RASSF10 Promoter Hypermethylation Is Frequent in Malignant Melanoma of the Skin but Uncommon in Nevus Cell Nevi

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The Ras association domain family (RASSF) consists of several tumor suppressor genes, which are frequently silenced in human cancers. We analyzed the epigenetic inactivation of *RASSF2* and *RASSF10* in malignant melanoma (MM) of the skin, including 5 MM cell lines, 28 primary MM, 33 metastases of MM, 47 nevus cell nevi (NCN), and 22 control tissues. The *RASSF2* promoter was epigenetically downregulated in two MM cell lines only, but not in any of the investigated tumor samples. In contrast, hypermethylation of the *RASSF10* promoter was found in all investigated cell lines, 19/28 (68%) of the primary MM and 30/33 (91%) of the MM metastases, 2/18 (11%) of the dysplastic NCN, and 0/29 (0%) of the non-dysplastic NCN (difference between MM and all nevi, $P<0.001$). *RASSF10* promoter hypermethylation correlated with a reduced *RASSF10* mRNA expression in 3/4 MM cell lines, and treatment with a DNA methylation inhibitor reactivated *RASSF10* transcription. Furthermore, immunohistological RASSF10 expression corresponds negatively to its promoter methylation state. In summary, RASSF10 proved to be a characteristically epigenetically silenced tumor suppressor in melanomagenesis, and analysis of *RASSF10* methylation status represents a new candidate tool to assist in discrimination between MM and NCN.

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INTRODUCTION

Malignant melanoma (MM) is a malignant tumor of the skin with a marked rising incidence (Parkin *et al.*, 2005). Several molecular pathways have been found to be deregulated in melanocytic tumors, including the Ras-RAF pathway, the $p16^{INK4a}$-RB pathway, and the PI3K-AKT pathway (Miller and Mihm, 2006; Dahl and Guldberg, 2007). Deregulation of these pathways is accomplished through activation of proto-oncogenes and inactivation of tumor suppressor genes. Besides mutation, the epigenetic silencing of tumor suppressor genes is a frequent event in malignant tumor (Jones and Baylin, 2002; Rothhammer and Bosserhoff, 2007). In MM, *pTEN* promoter hypermethylation but not *BRAF* mutations have negative survival effects (Lahtz *et al.*, 2010). This inactivation is achieved by aberrant methylation of the CpG island promoters in tumors (Jones and Baylin, 2007). In this context, Ras effectors are of special interest. The Ras association domain family (RASSF) is a group of Ras effectors. It consists of 10 different genes (*RASSF1–RASSF10*) that are expressed into different isoforms (van der Weyden and Adams, 2007; Richter *et al.*, 2009). Several of these genes (*RASSF1A, RASSF2A, RASSF5 [NORE1], RASSF6*) encode tumor suppressors, which are involved in proliferation, cell cycle regulation, microtubule stability, and apoptosis (Khokhlatchev *et al.*, 2002; Vos *et al.*, 2003; Allen *et al.*, 2007; Richter *et al.*, 2009, 2010; Schagdarsurengin *et al.*, 2010). Epigenetic inactivation of RASSF genes occurs at high frequencies in different tumors. The most prominent member of the family is the RASSF1A tumor suppressor, which is frequently epigenetically silenced in different cancers (Dammann *et al.*, 2000). *RASSF1A* is inactivated through hypermethylation of its CpG island promoter in primary MM (Spugnardi *et al.*, 2003; Furuta *et al.*, 2004; Hoon *et al.*, 2004; Reifenberger *et al.*, 2004; Rastetter *et al.*, 2007; Tellez *et al.*, 2009). In addition to *RASSF1A*, epigenetic silencing of *RASSF5* has been reported in different tumor entities (Tommasi *et al.*, 2002; Hesson *et al.*, 2003; Schagdarsurengin *et al.*, 2006, 2010; Steinmann *et al.*, 2009). In MM, methylation of *RASSF5* was not

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Abbreviations: ACTB, β-actin; aza, 5-aza-2′-deoxycytidine; COBRA, combined bisulfite restriction analysis; LN, lymph node; MM, malignant melanoma; NCN, nevus cell nevus; NM, nodular melanoma; RASSF, Ras association domain family; SM, skin metastasis; SSM, superficial spreading melanoma

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observed (Tommasi *et al.*, 2002). RASSF10 is a new member of the N-terminal RASSF family that also includes RASSF7, RASSF8, and RASSF9 (Falvella *et al.*, 2006; Sherwood *et al.*, 2008). Hypermethylation of *RASSF3*, *RASSF4*, *RASSF7*, and *RASSF8* occurs rarely in human cancers, and *RASSF6* and *RASSF9* contain no CpG island promoters (Dammann *et al.*, 2005; Richter *et al.*, 2009; Underhill-Day *et al.*, 2011). Recently, we have reported that *RASSF2* and *RASSF10* can be inactivated in some human cancers (Schagdarsurengin *et al.*, 2009, 2010; Steinmann *et al.*, 2009). *RASSF10* is frequently hypermethylated in childhood leukemias, thyroid cancer, and glioma (Hesson *et al.*, 2009; Schagdarsurengin *et al.*, 2009; Hill *et al.*, 2011). In MM, epigenetic silencing of *RASSF2* and *RASSF10* has not been investigated.

The aim of our study was to illuminate the epigenetic deactivation of *RASSF2* and *RASSF10* in cutaneous MM. Here, we report that *RASSF10* is—in contrast to benign melanocytic lesions—frequently hypermethylated in MM.

