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Frequent intra-tumoural heterogeneity of promoter hypermethylation in malignant melanoma

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Summary. To investigate intra-tumoural coexistence and heterogeneity of aberrant promoter hypermethylation of different tumour suppressor genes in melanoma, we analyzed the intra-tumoural distribution of promoter methylation of *RASSF1A*, *p16*, *DAPK*, *MGMT*, and *Rb* in 339 assays of 34 tumours (15 melanoma primaries, 19 metastases) by methylation-specific PCR, correlation to histopathology and RASSF1A expression. We detected promoter hypermethylation of at least one gene in 74% of tumours (30%, 52%, 33%, 20%, and 40% for *RASSF1A*, *p16*, *DAPK*, *MGMT* and *Rb*, respectively). 70% of the cases exhibited an inhomogeneous methylation pattern (17%, 45%, 33%, 20%, and 40% for *RASSF1A*, *p16*, *DAPK*, *MGMT* and *Rb*, respectively). Samples from the core of the tumours represented the methylation state of the whole tumours more accurately than the periphery. Local intra-tumoural correlation was found between the promoter hypermethylation state of *p16* and *Rb* or *p16* and *DAPK*, or epitheloid tumour cell type and *RASSF1A* or *p16* methylation. Mitosis rate and sex was correlated with methylation of *RASSF1A*. Histological results confirmed that promoter hypermethylation of *RASSF1A* led to aberrant expression patterns. We conclude that intra-tumoural inhomogeneity of promoter hypermethylation is frequent in melanoma and this supports the hypothesis of clonal instability during progression of melanomas. In prognosis studies, missing the intra-tumoural sample representativeness may result in a reduction of the sensitivities or specificities.

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Key words: Melanoma, Tumor suppressor, Intra-tumoral heterogeneity, Epigenetic inactivation

Introduction

Melanomas are highly malignant tumours deriving from melanocytic cells. The incidence of melanoma has increased more quickly in the last 50 years than that of any other cancers, except for lung cancer in women (Parkin et al., 2005). Melanomas develop slowly from normal melanocytes or precursor lesions. During tumour growth they typically acquire macro- and micro-morphological inhomogeneity with juxtaposition of different pigment patterns, regression zones, vascular arrangements, or tumour cell types. There is only scant knowledge on intra-tumoural differences in molecular alterations of melanomas. Variations of mutations between different metastases of melanoma have been reported (Morita et al., 1998; Nakayama et al., 2001; Lotem et al., 2003). Recently, we have shown that primary melanomas can be regarded as genetically instable resulting in polyclonality in relatively early stages of development (Helmbold et al., 2005). According to this result, different tumour cell clones tend to either spatial separation or combination within the same tumour areas. Different mutations and epigenetic changes are reported to take part in the pathogenesis of melanoma (Hussein, 2004; van Doorn et al., 2005). There is a nearly complete lack of further data on the mechanisms of developing clonal heterogeneity. Functional silencing of tumour suppressor genes by hypermethylation of their promoters seems to be a

Abbreviations: DAPK, death-associated protein kinase; MGMT, O6-methylguanine DNA methyltransferase; MSP, methylation specific PCR; RASSF1A, Ras association domain family protein 1A; Rb, retinoblastoma.

crucial event in tumourigenesis (Jones and Baylin, 2002). Promoters appear predominantly in CpG-rich DNA sequences termed CpG islands (Bird, 1986). Active promoters are unmethylated and their hypermethylation leads to deactivation with the consequence of under-expression of the affected tumour suppressor. Within a tumour cell, the methylation state is transferred clonally by DNA replication (mitosis) from the mother to the daughter cells. This epigenetic pathway of tumour suppressor gene inactivation was utilized by other authors in a multistage model of skin carcinogenesis (Fraga et al., 2004). Previously, we reported that aberrant methylation of the tumour suppressor *RASSF1A* is a frequent event in the pathogenesis of tumours, including melanoma (Dammann et al., 2000, 2005; Spugnardi et al., 2003). This observation was confirmed by others (Furuta et al., 2004; Hoon et al., 2004; Reifenberger et al., 2004). Hypermethylation of *p16*, *MGMT* and *DAPK* were previously reported in cutaneous melanoma (Straume et al., 2002; Hoon et al., 2004; van Doorn et al., 2005). It was reported that disruption of the Rb and p16 pathway is involved in tumourigenesis of sporadic melanoma (Maelandsmo et al., 1996; Kannan et al., 2003). To our knowledge epigenetic inactivation of Rb was not analyzed in primary cutaneous melanoma and a detailed investigation of aberrant promoter methylation of tumour suppressors in different histology-correlated intra-tumoural samples was previously not performed.

