Epigenetic Inactivation of RASSF1a in Uveal Melanoma

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PURPOSE. The RAS association domain family 1 (RASSF1) gene is a tumor-suppressor gene located on chromosome 3p21.3. The alternative transcript (RASSF1a) has been shown to be inactivated by hypermethylation in several human malignancies, including breast, prostate, and lung cancer, and in cutaneous melanoma. The purpose of this study was to evaluate the methylation status of RASSF1a in human uveal melanoma.

METHODS. The methylation status of the RASSF1a promoter region was analyzed using PCR in combination with melting curve analysis, sequencing, and restriction enzyme analysis. Eleven human uveal melanoma cell lines, normal melanocytes, 39 archival frozen tumor specimens, and a metastatic lesion of untreated primary uveal melanoma were studied. In addition, whether RASSF1a methylation correlates with patient survival and development of metastatic disease was investigated.

RESULTS. RASSF1a promoter methylation was detected in 10 of the 11 (91%) cell lines, in 19 of the 38 (50%) patients with primary uveal melanoma and in the metastatic lesion. A positive correlation was found between RASSF1a promoter methylation and development of metastatic disease (P = 0.041). A correlation with disease-free survival could not be established, but a positive trend was observed (P = 0.063).

CONCLUSIONS. These data show that RASSF1a methylation is a common epigenetic event in uveal melanoma development, potentially of clinical relevance. The presence of a methylated RASSF1a promoter region might therefore serve as a tumor marker and as a possible target for therapeutic intervention. (Invest Ophthalmol Vis Sci. 2007;48:486–490) DOI:10.1167/iovs.06-0781

Uveal melanoma is the most common primary intraocular neoplasm in adults. It accounts for 70% of all primary eye tumors and occurs at an annual incidence of 6 to 8 per million in white populations.1 Uveal melanoma develops without any obvious genetic or environmental predisposing factors.2 An effective starting point for the identification of genetic changes is to study the chromosomes involved in the progression and development of uveal melanoma. Cytogenetic studies have identified a number of chromosomal abnormalities in uveal melanoma, of which loss of chromosome 1p, gain of 6p and 18q, and loss of one copy of chromosome 3 (i.e., monosomy 3) commonly occur.3–7 Monosomy 3 is highly correlated with decreased survival and metastatic disease,8,9 suggesting the presence of a tumor suppressor gene (TSG) at this location. Despite this information, genetic analysis of this chromosome has not revealed specific TSGs as players in uveal melanoma pathogenesis.

Alternatively, hypermethylation of promoter-associated CpG islands has recently emerged as an important epigenetic mechanism leading to the transcriptional silencing of TSGs in cancer development,10 including the development of uveal melanoma.11–13 Recent findings in other malignancies have identified the Ras association domain family 1 (RASSF1) gene, located on chromosome 3p21.3, as an important regulator of cell proliferation.14–16 Hypermethylation of the CpG island in the promoter region of a major alternative transcript of this gene, RASSF1a, occurs frequently in various carcinomas, including those of the breast, prostate, and lung, and in cutaneous malignant melanoma.17–20

In this study, we investigated RASSF1a gene hypermethylation in uveal melanoma. We demonstrated that RASSF1a methylation of its promoter CpG island in uveal melanoma cell lines and primary tumors is a common event. Furthermore, we showed a correlation between RASSF1a hypermethylation and the development of metastatic disease. Consequently, we postulated that the methylation of RASSF1a is a prognostic tumor marker for uveal melanoma and may serve as a potential target for therapeutic intervention.

MATERIALS AND METHODS

Cell Lines, Primary Uveal Melanoma Specimens, and the Metastatic Lesion

In total, 11 cell lines derived from primary uveal melanomas (92.1, OCM-1, OCM-3, OCM-8, Mel-202, Mel-270, Mel-285, Mel-290) and uveal melanoma metastases (OMM-1, OMM-1.3, OMM-1.5) and a culture of normal uveal melanocytes (Mel-1a) were analyzed for promoter hypermethylation. Cell lines Mel-270, OMM-1.3, and OMM-1.5 represent a progression model because they were derived from a primary uveal melanoma and two of its liver metastases, respectively. All melanoma cell lines were cultured in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 3 mM t-glutamine (Gibco), 2% penicillin/streptomycin, and 10% FBS (Hyclone, Logan, UT). The melanocyte cell line (Mel-1a) was grown in F12 medium (Gibco).21 All cell cultures were incubated at 37°C in a humidified 5% CO2 atmosphere. Archival frozen tumor specimens of primary uveal melanoma came from 39 patients who attended the Leiden University Medical Center between 1988 and 1996. The metastatic lesion (adrenal gland metastasis) was derived from the patient with a primary uveal melanoma (tumor 31), from which cell line 92.1 was derived.22–25 All tumors were primary lesions with diameters greater than 12 mm and prominences greater than 6 mm; patients had not received any treatment before enucleation. The validity of the diagnosis, uveal melanoma, was confirmed histologically in all patients, and clinical and survival data were listed for use in this study (Table 1). The research protocol followed the tenets of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1964; ethical principles for medical research involving human subjects).