RESULTS

RNA expression of *RASSF2* and *RASS10* in melanoma cell lines

First, we analyzed the expression of *RASSF2* and *RASSF10* in five MM cell lines by reverse transcription-PCR (Figure 1). *RASSF2* mRNA was reduced in C8161 and MeWo (Figure 1a). Treatment of these two cell lines with 5-aza-2′-deoxycyticine (aza), a substance that inhibits *de novo* DNA methylation (Jones and Taylor, 1980), resulted in increased *RASSF2* mRNA levels (Figure 1a and b). Reduced expression of *RASSF10* was found in buf1280, C8161, and MeWo compared with IGR1 (Figure 1a and c). Treatment of these cell lines with aza significantly increased the expression of *RASSF10* for buf1280, C8161, and MeWo (Figure 1a and c). These results indicate that silencing of *RASSF2* and *RASSF10* can occur in MM cell lines.

Methylation of *RASSF2* promoter was not detected in primary MM

Subsequently, we analyzed the methylation status of the *RASSF2* promoter region in five MM cell lines (buf1280, C8161, IGR1, MeWo, and SK-Mel13), 23 primary MM, 28 metastases of MM, and 38 nevus cell nevi (NCN), as well as 3 control tissues by combined bisulfite restriction analysis (COBRA; Figure 2). Only the MM cell lines C8161 and MeWo were partially methylated at the *RASSF2* promoter (Figure 2a). All analyzed primary tumors, metastases, NCN, or control tissues were unmethylated for *RASSF2* (Figure 2b and c and Supplementary Tables S1 and S2 online). Our data suggest that epigenetic inactivation of RASSF2 occurred only in two MM cell lines, and this correlates with its expression data.

RASSF10 promoter hypermethylation occurs frequently in melanoma, is a rare event in dysplastic nevi, and was not found in non-dysplastic nevi

All five analyzed cell lines were at least partially methylated for the *RASSF10* promoter (Figure 3a). The methylation status of *RASSF10* for C8161, buf1280, and IGR1 was confirmed by pyrosequencing (Figure 4). High percentage of CpG methylation at six CpG was found in C8161 and buf1280, with an average of 80% and 72% methylation, respectively (Figure 4). For IGR1, which exhibits weaker methylation in COBRA, an average of 11% was revealed by pyrosequencing (Figure 4). The RASSF10 methylation status in MM cell lines matched with its expression data.

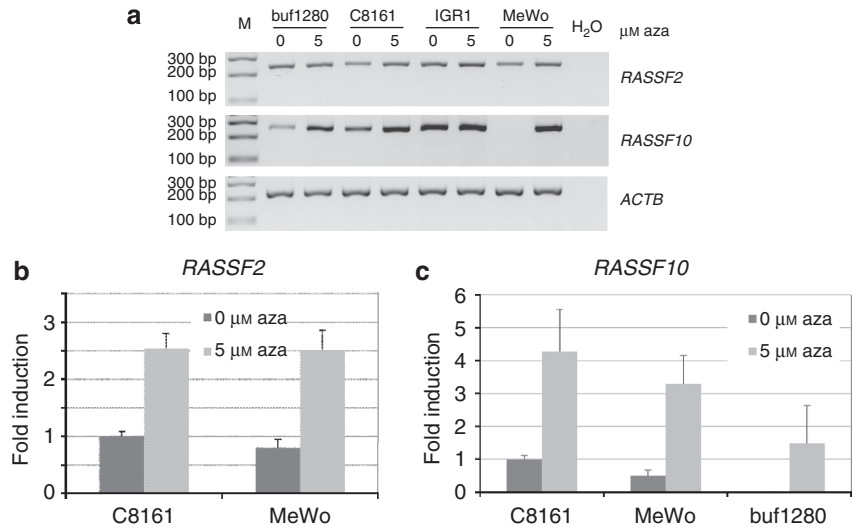

Figure 1. RNA expression of *RASSF2 and RASSF10* in malignant melanoma (MM) cell lines. MM cell lines (buf1280, C8161, IGR1, and MeWo) were treated for 4 days with the indicated concentrations of 5-aza-deoxycyticine (aza). RNA was isolated and analyzed by reverse transcription PCR (RT-PCR). (**a**) Products for *RASSF2* (236 bp) and *RASSF10* (243 bp) and a 100 bp ladder (M) were resolved on 2% gel. Expression of β-actin (*ACTB*; 225 bp) was determined as a control for RNA integrity. (**b**) Reexpression of *RASSF2* was analyzed by quantitative RT-PCR, normalized to *ACTB* expression, and plotted relative to the C8161 control (=1). (**c**) Expression of *RASSF10* was normalized to *ACTB* and plotted relative to C8161 (=1).

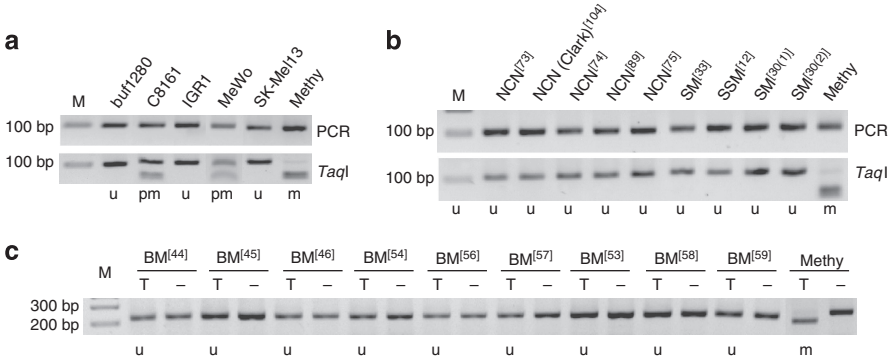

Figure 2. Methylation analysis of *RASSF2*. Methylation of the *RASSF2* promoter in (**a**) malignant melanoma (MM) cell lines, (**b**) nevus cell nevi (NCN), skin metastases of MM (SM), or primary MM (superficial spreading melanoma (SSM)), and (**c**) brain metastases of MM (BM). The methylation status of the *RASSF2* promoter was analyzed by combined bisulfite restriction analysis. PCR products (100 bp or 224 bp) from bisulfite-treated DNA were digested (*Taq*I, T) and mock digested (–) and resolved on 2% gel. *In vitro*-methylated DNA (Methy) was used as positive control. Sizes of a 100 bp ladder (M) are marked. Methy, methylation control; m, methylated; pm, partially methylated; u, unmethylated; numbers in brackets, case numbers of Supplementary Tables S1 and S2 online.

