Promoter Methylation Status of Multiple Genes in Uveal Melanoma

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PURPOSE. Aberrant promoter hypermethylation of CpG islands is thought to play an important role in the inactivation of tumor-suppressor genes (TSGs) in cancer. Studies of cutaneous melanoma have reported a high methylation rate for MGMT, DAPK, RAR-b2, and RASSF1A. In colon cancer, SOCS-1, IGF-2, RUNX3, Neurog1, and CACNA1G are commonly inactivated. The concomitant methylation of at least three of these genes may represent a distinct trait, the CpG island methylator phenotype (CIMP). The purpose of the present study was to investigate the role of epigenetic inactivation of multiple genes in uveal melanoma.

METHODS. Twenty samples of uveal melanoma were analyzed for the methylation status of nine candidate cancer-related genes: MGMT, DAPK, RAR-b2, RASSF1A, SOCS-1, IGF-2, RUNX3, Neurog1, and CACNA1G, using real-time quantitative methylation-specific polymerase chain reaction after sodium bisulfite modification.

RESULTS. Methylation rates of the genes commonly inactivated in cutaneous melanoma were 70% for RASSF1A, 5% for MGMT and DAPK, and 0% for RAR-b2. The rates for the CIMP-related genes were 25% for RUNX3, 5% for Neurog1 and CACNA1G, and 0% for SOCS-1 and IGF-2. None of the samples was CIMP-positive.

CONCLUSIONS. In this study uveal melanoma was negative for CIMP, with hypermethylation of RASSF1A. The negative CIMP phenotype and frequent RASSF1A methylation in uveal melanoma is in accord with its known lack of BRAF mutations. Given that mutations in genes of the RAS pathway are rarely observed in uveal melanoma, epigenetic inactivation of RASSF1A may be an alternative mechanism of tumorigenesis.

The low frequency of promoter methylation of TSGs commonly inactivated in cutaneous melanoma further stratifies the different tumorigenesis pathway in cutaneous and uveal melanoma. (Invest Ophthalmol Vis Sci. 2007;48:4403–4406) DOI:10.1167/iovs.07-0272

Uveal melanoma is the most common form of primary eye cancer in adults, with an annual incidence of six to seven cases per million.1 It accounts for 70% of all primary eye tumors. No obvious genetic or environmental predisposing factors are currently known for the development of uveal melanoma.2 Uveal and cutaneous melanomas originate from a common precursor cell, the melanocyte, which migrates from the neural crest to the respective site during the embryonic development period.3 The similar genetic background and some common histologic characteristics suggest a similar pathogenesis of these tumors.4,5 However, their genetic alterations differ: Although both show alterations of chromosomes 1 and 6, aberrations such as monosomy of chromosome 3 and gain of 8q are typically found in uveal melanoma but rarely in cutaneous melanomas. Conversely, alterations at the CDKN2 locus on 9p21 and mutations in the BRAF gene, common in cutaneous melanoma, are infrequent in uveal tumors.6,7 Unlike cutaneous melanoma, the molecular mechanisms leading to the development and progression of uveal melanoma are poorly understood.

Tumor-suppressor gene (TSG) inactivation is critical to the pathogenesis of cancer. Such loss of function may be mediated by irreversible processes such as gene deletion or mutation. Alternatively, TSGs may be inactivated via epigenetic processes, a reversible mechanism that promises to be more amenable to treatment.8 One such process involves methylation of discrete regions of the CpG islands, which may play a critical role in the control of gene expression. Aberrant DNA methylation of CpG islands has been widely observed in several human tumors.9 Studies in cutaneous melanoma have noted high methylation rates for retinoic acid receptor-b2 (RAR-b2), RAS association domain family protein 1A (RASSFIA), O6-methylguanine DNA methyltransferase (MGMT), and death-associated protein kinase (DAPK).10 Recent studies have suggested that the methylation of three of five commonly found promoter genes in colon cancer—namely, suppressor of cytokine signaling 1 (SOCS-1), insulin-like growth factor 2 (IGF-2), runt-related transcription factor 3 (RUNX3), neurogenin 1 (NEUROG1), and alpha1G T-type calcium channel gene (CACNA1G)—may represent a distinct trait, the CpG island methylator phenotype (CIMP).11,12

Fewer data are available on the mechanisms of tumorigenesis in uveal melanoma.13,14 Recently, analyses of uveal melanoma cell lines revealed that TIMP3 expression is regulated by methylation.15 Others reported promoter methylation inactivation of pRb cell cycle inhibitor p16 (p16NK4a) and of RASSFIA1,13,14,16,17

In the present study, to elucidate further the role of epigenetic events in uveal melanoma, we investigated the methylation status of genes commonly inactivated in cutaneous mel-
anoma and the newly described methylator phenotype TSG panel.

**MATERIALS AND METHODS**

The study protocol was approved by the national and institutional review boards. The research complied with the tenets of the Declaration of Helsinki.

**Tumor Specimens**

A search of the archives of the Ophthalmic Pathology Laboratory of the Hadassah-Hebrew University Medical Center, Jerusalem, from 1993 to 2004 yielded 20 samples of uveal melanoma.

