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

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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, CNM1, COL2A1, CPT1C, DDT4L, DERL3, DHR53, DPYS, EFEMP2, FAM62C, FAM78A, FJ133790, GBX2, GPR10, GPRASP1, HOXA9, HOXD11, HOXD12, HOXD13, p14ARF, PAX6, PRDX2, PTPRG, RAX, RECS1, SLC27A3, TGFBR2, 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, VMBC-MELG, A2058, C32T2G, and GAK were obtained from the Health Science Research Resources Bank (Sennan, Japan); G361, SK-MEL-28, and HMV-1 were obtained from the Cell Resource Center for Biomedical Research, Institute of Development (Sendai, Japan); COLO 679 and MAMc 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 metastic 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 melanoma 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...
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 5 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 HpaII, SacII, or NarI (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 reported (15). Genomic DNA (500 ng) restricted with BamHI (New England Biolabs) was denatured in 0.3 mol/L NaOH. In 3.1 mol/L NaHSO3 (pH 5) and 0.6 mol/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

<table>
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<tr>
<th>No.</th>
<th>Symbol</th>
<th>Gene Description</th>
<th>Accession no.</th>
<th>Chromosomal location</th>
<th>Accession no.</th>
<th>Map start position</th>
<th>Methylation in cell lines</th>
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<td>1</td>
<td>ABHD9</td>
<td>Abhydrolase domain containing 9</td>
<td>NM_024794</td>
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<td>2</td>
<td>BARHL1</td>
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<td>AL347355</td>
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<td>3</td>
<td>CLIC5</td>
<td>Chloride intracellular channel 5</td>
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<td>6p21.1</td>
<td>AL503363</td>
<td>58,000*</td>
<td>3/13</td>
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<td>4</td>
<td>CNNM1</td>
<td>Cyclin M1</td>
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<td>COL2A1</td>
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<td>AC004080</td>
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<td>3q22.3</td>
<td>AC096992</td>
<td>21,500*</td>
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<td>TMEM30B</td>
<td>Transmembrane protein 30B</td>
<td>NM_098644</td>
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<td>Unc-5 homologue C</td>
<td>NM_003728</td>
<td>4q23</td>
<td>AC106881</td>
<td>96,000*</td>
<td>3/13</td>
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</table>

**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.
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 SssI 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 (PE Biosystems, Foster City, CA). Primer sequences and PCR conditions for MSP and bisulfite sequencing are shown in Supplementary Table S1.

Immunohistochemical analysis. A goat polyclonal antibody raised against a peptide near the NH2 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 secondary 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 (HpaII, NarI, and SacII) were used, and resultantly, nine series of MS-RDA were done. A total of 584 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.

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. HpaII, NarI, or SacII, restriction enzyme used for MS-RDA. Closed boxes, exons. Arrowheads, MSP primers.
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, DDIR4L, DERL3, DHR53, DPYS, EFEMP2, FAM62C, FAM78A, FLJ33790, GBX2, GPR10, GPRASPI, HOXA9, HOXD11, HOXD12, HOXD13, p14ARF, PAX6, PRDX2, PTPRG, RASD1, RAX, RECSL1, 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. 2A). Complete methylation was observed for 30 of these genes, excluding FAM62C, HOXD13, p14ARF, and RASD1. Twelve genes (ABHD9, CNNM1, COL2A1, CPT1C, DDIR4L, HOXA9, HOXD12, PAX6, RAX, RECSL1, 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. 3A). 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.

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 was accompanied by reexpression of mRNA for the PRDX2 gene (Fig. 3B). 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).

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...
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). 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
metastatic sites), excluding 6 of 39 specimens. **TMEM22** was methylated in 8 of the 33 (24%) specimens (representative results in Fig. 2b). 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, DDI14L, DHR33, EFEMP2, FJ133790, GPRASP1, HOXA9, HOXD11, REC8L1, and TGFβ2**) 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 (**FJ133790, HOXD11, PTPRG, p14ARF, and REC8L1**) were previously reported in some types of cancers. Aberrant methylation of **FJ133790** 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. **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 at 29 of 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.

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References

Silencing of \textit{Peroxisiredoxin 2} and Aberrant Methylation of 33 CpG Islands in Putative Promoter Regions in Human Malignant Melanomas

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