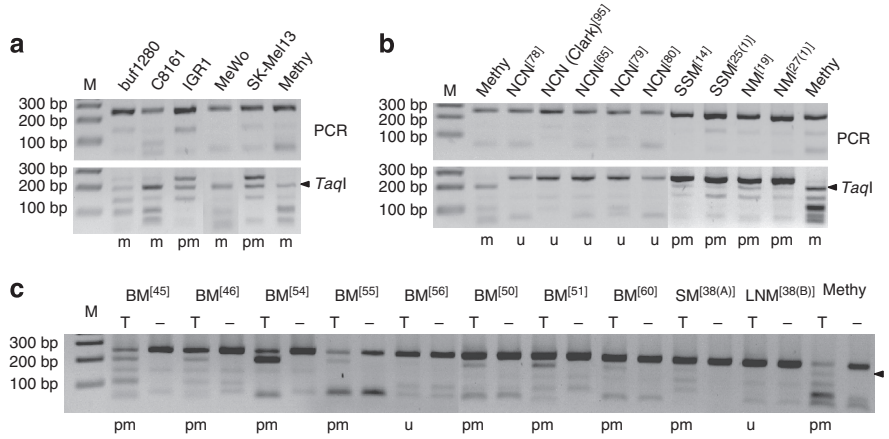

Figure 3. Methylation analysis of *RASSF10*. Methylation of the *RASSF10* promoter in (**a**) malignant melanoma (MM) cell lines, (**b**) nevus cell nevi (NCN) or primary MM (superficial spreading melanoma (SSM), nodular melanoma (NM)), and (**c**) MM metastasis. PCR products (241 bp) were digested (*Taq*I, T) and mock digested (–) and resolved on 2% gel. The arrowheads indicate a digest product at 192 bp. Sizes of a 100 bp ladder (M) are marked (Methy, methylation control; m, methylated; pm, partially methylated; u, unmethylated; numbers in brackets, case numbers of Supplementary Tables S1 and S2 online). BM, brain metastasis; LNM, lymph node metastasis; SM, skin metastasis.

Subsequently, we analyzed the methylation of the *RASSF10* in primary MM, MM metastases, NCN, and controls by COBRA (Figure 3 and Supplementary Tables S1 and S2 online). In 19 out of 28 (68%) primary MM, the promoter of *RASSF10* was partially methylated and a restriction product with 192 bp was detected (Figure 3b and Supplementary Tables S1 and S2 online). Methylation of selected MM samples was confirmed by pyrosequencing (Figure 4). Moreover, methylation of *RASSF10* was found in 10 out of 10 (100%) skin metastases of MM, 5 out of 6 (83%) lymph node (LN) metastases of MM, and 15 out of 17 (88%) brain metastases of MM (Figure 3c and Supplementary Tables S1 and S2 online). In contrast, none of the 29 (0%) non-dysplastic NCN showed methylation of *RASSF10*. We analyzed a group of dysplastic (Clark) NCN with intermediate dignity between completely benign NCN and MM. In all, 2 out of 18 (11%) Clark NCN showed methylation of *RASSF10* by COBRA (Figure 3b and Supplementary Tables S1 and S2 online). By pyrosequencing, the background was under 10% hypermethylated cells in all samples that were negative by COBRA (Figure 4).

Statistical analysis revealed highly a significant difference of *RASSF10* promoter methylation state between all MM (primaries and metastases) and NCN (48/60 vs. 2/47 methylated tumors, $P<0.001$, Fisher's exact test, Figure 5). The subgroup analysis between MM primaries alone and NCN was significant as well (19/28 vs. 2/47 methylated tumors, $P=0.014$). In the total of 75 primary melanocytic tumors (28 primary MM, 47 NCN), the sensitivity to detect MM by COBRA of the *RASSF10* promoter was 67.9%, and the specificity of this analysis was 95.7%. Furthermore, we found a slight but significant difference of *RASSF10* promoter state between MM primaries and metastases (19/28 vs. 30/33 methylated tumors, $P=0.025$, two-tailed significance, Mann–Whitney test). In contrast, there was no significant difference in *RASSF10* promoter state detectable between