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DNA Extraction and Sodium Bisulfite Modification

With the use of a column-based extraction kit (Qiagen Genomic tip 100/G; Qiagen Benelux BV, Venlo, The Netherlands), DNA was extracted from cell lines, frozen tumor material, and cultured uveal melanocytes according to the kit manufacturer's guidelines. Genomic DNA was modified with sodium bisulfite (EZ Methylation Gold Kit; Zymo Research Corporation, Orange, CA.). Enzymatically methylated human DNA (Chemicon Europe Ltd., Hampshire, UK) was used as a positive control in all experiments.

Methylation Analysis

Methylation status of the promoter region was determined by polymerase chain reaction with specific primers and by melting temperature analysis and was further validated with restriction digestion analysis. Primers were designed using bisulfite-converted DNA sequences (Beacon Designer Software version 5.0; Premier Biosoft International, Palo Alto, CA.) and amplified a region of the \textit{RASSF1a} gene promoter CpG island (NCBI accession *605082; bases 353–618). Primers are shown in Table 2. PCR was carried out in a final volume of 21 
\mu L containing 10 
\mu L mix (IQ SYBR Green Supermix; Bio-Rad Laboratories BV, Veenendaal, The Netherlands), 1 
\mu L (10 pmol/\mu L) of each primer, 8 
\mu L H\textsubscript{2}O, and 1 
\mu L bisulfite-converted DNA. PCR was initiated by hot start, followed by 40 cycles at 96°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and with a final melting curve from 70°C to 97°C with an increase in temperature of 0.2°C every 10 seconds. The fluorescence of SYBR Green was measured once per cycle to monitor template amplification.

Melting Temperature Analysis

PCR amplification of bisulfite-treated DNA and subsequent melting analysis (iQ5 Real-time PCR Detection System; Bio-Rad Laboratories BV) allowed detection of heterogeneous methylation (in which the content and distribution of methylated cytosines differed between different molecules in the same sample). DNA melting peaks were acquired by measuring the fluorescence of SYBR Green during a linear temperature transition from 70°C to 97°C at 0.2°C each 10 seconds with accompanying software (Bio-Rad Laboratories BV).

Restriction Digestion Analysis and Sequence Analysis

After amplification with specific primers for methylated DNA, the PCR-amplified fragments were digested using 4 U restriction enzyme