In this study, we investigated the intra-tumoural differences regarding the methylation state of the promoter CpG islands of five different genes involved in the pathomechanism of melanoma development: *Ras Association Domain Family 1 (RASSF1A)*, *p16 Inhibitor of CyclinD Dependant Kinase 4 (p16)*, *Death-Associated Protein Kinase (DAPK)*, *O6-Methylguanine DNA Methyl Transferase (MGMT)*, and the *Retinoblastoma Susceptibility Gene (Rb)*. In contrast to other studies, we focused our investigation on as complete as possible a representation of the whole tumours. Spatial assignment of the different samples to macro-morphology and histological tumour parameters was guaranteed by serial sectioning. Here, we report that hypermethylation of all five investigated tumour suppressor promoters occurred frequently in melanoma. In several tumour samples, heterogeneous methylation profiles were detected and this observation may result from a polyclonal transformation of melanoma.

Material and methods

Patients and tumours

We studied 101 samples of 34 tumours (15 melanomas primaries, and 19 metastases of malignant melanomas) of 23 patients randomly selected from the melanoma register of the Martin Luther University Halle-Wittenberg, Germany. Details of the material are given in Table 2. The study received approval by the local Ethics Committee of the Medical Faculty at the University of Halle. Controls were analyzed from same cases using histologically-controlled non-tumoural epidermal and dermal tissue.

Intra-tumoural sampling

Tumours were prospectively sampled. After resection, tumours were transported in buffered 4% formaline and totally serially sectioned within two to five hours after resection by a dermatopathologist. All tumours were totally cut into 3-6 mm broad serial sections that represented the total melanoma. In this process, it was guaranteed that each macroscopic different surface area was represented in at least one section. Each section was divided into two corresponding subsections that shared one identical cutting edge. The bigger subsection (2.5-5 µm broad) was further fixed in formaline overnight, paraffin embedded, and prepared for histological investigations in order to establish the diagnoses and to perform immunohistology. The corresponding subsection (0.5 to 1 µm broad) was exempted from visible non-tumoural tissue by a fine scalpel using a 10x magnifying glass (Zeiss, Jena, Germany), immediately snap frozen in liquid nitrogen, and stored at -70°C for DNA isolation. A drawing of each tumour was made for documentation of different sections. By this method, a minimum of two subsections was analyzed in very small melanoma primaries; the maximum was ten subsections in a melanoma primary with a larger surface. For metastases, single samples of each metastasis were obtained (in case of very small metastases), but the whole case had to contain multiple samples (from a single and/or different metastases). For analysis, samples were assigned according to their intratumoural position to edge (lateral) or core (more central) location. Edge position was defined as to be within the outer most lateral 2 mm margin of the tumour. All other samples were assigned to a core position. In tumours with ≥ 3 analysed samples, one to two samples could be identified as edge originating. In all tumours, at least one sample originated from the core.