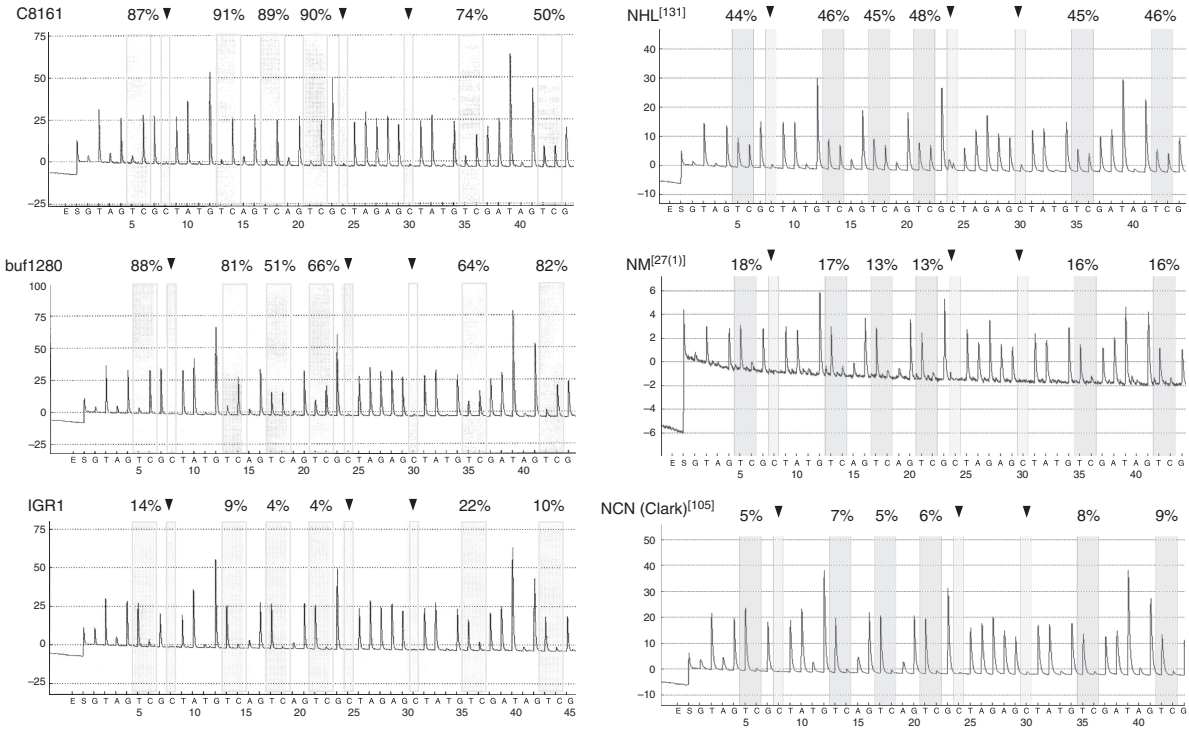

Figure 4. Methylation analysis of the *RASSF10* promoter by pyrosequencing. PCR products obtained from bisulfite PCR of malignant melanoma (MM) cell lines (C8161, buf1280, and IGR1), non-Hodgkin lymphoma (NHL, diffuse large B-cell lymphoma of the skin), nodular MM (NM), and dyplastic (Clark) nevus cell nevi (NCN) were analyzed by pyrosequencing. The frequency of CpG methylation is indicated (arrowheads, bisulfite controls; numbers in brackets, case numbers of Supplementary Tables S1 and S2 online).

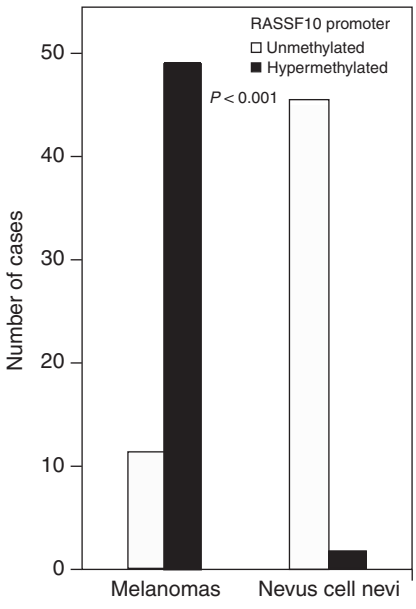

Figure 5. Comparison between malignant melanoma (primaries, metastases) and nevus cell nevi (including dysplastic Clark nevi). Bar chart indicates the number of tumors with unmethylated or hypermethylated RASSF10 promoter regions. Fisher's exact test.

in situ and invasive MM primaries (4/6 vs. 15/22), or different types of metastases (skin and LN metastases versus brain metastases, 15/16 vs. 15/17, respectively). In summary, our data suggest that epigenetic silencing of *RASSF10* is a frequently found in cutaneous MM and MM metastases, rarely found in dysplastic NCN, and was not detectable in non-dyplastic NCN.

Immunohistological investigations on RASSF10 expression

The used noncommercial anti-RASSF10 antibody was not suitable for working on paraffin-embedded material (data not shown). Thus, we analyzed cryosections of five MM metastases (all with hypermethylated *RASSF10* promoter, COBRA) and five NCN (all with unmethylated *RASSF10* promoter; Figure 6). Although RASSF10 expression was strong in two out of five NCN and weak in three out of five NCN, focal strong RASSF10 expression was detectable in one MM, weak diffuse expression in one MM, and no expression was detectable in three out of five MM (Figure 6g–k). We conclude that RASSF10 expression corresponds negatively to its promoter methylation state, but there is no strong correlation between promoter methylation and negative gene expression.

RASSF10 promoter hypermethylation and clinical data

In the group of patients who suffered from primary MM with hypermethylated *RASSF10* promoter, 9/28 died during the follow-up. In contrast, there was no death reported in the unmethylated MM group (nine patients). This tendency proved no significance in univariate Cox regression owing to the asymmetry of the two groups together with a relative

