with unmethylated DNA molecules only and three specimens with methylated DNA molecules. All of the three specimens with methylation lacked immunoreactivity for Prx II, whereas two of the four specimens without methylation retained immunoreactivity in their cytoplasm (Fig. 5). These results showed that Prx II is lacking in melanomas due to silencing by promoter methylation and other mechanisms.

TMEM22 as a potential melanoma marker. As a potential marker to detect melanoma cells, methylation of *TMEM22*, one of the genes most frequently methylated in melanoma cell lines, was analyzed in 33 surgical melanoma specimens (17 primary and 16

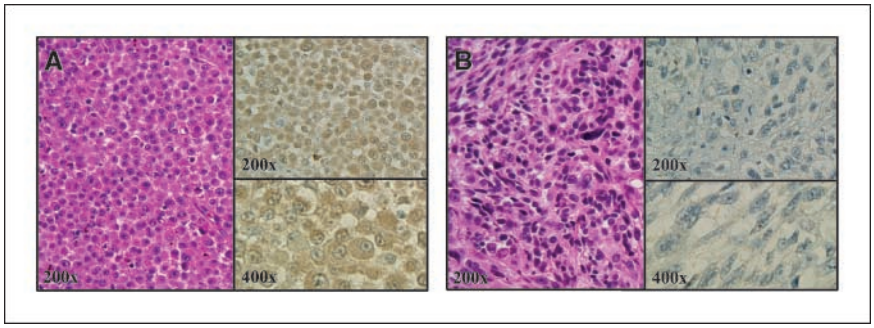

Figure 5. Representative immunohistochemical analysis of Prx II in surgical melanoma specimens. *A,* a skin metastasis (specimen 36) without promoter methylation. *B,* a primary site (specimen 19) with promoter methylation. Regions with little melanin granules were used for the immunohistochemical analysis. Prx II immunoreactivity was observed in the cytoplasm and was detected in two of four melanoma specimens without methylation and in none of three melanoma specimens with methylation.

metastatic sites), excluding 6 of 39 specimens. *TMEM22* was methylated in 8 of the 33 (24%) specimens (representative results in Fig. 2*b*). To detect metastatic melanoma cells in lymph nodes, the absence of methylation in normal lymph nodes is essential. We analyzed *TMEM22* methylation in five normal lymph node specimens, but no methylation was detected. This indicated that some of the frequently methylated genes are useful as markers to detect metastatic melanoma cells in lymph nodes.

Discussion

Gene silencing of *PRDX2* in human malignant melanomas was here identified by a genome-wide screening using MS-RDA. *PRDX2* product, Prx II, was recently shown to function as a negative regulator of PDGF signaling, a potent mitogenic signal pathway (19). Although Prx II is a 2-Cys thioredoxin peroxidase and a cellular antioxidant, it interacts with activated PDGF receptor β, unlike other peroxiredoxins, and suppresses its phosphorylation (19). Down-regulation of Prx II was recently shown to be associated with progression of melanomas (20) and urinary bladder cancers (21). Molecular mechanisms for the down-regulation have been unknown, but our study here showed that gene silencing by promoter methylation is one of the mechanisms. At the same time, because two of the four surgical melanoma specimens without methylation also lacked Prx II immunoreactivity, involvement of other mechanisms, such as mutation and chromosomal losses, should be considered.

Including *PRDX2*, CGIs in putative promoter regions of 34 genes were found to be methylated in melanoma cell lines, and CGIs of 30 genes were completely methylated at least in one cell line. Methylation of 18 CGIs consistently repressed expression of their downstream genes, supporting that the regions analyzed were promoter regions. Among the 18 genes, six genes, including *PRDX2*, were abundantly expressed in HEMs, whereas the other 12 genes were not. This finding was in accordance with our previous studies in pancreatic and breast cancers (17, 18), where most of the genes with methylation of their putative promoter CGIs in cancers had little expression in their normal counterpart tissues. This supported a hypothesis that gene transcription is an important factor to keep promoter CGIs unmethylated (3, 22, 23) and suggested that a significant number of genes are methylated as "bystanders" in tumors. Among the six genes abundantly expressed in HEMs, expression of only *PRDX2* was restored by demethylation of the putative promoter CGIs. For the other five genes, it was suggested that their transcription was first repressed, and that the repression was followed by methylation of the putative promoter CGIs because their expression was not restored by demethylation of the CGIs. Therefore, a possibility that silencing of these five genes was causally involved in melanoma development and progression seemed low. Methylation of 12 (*CNNM1, COL2A1, CPT1C, DDIT4L, DHRS3, EFEMP2, FLJ33790, GPRASP1, HOXA9, HOXD11, REC8L1*, and *TGFB2*) of the 30 CGIs did not consistently repress their downstream genes. This could have been due to leaky expression even in the presence of methylation of CGIs in promoter regions or improper localization of the promoter regions simply based upon the 5′ transcription start sites of genes.

Among the 34 CGIs specifically methylated in melanoma cell lines, 29, including *PRDX2*, were novel, and five (*FLJ33790, HOXD11, PTPRG, p14ARF*, and *REC8L1*) were previously reported in some types of cancers. Aberrant methylation of *FLJ33790* and *HOXD11* was identified in breast cancers by MS-RDA (18), and that of *REC8L1* was identified in lung and ovarian cancers by MS-RDA.[4] *PTPRG*, a putative tumor suppressor gene, was reported to be methylated in cutaneous T-cell lymphomas (24). Methylation of *p14ARF* is reported in many cancers (1).