Bisulfite modification of genomic DNA and MSP analyses

Frozen subsections were used for DNA isolation. Genomic DNA was extracted from frozen tissues by a standard phenol/chloroform procedure. Methylation of the promoter regions was determined by bisulfite modification of genomic DNA (Clark et al., 1994; Dammann et al., 2000). Promoter methylation of tumour suppressor genes was investigated by methylation-specific PCR (MSP) with the primers and conditions listed in Table 1 (Herman et al., 1996; Schagdarsurengin et al., 2002). Briefly, PCR was performed with methylation-specific primers and unmethylation-specific primers using 100 ng of the bisulfite-modified genomic DNA as templates for 35 to 45 cycles at 95°C for 30 s,

Tan (54°C to 60°C) for 30 s and 72°C for 30 s in 25 µL containing 25 mM NTPs, 20 pmol primers and 1 unit of Taq polymerase (Invitek, Berlin, Germany). 10 µL of PCR products were analyzed on a 2% Tris-borate EDTA agarose gel. All visible PCR products obtained by methylation-specific PCR amplification were classified as methylated samples. To confirm the results, each PCR was repeated with a different bisulfite modified DNA isolated from the same section.

Immunostaining

5 µm paraffin sections were deparaffinized in graded ethanol, the endogenous peroxidase was blocked (0.3% H_2O_2 in methanol), and an antigen demasking was performed by microwaving. Affinity-purified monoclonal mouse anti-human RASSF1A (eBioscience, San Diego, CA), mouse monoclonal IgG1 anti-p16 INK 4a (Clone 16P04/JC2, Neomarkers, Freemont, CA, USA), rabbit anti-human Rb (C-15/sc50, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), or mouse monoclonal IgG1 anti-human DAPK (Clone 17, BD Biosciences, Heidelberg, Germany) were applied at concentrations of 1:500, 1:500, 1:200, or 1:100, respectively (30 min, 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 counter-stained with hemalaun.

Histological analysis

Histological slides were independently re-reviewed by two dermatohistopathologists (authors W. Ch. M. and P. H.) for confirmation of the diagnosis according to histopathological diagnostic standards (Tronnier et al., 1997). Immunostained sections were evaluated using previously-published criteria (Blessing et al., 1998). Tumour thickness, Clark's level, ulceration, histological melanoma type, standardized tumour architecture criteria, or nevus association was determined in each primary tumour. Mitotic rate, predominant cell type (Azzola et al., 2003), host lymphocytic response (Spatz et al., 1998) and tumour regression (Barnhill and Mihm, 1993) were determined in each sample.

To ensure that each sample consisted of melanoma tissue, we approximated the relative number of melanoma cells by counting in the corresponding HE sections. These sections shared the same cutting edge with the cryo-conserved material. In the presented study, the percentage of melanoma cells in the total material was 51±34%.

Statistics

All statistical correlations were performed by SPSS 12.0.1 (Chicago, Il, USA).

Results

Frequencies of promoter hypermethylation

The promoter hypermethylation of five tumour-related genes (*RASSF1A*, *p16*, *DAPK*, *MGMT* and *Rb*) was investigated in primary melanomas and cases with melanoma metastases by methylation-specific PCR (Fig. 1 and Table 2). 379 single methylation assays (339 of histologically-proved tumour tissue and 40 of normal inter-individual controls) of 119 samples (101 tumour samples and 18 controls) were evaluated (Table 2). In the tumour samples, we found three different patterns: 1) completely unmethylated promoters (n=259 of 339 assays), 2) completely methylated promoters (n=12), or 3) both unmethylated and methylated promoters in the same sample (n=68) (Fig. 1). The second and third patterns (total or partial methylation, n=80) were summarized as hypermethylated for further statistical analysis (Table 2). All results were confirmed by methylation-specific PCR with a different bisulfite-modified DNA isolated from the same sample section. We found hypermethylation of at least one promoter in 25 out of 34 (74%) of the single tumours and in 18 out of 23 (78%) of the cases (Table 2). The proportion of promoter hypermethylation differed between the

Table 1. Primers and conditions for methylation-specific PCR.