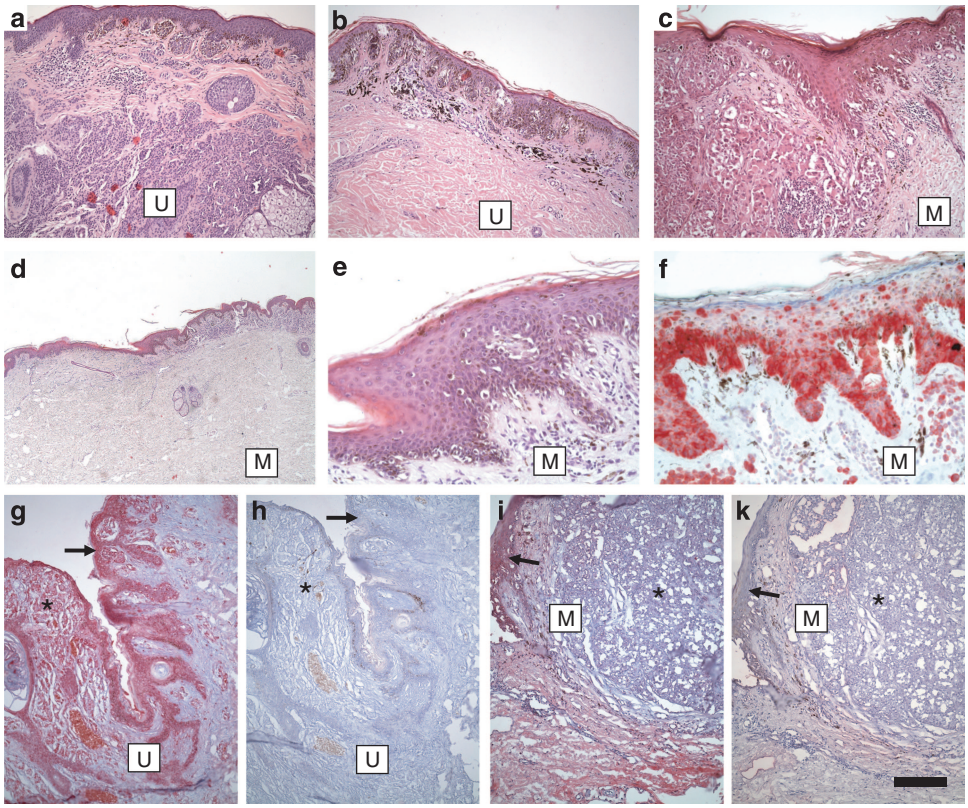

Figure 6. Microphotographs of selected samples (case numbers of Supplementary Tables S1 and S2 online); RASSF10 promoter state: M, hypermethylated; U, unmethylated (□). (**a**) Compound nevus (case 79). (**b**) Dysplastic (Clark) nevus (case 96). (**c**) Superficial spreading melanoma (case 18). (**d**–**f**) Clark nevus with initial transition into malignant melanoma (MM; case 7). (**f**) Focal intraepidermal transmigration of melanocytes (melan A immunostaining). (**g**–**k**) Anti-RASSF10 antibody (red). (**g**) Compound nevus with unmethylated *RASSF10* promoter, RASSF10 expression in nevus tissue (*), epidermis (arrow), and connective tissue cells. (**h**) Isotype control. (**i**) Skin metastasis of MM with hypermethylated *RASSF10* promoter without expression of RASSF10 gene product (*), epidermis (arrow), and connective tissue cells are RASSF10 positive. (**k**) Isotype control. Bar = 50 μm (**a**–**c**, **g**–**k**); 100 μm (**d**); 25 μm (**e**, **f**).

small sample size and a relatively short follow-up time. Further details of survival and recurrence states of the patients are given in Supplementary Tables S1 and S2 online. Furthermore, we found no correlation of sex, age, tumor thickness or ulceration of the primaries, mitotic index (number of mitoses in 10 microscopic high-power fields), inflammation (no/few versus medium/high inflammatory infiltration), predominating tumor cell type (epitheloid versus other), or the percentage of tumor tissue with *RASSF10* promoter state (age and tumor thickness, Spearman correlation, other factors, Fisher's exact test). The distribution of age was asymmetric with nonsignificant higher ages in the *RASSF10* hypermethylated melanocytic samples (Supplementary Figure S2 online). Thus, we studied the predictive power of age and that of the dignity diagnosis together in a logistic regression model (Supplementary Table S4 online). We found no predictive influence of age but a high impact of the diagnosis (NCN versus MM) on *RASSF10* promoter methylation state.

DISCUSSION

The RASSF family consists of 10 members, which encode a Ras association domain. RASSF10 was recently identified as a member that harbors a N-terminal Ras association domain and lacks the Sav-Ras-Hippo interaction domain that is found in the classical RASSF members 1–6 (Sherwood *et al.*, 2008; Richter *et al.*, 2009; Underhill-Day *et al.*, 2011). Here we report that *RASSF10* is frequently hypermethylated in cutaneous MM, but not in NCN. To the best of our knowledge, this is the first study that reports intensive hypermethylation of the *RASSF10* promoter in MM and its metastases. Previously, it has been reported that *RASSF10* is epigenetically inactivated in thyroid, childhood acute lymphocytic leukemias (ALL), and glioma (Hesson *et al.*, 2009; Schagdarsurengin *et al.*, 2009; Hill *et al.*, 2011). We have previously shown that primary thyroid cancers of patients with cancerous LNs were significantly hypermethylated for *RASSF10* compared with those with non-affected LNs (Schagdarsurengin *et al.*, 2009). In childhood ALL, *RASSF10* was methylated in 16% of B-ALL and 88% of T-ALL (Hesson *et al.*, 2009). Recently, its has been reported that *RASSF10* was frequently methylated in grade II–III astrocytomas and grade IV primary glioblastomas (68%), but was unmethylated in grade I astrocytomas and in age-matched control brains (Hill *et al.*, 2011). In secondary glioblastomas, *RASSF10* methylation was an independent prognostic factor associated with worst progression-free survival and overall survival, and occurred at an early stage in their development (Hill *et al.*, 2011).