Among the 34 CGIs, methylation of 12 CGIs was detected in ≥9 of the 13 melanoma cell lines. Because aberrant methylations can be detected rapidly and sensitively using MSP, the aberrant methylation itself can be used as biomarkers to detect melanoma cells in sentinel lymph node biopsy and other samples. Therefore, we analyzed methylation of *TMEM22*, methylated at a high incidence in melanoma cell lines, in surgical melanoma specimens and normal lymph nodes. Its specific methylation in melanoma specimens at an incidence of 24% supported its potential as a biomarker and warranted further analysis involving a large number of clinical specimens. When we focus on the aspect of methylation as biomarkers, methylation of candidate CGIs does not necessarily cause gene silencing but must be specific to melanoma cells. In this sense, screening of CGIs outside promoter regions could be considered.

In conclusion, we showed that *PRDX2* is silenced by methylation of a CGI in its promoter region, and the silencing was suggested to be involved in melanoma progression by augmenting PDGF signaling.

Acknowledgments

Received 1/16/2006; revised 4/4/2006; accepted 4/13/2006.

Grant support: Third-term Comprehensive 10-Year Strategy for Cancer Control; Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology, Japan; and Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare.

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.

[4] Unpublished data.

References

1. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–28.
2. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349:2042–54.
3. Ushijima T, Okochi-Takada E. Aberrant methylations in cancer cells: where do they come from? Cancer Sci 2005;96:206–11.
4. Laird PW. The power and the promise of DNA methylation markers. Nat Rev Cancer 2003;3:253–66.
5. Miyamoto K, Ushijima T. Diagnostic and therapeutic applications of epigenetics. Jpn J Clin Oncol 2005;35:293–301.
6. Tucker MA, Goldstein AM. Melanoma etiology: where are we? Oncogene 2003;22:3042–52.
7. Furuta J, Umebayashi Y, Miyamoto K, et al. Promoter methylation profiling of 30 genes in human malignant melanoma. Cancer Sci 2004;95:962–8.
8. Worm J, Christensen C, Gronbaek K, Tulchinsky E, Guldberg P. Genetic and epigenetic alterations of the APC gene in malignant melanoma. Oncogene 2004;23:5215–26.
9. Hoon DS, Spugnardi M, Kuo C, Huang SK, Morton DL, Taback B. Profiling epigenetic inactivation of tumor suppressor genes in tumors and plasma from cutaneous melanoma patients. Oncogene 2004;23:4014–22.
10. Ushijima T, Morimura K, Hosoya Y, et al. Establishment of methylation-sensitive-representational difference analysis and isolation of hypo- and hypermethylated genomic fragments in mouse liver tumors. Proc Natl Acad Sci U S A 1997;94:2284–9.
11. Kaneda A, Takai D, Kaminishi M, Okochi E, Ushijima T. Methylation-sensitive representational difference analysis and its application to cancer research. Ann N Y Acad Sci 2003;983:131–41.
12. Ushijima T. Detection and interpretation of altered methylation patterns in cancer cells. Nat Rev Cancer 2005;5:223–31.

13. Lagonigro MS, De Cecco L, Carninci P, et al. CTAB-urea method purifies RNA from melanin for cDNA microarray analysis. Pigment Cell Res 2004;17:312–5.
14. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci U S A 2002;99:3740–5.
15. Kaneda A, Kaminishi M, Sugimura T, Ushijima T. Decreased expression of the seven ARP2/3 complex genes in human gastric cancers. Cancer Lett 2004;212:203–10.
16. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996;93:9821–6.
17. Hagihara A, Miyamoto K, Furuta J, et al. Identification of 27 5′ CpG islands aberrantly methylated and 13 genes silenced in human pancreatic cancers. Oncogene 2004;23:8705–10.
18. Miyamoto K, Fukutomi T, Akashi-Tanaka S, et al. Identification of 20 genes aberrantly methylated in human breast cancers. Int J Cancer 2005;116:407–14.
19. Choi MH, Lee IK, Kim GW, et al. Regulation of PDGF signalling and vascular remodelling by peroxiredoxin II. Nature 2005;435:347–53.
20. Carta F, Demuro PP, Zanini C, et al. Analysis of candidate genes through a proteomics-based approach in primary cell lines from malignant melanomas and their metastases. Melanoma Res 2005;15:235–44.
21. Memon AA, Chang JW, Oh BR, Yoo YJ. Identification of differentially expressed proteins during human urinary bladder cancer progression. Cancer Detect Prev 2005;29:249–55.
22. De Smet C, Loriot A, Boon T. Promoter-dependent mechanism leading to selective hypomethylation within the 5′ region of gene MAGE-A1 in tumor cells. Mol Cell Biol 2004;24:4781–90.
23. Song JZ, Stirzaker C, Harrison J, Melki JR, Clark SJ. Hypermethylation trigger of the glutathione-*S*-transferase gene (GSTP1) in prostate cancer cells. Oncogene 2002;21:1048–61.
24. van Doorn R, Zoutman WH, Dijkman R, et al. Epigenetic profiling of cutaneous T-cell lymphoma: promoter hypermethylation of multiple tumor suppressor genes including BCL7a, PTPRG, and p73. J Clin Oncol 2005;23:3886–96.

Silencing of *Peroxiredoxin 2* and Aberrant Methylation of 33 CpG Islands in Putative Promoter Regions in Human Malignant Melanomas

Junichi Furuta, Yoshimasa Nobeyama, Yoshihiro Umebayashi, et al.

Cancer Res 2006;66:6080-6086.