Gene	Forward primer (5´ to 3´)	Reverse primer (5´ to 3´)	Tan in °C	Cycles	Product size in bp
RASSF1A	M: GTGTTAACGCGTTGCGTATC	M: AACCCCGCGAACTAAAAACGA	60	34	93
	U: TTTGGTTGGAGTGTGTTAATGTG	U: CAAACCCCACAAACTAAAAACAA	60	34	105
p16	M: TTATTAGAGGGTGGGGCGGATCGC	M: GACCCCGAACCGCGACCGTAA	65	38	150
	U: TTATTAGAGGGTGGGGTGGATTGT	U: CAACCCCAAACCACAACCATAA	60	38	151
DAPK	M: GGATAGTCGGATCGAGTTAACGTC	M: CCCTCCCAAACGCCGA	54	40	98
	U: GGAGGATAGTTGGATTGAGTTAATGTT	U: CAAATCCCTCCCAAACACCAA	54	40	106
MGMT	M: TTTCGACGTTCGTACCTTTTCGC	M: GCACTCTTCCGAAAACGAAACG	59	40	81
	U: TTTGTGTTTTGATGTTTGTAGGTTTTTGT	U: AACTCCACACTCTTCCAAAAACAAAACA	59	40	93
Rb	M: GGGAGTTTCGCGGACGTGAC	M: ACGTCGAAACACGCCCCG	59	45	180
	U: GGGAGTTTTGTGGATGTGAT	U: ACATCAAAACACACCCCA	59	45	180

M: methylation specific primer; U: unmethylation specific primer; Tan: annealing temperature.

investigated genes (Table 2). We detected the highest rate of cases with promoter hypermethylation for *p16* (52%), followed by *Rb* (40%), *DAPK* (33%), *RASSF1A* (30%), and *MGMT* (20%) (Table 2). We found no significant difference (Chi-square test) between melanoma primaries and metastatic cases: 73% of the studied primaries and 88% of the metastatic cases revealed hypermethylation of at least one promoter of the investigated genes (Table 2). The normal controls were completely unmethylated for all analyzed gene promoters.

Intra-tumoural homogeneity of hypermethylation

The majority (16 out of 23, 70%) of the investigated cases showed an inhomogeneous methylation pattern in at least one of the promoters (Table 2). Of these, the promoter methylation pattern of *RASSF1A* showed highest homogeneity (only 17% of cases were inhomogeneous) and methylation of p16 was most inhomogeneous (45%) (Table 2). The frequencies of inhomogeneous promoter methylation of the other genes were between these two values: 33% for *DAPK*, 20% for *MGMT* and 40% for *Rb*.

Sensitivity of methylation analyses

In the cases with at least one hypermethylated promoter (n=18), the sensitivity to discover case-specific promoter hypermethylation of all investigated genes in a single random sample was 0.51 (95% CI, 0.40 to 0.62). The difference between melanoma primaries (0.57, 95%

Table 2. Patients, tumour features, and promoter methylation in 15 primary melanomas and 8 cases with single or multiple melanoma metastases.