Here we reveal that hypermethylation of *RASSF10* occurs frequently in MM, and treatment of three MM cell lines with an inhibitor of DNA methyltransferases (aza) reactivated its expression. In IGR1 and SK-MEL13 cells, which exhibit low methylation rate at the *RASSF10* promoter, aza treatment resulted in a reduced *RASSF10* expression (data not shown). This reduced transcription rate could be attributed to the cytotoxic effect of aza on melanoma cells (Cortvrindt *et al.*, 1987). For the cell lines (buf1280, MeWo and C8161) with high percentage of methylation, a significant reexpression of *RASSF10* occurred. This indicates that *RASSF10* methylation causes its epigenetic silencing in MM. Immunohistologically, RASSF10 expression corresponds to the *RASSF10* promoter state, but we have not found strong correlation between promoter methylation and negative gene expression, potentially because of partial methylation in the investigated MM or regulation of the RASSF10 expression by other factors. Thus, it would be important to analyze the expression of RASSF10 in normal melanocytes. In our series of immunohistological experiments, intraepidermal melanocytes seemed to be positive with respect to RASSF10 expression (Figure 6). We were not able to overcome the problem of separation of intraepidermal melanocytes from the surrounding RASSF10+ epidermal keratinocytes by the available antibody (Supplementary Figure S1 online and data not shown). We used a noncommercial RASSF10 antibody that proved in our hands as unsuitable to analyze paraffin-embedded material. The necessity to use cryosections and the lack of a strong correlation between promoter methylation state and the immunohistological results imply that the antibody alone is not a sufficient tool for analysis of great series of samples.

We have also analyzed the epigenetic inactivation of *RASSF2*, a member of the classic RASSFs, which encode a C-terminal Ras association domain and Sav-Ras-Hippo domain. Several classic RASSF members are frequently epigenetically silenced in cancer (e.g., RASSF1A, RASSF2, RASSF5) and encode prominent tumor suppressors (van der Weyden and Adams, 2007; Richter *et al.*, 2009). Here, we report that the epigenetic inactivation of *RASSF2* occurs only in MM cell lines, but was not found in primary tumors. This indicates that hypermethylation of *RASSF2* occurs late in melanomagenesis or may arise when cancer cell lines are propagated. It has been shown that methylation of CpG sites is induced by cell culturing (Antequera *et al.*, 1990; Allegrucci *et al.*, 2007).

The biological functions of the N-terminal RASSFs are largely unknown, and the role of these RASSFs in tumorigenesis is still under investigation. Interestingly, *RASSF7, RASSF8*, and *RASSF10* map near various RAS isoforms (Sherwood *et al.*, 2008). In cell culture experiments, overexpression of RASSF10 mediated a reduction in the colony-forming ability of glioma cell lines (Hill *et al.*, 2011). Thus, it will be interesting to conduct functional studies in MM cell lines. Our own unpublished data in prostate cancer show that RASSF10 overexpression inhibits cell growth and induces apoptosis of a prostate cancer cell line. Hill *et al.* (2011) have generated and characterized a RASSF10-specific antibody and demonstrated that RASSF10 subcellular localization is cell cycle dependent, with RASSF10 colocalizing to centrosomes and associated microtubules during mitosis. Although CpG island promoters have been predicted for *RASSF7* and *RASSF8* (van der Weyden and Adams, 2007; Richter *et al.*, 2009), it has not been reported yet whether promoter hypermethylation of *RASSF7* and *RASSF8* occurs in MM.

In summary, our results show that hypermethylation of *RASSF10* promoter occurs frequently in MM, but was not found in NCN. These data suggest that *RASSF10* may encode a new melanoma-specific tumor suppressor gene. Further studies are necessary to elaborate the tumor suppressor function of RASSF10 in melanomagenesis. Furthermore, RASSF10 promoter methylation analysis is a candidate biomarker for an easy applicable tool to differentiate between benign and malignant melanocytic tumors in routine histology.

MATERIALS AND METHODS

Tissues and cell lines

We analyzed 140 samples of 118 tumors, 6 normal skin tissues, and 6 normal LNs of 119 patients (age 54.3 ± 20.4 years/mean ± SD; ratio male/female = 1.7), as well as 5 MM cell lines (Supplementary Tables S1 and S2 online). The material included 31 samples of 28 primary MM (Tis/T1/T2/T3/T4/unclassified (Balch *et al.*, 2009) *n* = 6/10/1/4/6/1) of 28 patients (age 67.7 ± 15.4 years/mean ± SD), 38 samples of 33 MM metastases (10 skin metastases, 6 LN metastases, 17 brain metastases; age 66.2 ± 15.7 years/mean ± SD (*n* = 15, age data of patients with brain metastases not available)), 47 samples of 47 NCN (2 junctional NCN, 14 dermal NCN, 13 compound NCN, 18 dysplastic Clark NCN; age 40.2 ± 17.1 years/mean ± SD), and 24 samples of control tissues (6 normal skin samples, 6 normal LNs, 7 basal cell carcinomas, 1 spiradenoma, 1 keratoakanthoma, as well as 1 diffuse large cell B-cell lymphoma of the skin; age 63.9 ± 15.8 years/mean ± SD). Diagnoses were established and controlled in each single tumoral or normal control sample according to histopathological standards by two experienced dermatopathologists (authors P.H. and W.C.M.; Figure 6). The proportion of tumor tissue was 55.4 ± 30.8%, mean ± SD (range 5–95%). The study was conducted according to the Declaration of Helsinki Principles. All patients signed informed consent at initial clinical investigation. The study was approved by local ethics committees.

Five human MM cell lines—buf1280, C8161, IGR1, MeWo, and SK-Mel13—were obtained from the Medical Faculty of the University of Halle (generous gift from Dr B Seliger and Dr J Wohlrab) and cultured in the recommended medium (Seliger *et al.*, 2001; Klapperstuck *et al.*, 2009). Genomic DNA was extracted from frozen tissues and cultured cells by a standard proteinase K and phenol/chloroform procedure (Dammann *et al.*, 2003).