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Tumor ID & Cell Type & Survival (mo) & Present Status & Hypermethylated RASSF1a \\
\hline
UM 1 & Spindle & 198 & Alive & Present \\
UM 2 & Mixed & 29 & Dead, metastases & Present \\
UM 3 & Mixed & 95 & Dead, metastases & Present \\
UM 4 & Spindle & 31 & Dead, metastases & Present \\
UM 5 & Spindle & 50 & Dead, metastases & Present \\
UM 6 & Epithelioid & 57 & Dead, unknown & Not present \\
UM 7 & Mixed & 63 & Dead, unknown & Not present \\
UM 8 & Spindle & 179 & Alive & Not present \\
UM 9 & Mixed & 151 & Alive & Not present \\
UM 10 & Mixed & 124 & Alive & Not present \\
UM 11 & Epithelioid & 50 & Dead, other cause & Not present \\
UM 12 & Mixed & 15 & Dead, metastases & Present \\
UM 13 & Epithelioid & 128 & Alive & Present \\
UM 14 & Epithelioid & 50 & Dead, metastases & Not present \\
UM 15 & Epithelioid & 21 & Dead, unknown & Failed \\
UM 16 & Mixed & 42 & Dead, metastases & Present \\
UM 17 & Mixed & 23 & Dead, metastases & Present \\
UM 18 & Epithelioid & 115 & Dead, unknown & Present \\
UM 19 & Epithelioid & 35 & Dead, metastases & Present \\
UM 20 & Spindle & 12 & Dead, metastases & Present \\
UM 21 & Spindle & 15 & Dead, metastases & Present \\
UM 22 & Mixed & 187 & Alive & Present \\
UM 23 & Epithelioid & 167 & Dead, metastases & Present \\
UM 24 & Spindle & 2 & Lost to follow-up & Not present \\
UM 25 & Spindle & 145 & Alive & Not present \\
UM 26 & Mixed & 151 & Alive & Present \\
UM 27 & Mixed & 15 & Dead, other cause & Not present \\
UM 28 & Mixed & 29 & Dead, other cause & Not present \\
UM 29 & Mixed & 122 & Alive & Not present \\
UM 30 & Mixed & 23 & Dead, unknown & Not present \\
UM 31 & Epithelioid & 35 & Dead, metastases & Present \\
UM 32 & Spindle & 24 & Dead, metastases & Not present \\
UM 33 & Spindle & 30 & Dead, metastases & Not present \\
UM 34 & Mixed & 15 & Dead, metastases & Present \\
UM 35 & Spindle & 101 & Alive & Present \\
UM 36 & Mixed & 34 & Dead, metastases & Present \\
UM 37 & Epithelioid & 36 & Dead, unknown & Not present \\
UM 38 & Mixed & 24 & Dead, unknown & Not present \\
UM 39 & Spindle & 94 & Alive & Not present \\
\hline
\end{tabular}
\caption{Clinical and Survival Data}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
Primer & Sequence \\
\hline
RASSF1a forward & 3'-GAAGGAGGGAAGGAAGGTTAAGG-5' \\
RASSF1a reverse & 5'-GGAGTTTGAATTATTTGAGTGGCGG-3' \\
\hline
\end{tabular}
\caption{Primers Used in PCR}
\end{table}
RASSF1a (Fermentas GmbH, St. Leon Rot, Germany) directly added to the PCR mixture (under conditions specified by the manufacturer). The RASSF1a amplicon of methylated DNA contains one RSA1 recognition site and is dependent on CT conversion and methylation of a CpG. The recognition site 5'-GTAC-3' only appears when the first C in the GCAC sequence is converted to thymine, whereas the second must be methylated and remains C. Amplified products were analyzed by electrophoresis on a 2% agarose gel. DNA bands were excised from the gel, purified with a gel extraction kit (Nucleospin Extract II; Macherey-Nagel GmbH & Co., Düren, Germany), and sequenced on a DNA sequencing system (ABI PRISM 3700; Applied Biosystems, Foster City, CA).

**Statistical Analysis**

Survival analysis for RASSF1a promoter methylation was performed with Kaplan-Meier analysis and log rank test (SPSS version 12.0.1 for Windows; SPSS Inc., Chicago, IL). For comparison between the presence or absence of RASSF1a methylation and metastatic disease, the \( \chi^2 \) test was performed.

**RESULTS**

**Methylation Analysis in Cell Lines, Primary Tumor Tissue, and Metastasis**

To evaluate whether RASSF1a methylation may play a role in uveal melanoma, we analyzed the methylation status of the RASSF1a promoter region in a panel of uveal melanoma cell lines and archival fresh frozen tumor tissue using melting temperature analysis, sequencing, and restriction enzyme analysis. In unmethylated DNA, all cytosines in a CpG dinucleotide are converted to thymines, but in methylated DNA such cytosines remain unchanged. Replacement of cytosines by thymines results in change of a CG base pair to a TA base pair in double-stranded DNA. Subsequently, the temperature at which DNA becomes single stranded is higher in methylated DNA. PCR performed on sodium bisulfite–converted DNA isolated from uveal melanoma cell lines showed a melting peak at 83.6° to 84.6°C in 10 of 11 cell lines (all except Mel-290), which coincided with the peak of the positive control (full methylation). In cell line Mel-290, a melting peak was observed at 81.2° to 81.4°C, similar to the normal cultured melanocytes, 81.2° to 81.4°C, similar to the normal cultured melanocytes, indicating an unmethylated promoter (Fig. 1). This result is consistent with the fact that the amplified RASSF1a amplicon contains 20 CpGs (primer design software predicts a melting temperature difference of 3.4°C).

In addition, 39 primary tumors and a metastatic lesion in patient 31 were analyzed in the same manner as the cell lines. One tumor sample failed repeatedly in the PCR and was excluded (sample 15). Of the remaining 38 primary tumor samples, 19 (50%) included a methylated RASSF1a promoter region. Nine of these samples and the metastatic lesion showed an additional melting peak at 81.2° to 81.4°C, indicating the presence of methylated and unmethylated DNA in the same sample and signaling the presence of heterogeneous cell populations (Fig. 1).