Case	Age/ Sex	Tumour Type	TT (mm), Ulceration (U)	Mitosis per 10 HPF (range)	Number of samples	Gene promoter methylation state[z]				
						RASSF1A	*p16*	*DAPK*	*MGMT*	*Rb*
1	54/m	SSM	in situ		3	-/-/-		-/-/-	-/-/*	-/+/+
2	50/m	SSM	0.35	0	2	-/+	-/-			
3	76/f	SSM	0.45	2	3	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
4	52/f	SSM	0.45 (U)	1-3	2	-/-	*/-	-/-	-/-	-/-
5	45/m	SSM	0.56	0	2	-/-	-/-	-/-	-/-	-/-
6	53/m	SSM	0.68	0-2	2	-/-	-/+	-/+	-/-	-/-
7	51/f	SSM	0.90	0-1	3	-/-/-	+/+/-			
8	47/m	Naevoid	1.70 (U)	0-3	2	-/-	*/-	-/-	-/-	-/-
9	64/m	SSM	2.00	1-3	4	-/-/-/-	-/+/-/-	*/+/-/-	-/-/-/-	*/-/-/-
10	66/m	NM	2.10 (U)	1-8	3	-/*/-	+/+/-			
11	74/m	SSM	2.20	3-7	3	-/-/-	-/-/+	+/-/-	-/-/-	-/-/-
12	37/f	SSM+Nod.	5.60 (U)	1x –137y	3	+/+/+	-/*			
13	92/f	SSM+Nod.	7,05 (U)	4-16	5	+/+/+/+/+	+/+/+/+/+	-/-/-/-/-	-/-/-/-/-	-/+/+/-/-
14	83/f	NM	9.50 (U)	0-4	5	-/-/-/-/*	-/+/+/+/*			
15	92/f	LMM	10.10 (U)	3-17	10	-/-/-/-/-/-/-/-/-/-	-/-/-/-/-/-/-/-/-/*	-/-/-/*/-/-/-/-/-/*	*/+/+/*/+/+/+/+/-/-/*	-/-/-/*/-/-/*/*/*/*
16	51/f	Meta (LN)		1-4	6	+/-/+/+/+/-	+/+/+/-/+/+	-/-/+/-/-/*	-/-/-/-/-/-	-/-/+/-/+/-
17a	65/m	Meta (LN)		12	4	-/-/*/*	-/-/+/+	-/-/*/-	-/-/*/-	-/-/*/-
b		Meta (LN)			2	-/-	+/+	-	*/-	-/+
c		Meta (LN)		15	1	-	+	-	+	+
d		Meta (LN)			2	-/-	-/+	-/+		-/-
18	92/f	Meta (SC)		4-26	5	+/+/+/+/-	*/*/*/+/*	*/-/*/-/-	*/+/-/-/-	*/-/*/*/+
19a	57/f	Meta (SC)		3-14	2	-/-	*/-	-/-	-/-	+/-
b		Meta (SC)		5	1	-	-	-	-	-
c		Meta (SC)		22	1	+	-	-	-	-
20a	61/f	Meta (SC)		5-38	4	-/-/-/*	-/-/-/*	-/-/-/*	-/-/-/-	-/-/-/-
b		Meta (SC)		4	1		-	-	-	-
21	81/f	Meta (SC)		4-14	4	+/+/+/+	*/-/*/-			
22a	79/m	Meta (SC)		3-8	3	-/-/-	+/+/*			
b		Meta (SC)		2	2	-/-	+/+			
c		Meta (SC)		3	1	-	+			
d		Meta (SC)		1	1		+			
e		Meta (SC)		1	1	-	+			
f		Meta (SC)		0	1	-	-			
23	84/m	Meta (SC)		0-26	5	-/-/-/*/-	+/-/-/*/+			

Legend: a, b, c, d, e and f indicate different metastases in same case; m, male; f, female; SSM, superficial spreading melanoma; Naevoid, nevus cell nevus-associated melanoma (nevus was excluded from analysis); SSM+Nod., superficial spreading melanoma with nodular part; NM nodular melanoma; LMM, Lentigo maligna-melanoma; Meta (LN), lymph node metastasis; Meta (SC), subcutaneous metastasis; TT, tumour thickness; U, ulcerated; HPF, high power field (microscopic 40x field); xn corial nests; ynodular part; +, hypermethylated; -, unmethylated; *non evaluable; z sort order of the symbols (+/-/*) correspond to intra-tumoural serial sampling and - from left to the right - to the sample numbers used in Fig. 1.

CI 0.42 to 0.73) and metastases (0.44, 95%CI 0.28 to 0.60) was not significant (p=0.228, Chi-square test, n=83). Samples from the core of the tumours (central samples) tended to represent the methylation status of the whole tumour much more accurately than samples from the tumour edges (peripheral samples): The sensitivities to detect case-specific promoter hypermethylation of all investigated genes in a single random sample were 0.71 (95% CI 0.58 to 0.84) or 0.16 (95% CI 0.02 to 0.30) for central or peripheral samples, respectively (p<0.001, n=83) if tumours with a minor number of samples (≤ 2) were included in analysis.