RNA expression analysis of *RASSF2* and *RASSF10*

MM cancer cell lines were treated for 4 days with 5 μM of aza (Sigma, Taufkirchen, Germany). RNA was isolated using TRIzol-Reagent (Invitrogen, Karlsruhe, Germany; Dammann *et al.*, 2003). To eliminate DNA contamination, 1 μg RNA was incubated with 1 U DNase I (Fermentas GmbH, St Leon-Rot, Germany), 1 μl 10 × DNase I buffer, and 10 μl DEPC-treated water. After 15 minutes of incubation at room temperature, DNase was inactivated by incubating at 65 °C for

15 minutes. The residue of the mixture was directly reverse transcribed using poly-dT primers and random hexamers in 20 µl of reverse transcriptase-mix (Promega, Heidelberg, Germany) for 1 hour at 42 °C. Subsequently, 2 µl of complementary DNA was amplified for 34, 45, and 25 cycles with primers for *RASSF2*, *RASSF10*, and β-actin (*ACTB*), respectively, or analyzed by real-time PCR (Rotor-Gene 2000, Corbett Life Science, Sydney, Australia) with Syber Green. Results were normalized to *ACTB*. PCR products were separated on 2% Tris-borate EDTA agarose gels. Primer pairs for *RASSF2*, *RASSF10*, and *ACTB* are listed in Supplementary Table S3 online.

Methylation analysis

Methylation of the *RASSF10* or *RASSF2* promoter regions was determined by combined bisulfite restriction analysis (COBRA) and bisulfite pyrosequencing (Xiong and Laird, 1997). Briefly, 100 ng of bisulfite-treated DNA was amplified with 10 pmol of primers in a reaction buffer containing 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 10 pmol of each primer, and 1.5 U Taq polymerase (InViTek GmbH, Berlin, Germany) for 45 cycles. Primer pairs for *RASSF10* and *RASSF2* are listed in Supplementary Table S3 online. A measure of 20–50 ng of PCR products were digested with 10 U of *Taq*I (Fermentas GmBH) and analyzed on a 2% Tris-borate EDTA agarose gel. Pyrosequencing was used for quantitative methylation analysis of six CpGs at the RASSF10 promoter. Therefore, DNA was amplified with a biotinylated reverse primer and sequenced with an internal primer (Supplementary Table S3 online). Pyrosequencing was performed in PyroMark Q24 according to the PyroMark Gold Q24 Reagents Handbook (Qiagen, Hilden, Germany). Methylation percentage for each CpG site was determined.

Immunostaining

Paraffin sections (2 µm) were deparaffinized in graded ethanol, the endogenous peroxidase was blocked (0.3% H_2O_2 in methanol), and heat-induced epitope retrieval was performed by microwaving. Monoclonal mouse anti-human MART-1/Melan-A, Clone A103 (Dako, Glostrup, Denmark) was applied at a concentration of 1:50 (30 minutes, room temperature). Cryosections (3 µm) of shock-frozen tissue material were incubated with polyclonal rabbit anti-RASSF10 (raised against RASSF10 peptide sequence, kindly gifted by Dr F Latif, Medical and Molecular Genetics, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, Institute of Biomedical Research, University of Birmingham, Birmingham, West Midlands B15 2TT, UK; Hill *et al.*, 2011) at a concentration of 1:500 (30 minutes, room temperature). Further steps were performed with the Elite-ABC-Kit (Vector, Burlingame, CA) and the AEC Substrate System (Dako). Finally, all sections were routinely counterstained with hemalaun. In RASSF10 staining, 0.5 µg ml^{-1} primary rabbit antibody isotype control (Zymed, South San Francisco, CA) was used for negative control.

Statistical evaluation

Statistical analysis was carried out using SPSS17 (SPSS, München, Germany). Categorical variables were plotted into contingency tables and evaluated using Fisher's exact test. Linear logistic regression analysis was performed to study the influence of the patient's age on the *RASSF10* methylation state. All reported *P*-values are two-sided and considered significant for $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Allegrucci C, Wu YZ, Thurston A *et al.* (2007) Restriction landmark genome scanning identifies culture-induced DNA methylation instability in the human embryonic stem cell epigenome. *Hum Mol Genet* 16:1253–68

Allen NP, Donninger H, Vos MD *et al.* (2007) RASSF6 is a novel member of the RASSF family of tumor suppressors. *Oncogene* 26:6203–11

Antequera F, Boyes J, Bird A (1990) High levels of *de novo* methylation and altered chromatin structure at CpG islands in cell lines. *Cell* 62: 503–14

Balch CM, Gershenwald JE, Soong S-j *et al.* (2009) Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol* 27:6199–206

Cortvrindt R, Bernheim J, Buyssens N *et al.* (1987) 5-Azacytidine and 5-aza-2′-deoxycytidine behave as different antineoplastic agents in B16 melanoma. *Br J Cancer* 56:261–5

Dahl C, Guldberg P (2007) The genome and epigenome of malignant melanoma. *APMIS* 115:1161–76

Dammann R, Li C, Yoon JH *et al.* (2000) Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet* 25:315–9

Dammann R, Schagdarsurengin U, Liu L *et al.* (2003) Frequent RASSF1A promoter hypermethylation and K-ras mutations in pancreatic carcinoma. *Oncogene* 22:3806–12

Dammann R, Schagdarsurengin U, Seidel C *et al.* (2005) The tumor suppressor RASSF1A in human carcinogenesis: an update. *Histol Histopathol* 20:645–63