**DNA Extraction**

The archival formalin-fixed, paraffin-embedded tissues were used for DNA isolation, as previously described. In brief, hematoxylin-eosin stained 10-µm-section slides were first reviewed by a pathologist. Areas with an estimated content of >75% tumor cells were separated by microdissection from five consecutive 10-µm unstained paraffin-embedded sections of each block, with a no. 11 surgical blade. After deparaffinization, the microdissected tissues were incubated overnight in 1% SDS and proteinase K 0.5 mg/mL. DNA was purified by phenol-chloroform extraction and ethanol precipitation and dissolved in 50 µL of distilled water as described previously.

**Bisulfite Modification**

The bisulfite treatment was performed as described previously. In brief, 1 µg of genomic DNA was denatured with NaOH and bisulfite treated for 16 hours at 50°C. DNA samples were then purified (Wizard DNA Clean-Up System; Promega, Madison, WI), treated again with NaOH, ethanol-precipitated, and resuspended in water.

**Real-Time Quantitative Methylation-Specific Polymerase Chain Reaction**

After sodium bisulfite conversion, genomic DNA was analyzed (Prism 7900 Sequence Detection System; Applied Biosystems, Inc. [ABI], Foster City, CA), as previously described. Amplifications were performed in 96-well plates. Each plate consisted of patient samples and water blanks, as well as positive and negative control samples. DNA extracted from leukocytes, derived from healthy individuals, were methylated in vitro with SSSI methyltransferase (New England Biolabs Inc., Beverly, MA), to generate completely methylated DNA and served as the positive control. Serial dilutions of this DNA were used to construct the standard curves on each plate. The relative degree of methylation of each DNA sample was defined as the ratio between the value of the gene of interest and the value of the internal reference gene (gene of interest/reference gene × 1000), as described previously. Eleven sets of primers and probes designed specifically for bisulfite-converted DNA were used to investigate the methylation status of nine candidate genes (Table 1)—namely, methylated sets for RASSF1A, RAR-b2, MGMT, DAPK, SOCS-1, IGF-2, RUNX3, NEUROG1, and CACNA1G, and reference sets for β-actin (ACTB) and ALU-C4, to normalize the input DNA (Fig. 1).

**RESULTS**

**Detection of TSG Methylation Status**

Our analysis of 20 samples of uveal melanoma revealed methylation rates of 70% for RASSF1A, 5% for MGMT, 5% for DAPK, and 0% for RAR-b2 (Table 2).
None of the samples was CIMP positive. The methylation rates of the CIMP-related genes were 25% for \textit{RUNX3}, 5% for \textit{NEUROG1} and \textit{CACNA1G}, and 0% for \textit{SOCS-1} and \textit{IGF-2} (Table 2).

### DISCUSSION

In this study, uveal melanoma was negative for CIMP, with hypermethylation of \textit{RASSF1A}. CIMP is common in colon cancer, in particular in tumors with microsatellite instability, and many of these tumors harbor V600E \textit{BRAF} mutations.\textsuperscript{12} Therefore, the negative CIMP and frequent \textit{RASSF1A} methylation in uveal melanoma may be related to its lack of \textit{BRAF} mutations, as previously reported.\textsuperscript{6}

Epigenetic silencing of \textit{RAR-b2}, \textit{RASSF1A}, \textit{MGMT}, and \textit{DAPK} was reported in cutaneous melanoma as a common and probably important mechanism for tumor formation. Therefore, we investigated the role of epigenetic inactivation of these genes in uveal melanoma. We found that \textit{RAR-b2}, \textit{MGMT},\textit{TABLE 2. Methylation Status of Multiple Tumor Suppressor Genes in Uveal Melanoma

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| Frequency | 5% | 0% | 5% | 25% | 0% | 0% | 5% | 5% | 70% |

\textsuperscript{1} = \textbf{Representative positive \textit{NEUROG1}- and SSSI-treated DNA amplification curves. Samples were run in duplicate. The amplification curve of \textit{ALU-C4} represents input DNA, and the amplification of \textit{NEUROG1} indicates the presence of aberrant promoter methylation. The relative proportion of methylation was calculated.}
and DAPK promoter hypermethylation is uncommon in uveal melanoma. The low frequency of promoter methylation of these genes further stratifies the different tumorigenesis pathways in these tumors.

Our analysis revealed promoter hypermethylation for the RASSF1A gene in a significant proportion of the samples, in accord with a recently published study.\textsuperscript{17} Tumor biopsy and cancer cell line investigations have shown that inactivation of RASSF1A is extremely common in many human cancers,\textsuperscript{21} such as breast,\textsuperscript{22} head and neck,\textsuperscript{23} and lung\textsuperscript{24} cancers and in cutaneous melanoma.\textsuperscript{25} The epigenetic mechanism of aberrant methylation of this promoter gene is the rule, as opposed to somatic inactivating mutation, which is a rare event.\textsuperscript{21,25} RASSF1A lies on the p21.3 region of chromosome 3 which is frequently rearranged in uveal melanoma, making it a candidate TSG in this tumor.\textsuperscript{26,27} Further studies are needed to elucidate its role.

In summary, uveal melanoma is not characterized by the CpG island methylator phenotype. RASSF1A inactivation is a common event in this tumor. RASSF1A methylation alone may not be responsible for development of the tumor, and other TSGs may be methylated as well.

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**References**