**Restriction Digestion Analysis and Sequence Analysis**

Amplified PCR fragments were digested using the restriction enzyme RSA1. Incubation with RSA1 and gel analysis of the fragments confirmed the methylation status as already observed with the melting curve analysis. Ten of 11 cell lines showed bands representative for methylated DNA; again, hypermethylation was not observed in the melanocyte culture Mel-1a (normal control; Fig. 2). Of the 38 primary uveal melanomas, 19 samples were found to be methylated by restriction enzyme analysis. These were in exact correspondence with those found to be methylated in melting curve analysis. As a final check, methylation of RASSF1a was confirmed by direct sequencing of the RASSF1a product in all samples (Fig. 3 depicts an example of cell lines Mel-270 and Mel-290).

**Survival Analysis**

To assess whether the presence of hypermethylated RASSF1a promoter was correlated with a clinical parameter, correlations between methylation status and melanoma-associated survival and development of metastatic disease were evaluated. Of the initial group of 39 patients, one was lost after 2 months of follow-up. Mean survival of the remaining 38 patients was 68 months (range, 12–198 months). Development of metastatic disease correlated with the presence of a hypermethylated RASSF1a promoter region \( (P = 0.041). \) An association between disease-free survival and the presence of a hypermethylated RASSF1a promoter could not be established, though a positive trend was observed \( (P = 0.063; \log \text{rank test}) \), as shown in Figure 4.

**DISCUSSION**

Epigenetic modification of gene expression is important in tumor development. Methylation of TSG is now commonly analyzed in tumors and even rivals mutation and deletion as the main mechanism in tumor development in certain tumors. RASSF1a is an example of a potential TSG that has been studied in many tumors in which methylation correlates with reduced expression.

The frequent methylation of the RASSF1a gene in uveal melanoma cell lines (91%) and also in primary tumor tissue (50%) suggests that RASSF1a also plays a role in uveal mel-
RASSF1a gene on chromosome 3p21.3 could be of utmost importance for uveal melanoma. Although methylation of RASSF1a may not be held wholly responsible for uveal melanoma development, it could be a contributing factor for uveal melanoma tumor formation and progression. RASSF1a methylation could be the second hit in a classic TSG inactivation in uveal melanoma with monosomy 3. Loss of one RASSF1a copy because of monosomy 3, in combination with hypermethylation of the other copy, could promote progression through the G1/S phase of the cell cycle, stimulating the formation of uveal melanoma tumor.

The high frequency of RASSF1A methylation in uveal melanoma cell lines compared with primary tumors has also been observed in cell lines derived from breast and ovarian cancer. In addition, the methylation of p16^INK4A in uveal melanoma was shown to be more common in uveal melanoma cell lines, in contrast to primary tumors. This observation might indicate that tumors with RASSF1A methylation are more oncogenic and are likely to be established as cell lines and is consistent with the observation that, in contrast to the cell lines, one third of the positive tumors presented methylated and unmethylated DNA, suggesting that heterogeneity of the primary tumor is lost in the cell line cultures.

An obvious example of tumor heterogeneity is shown in Figure 1. Primary tumor sample 11 seems to be unmethylated, but the tiny shoulder at the position of methylated DNA suggests the presence of a minimal amount of methylated DNA. An attempt to validate this minor methylated DNA fraction through digestion analysis was inconclusive. Future analysis using more sensitive and advanced techniques should be conducted to clarify the possible presence of small quantities of methylated DNA in such samples.

Although RASSF1a methylation alone may not be responsible for uveal development, it could be a contributing factor for uveal melanoma tumor formation and progression. The three cell lines derived from one patient (OMM-1.3 and OMM-1.5 are from two different metastases, and Mel-270 is from the primary tumor) were all methylated. The metastatic lesion derived from the same patient bearing the RASSF1a methylated tumor 31 showed two melting peaks, indicating a mixture of methylated and unmethylated DNA probably as a result of contamination of the sample with...
normal tissue. Furthermore, RASSF1a promoter hypermethylation was found to be associated with the development of metastatic disease \( (P = 0.041) \), and the presence of methylation in the primary tumor and its metastasis indicates a role for methylated RASSF1a in tumor progression. A correlation with decreased survival could not be established in this study, but a positive trend was observed \( (P = 0.063) \).

Based on these findings, we propose that the epigenetic regulation of the RASSF1a gene through promoter hypermethylation may play an important role in the development and progression of uveal melanoma, which once again adds to the proposed high frequency of methylation in uveal melanoma compared with normal tissue. We also propose that RASSF1a might serve as a tumor marker in uveal melanoma.

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References