Falvella FS, Manenti G, Spinola M *et al.* (2006) Identification of RASSF8 as a candidate lung tumor suppressor gene. *Oncogene* 25:3934–8

Furuta J, Umebayashi Y, Miyamoto K *et al.* (2004) Promoter methylation profiling of 30 genes in human malignant melanoma. *Cancer Sci* 95:962–8

Hesson L, Dallol A, Minna JD *et al.* (2003) NORE1A, a homologue of RASSF1A tumour suppressor gene is inactivated in human cancers. *Oncogene* 22:947–54

Hesson LB, Dunwell TL, Cooper WN *et al.* (2009) The novel RASSF6 and RASSF10 candidate tumour suppressor genes are frequently epigenetically inactivated in childhood leukaemias. *Mol Cancer* 8:42

Hill VK, Underhill-Day N, Krex D *et al.* (2011) Epigenetic inactivation of the RASSF10 candidate tumor suppressor gene is a frequent and an early event in gliomagenesis. *Oncogene* 30:978–89

Hoon DS, Spugnardi M, Kuo C *et al.* (2004) Profiling epigenetic inactivation of tumor suppressor genes in tumors and plasma from cutaneous melanoma patients. *Oncogene* 23:4014–22

Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 3:415–28

Jones PA, Baylin SB (2007) The epigenomics of cancer. *Cell* 128:683–92

Jones PA, Taylor SM (1980) Cellular differentiation, cytidine analogs and DNA methylation. *Cell* 20:85–93

Khokhlatchev A, Rabizadeh S, Xavier R *et al.* (2002) Identification of a novel Ras-regulated proapoptotic pathway. *Curr Biol* 12:253–65

Klapperstuck T, Glanz D, Klapperstuck M *et al.* (2009) Methodological aspects of measuring absolute values of membrane potential in human cells by flow cytometry. *Cytometry A* 75:593–608

Lahtz C, Stranzenbach R, Fiedler E *et al.* (2010) Methylation of PTEN as a prognostic factor in malignant melanoma of the skin. *J Invest Dermatol* 130:620–2

Miller AJ, Mihm MC Jr (2006) Melanoma. *N Engl J Med* 355:51–65

Parkin DM, Bray F, Ferlay J *et al.* (2005) Global cancer statistics, 2002. *CA Cancer J Clin* 55:74–108

Rastetter M, Schagdarsurengin U, Lahtz C *et al.* (2007) Frequent intra-tumoural heterogeneity of promoter hypermethylation in malignant melanoma. *Histol Histopathol* 22:1005–15

Reifenberger J, Knobbe CB, Sterzinger AA *et al.* (2004) Frequent alterations of Ras signaling pathway genes in sporadic malignant melanomas. *Int J Cancer* 109:377–84

Richter AM, Pfeifer GP, Dammann RH (2009) The RASSF proteins in cancer; from epigenetic silencing to functional characterization. *Biochim Biophys Acta* 1796:114–28

Richter AM, Schagdarsurengin U, Rastetter M *et al.* (2010) Protein kinase A-mediated phosphorylation of the RASSF1A tumour suppressor at Serine 203 and regulation of RASSF1A function. *Eur J Cancer* 46:2986–95

Rothhammer T, Bosserhoff AK (2007) Epigenetic events in malignant melanoma. *Pigment Cell Res* 20:92–111

Schagdarsurengin U, Gimm O, Dralle H *et al.* (2006) CpG island methylation of tumor-related promoters occurs preferentially in undifferentiated carcinoma. *Thyroid* 16:633–42

Schagdarsurengin U, Richter AM, Hornung J *et al.* (2010) Frequent epigenetic inactivation of RASSF2 in thyroid cancer and functional consequences. *Mol Cancer* 9:264

Schagdarsurengin U, Richter AM, Wohler C *et al.* (2009) Frequent epigenetic inactivation of RASSF10 in thyroid cancer. *Epigenetics* 4:571–6

Seliger B, Ritz U, Abele R *et al.* (2001) Immune escape of melanoma: first evidence of structural alterations in two distinct components of the MHC class I antigen processing pathway. *Cancer Res* 61: 8647–50

Sherwood V, Manbodh R, Sheppard C *et al.* (2008) RASSF7 is a member of a new family of RAS association domain-containing proteins and is required for completing mitosis. *Mol Biol Cell* 19:1772–82

Spugnardi M, Tommasi S, Dammann R *et al.* (2003) Epigenetic inactivation of RAS association domain family protein 1 (RASSF1A) in malignant cutaneous melanoma. *Cancer Res* 63:1639–43

Steinmann K, Sandner A, Schagdarsurengin U *et al.* (2009) Frequent promoter hypermethylation of tumor-related genes in head and neck squamous cell carcinoma. *Oncol Rep* 22:1519–26

Tellez CS, Shen L, Estecio MR *et al.* (2009) CpG island methylation profiling in human melanoma cell lines. *Melanoma Res* 19:146–55

Tommasi S, Dammann R, Jin SG *et al.* (2002) RASSF3 and NORE1: identification and cloning of two human homologues of the putative tumor suppressor gene RASSF1. *Oncogene* 21:2713–20

Underhill-Day N, Hill V, Latif F (2011) N-terminal RASSF family: RASSF7-RASSF10. *Epigenetics* 6:284–92

van der Weyden L, Adams DJ (2007) The Ras-association domain family (RASSF) members and their role in human tumourigenesis. *Biochim Biophys Acta* 1776:58–85

Vos MD, Ellis CA, Elam C *et al.* (2003) RASSF2 is a novel K-Ras-specific effector and potential tumor suppressor. *J Biol Chem* 278: 28045–51

Xiong Z, Laird PW (1997) COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res* 25:2532–4