Correlation between methylation states of different genes

There was significant intra-sample correlation between the promoter methylation states of the *p16* and the *Rb* CpG islands (r=0.483, p=0.001, n=46, Spearman

Table 3. Logistic regression models of the influence of different covariates on promoter hypermethylation of the *RASSF1A* (n=70) or *p16* gene (n=77) in the tumour samples. Age, sex, tumour cell type, mitosis rate, inflammatory infiltrate, tumour regression, tumour vascularization, TT, Clark level, and ulceration (the last three only in primaries) were included in a backward-step regression model that eliminated non-significant factors resulting in identification of three independent covariates. The table shows only significant covariates of the last step.[a]

		RASSF1A promoter hypermethylation[b]		*p16* promoter hypermethylation[c]	
Covariate	Stratification	Odds ratio (CI)	Significance	Odds ratio (CI)	Significance
Tumour cell type					
	epitheloid	11.6 (1.2 – 109.9)	p = 0.032	8.6 (1.8 – 40.8)	p = 0.007
	non-epitheloid	1		1	
Mitosis rate					
	≥ 8/10 HPF	5.1 (1.4 – 18.1)	p = 0.013		n.s.
	< 8/10 HPF	1			
Sex of the patient					
	female	5.1 (1.3 – 19.9)	p = 0.018		n.s.
	male	1			

Legend: [a]For each parameter, odds ratio refers to the category with lowest risk for hypermethylation development (odds ratio = 1). I.e. samples with predominant epitheloid tumour cells have a 11.6 times higher odds ratio than patients with non-epitheloid tumour cells to develop *RASSF1A* promoter hypermethylation; bn=70 cases; cn=77 cases; CI, confidence interval; HPF, microscopic high power field (x 40 objective).

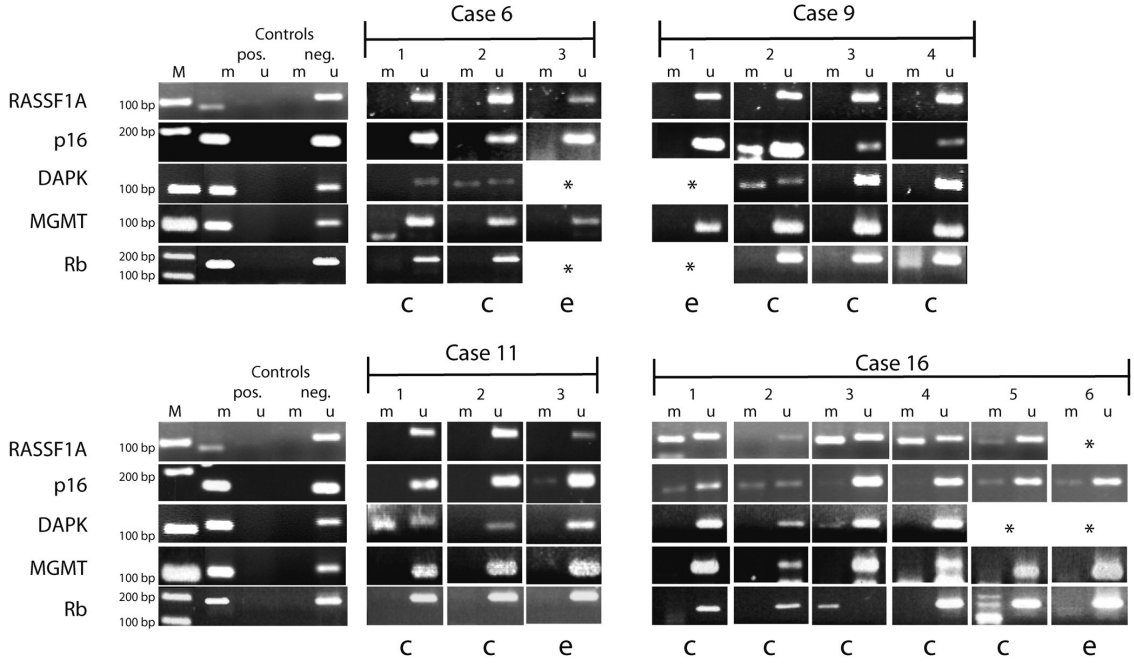

Fig. 1. Representative results of methylation-specific PCR (MSP) of five indicated genes (*RASSF1A, p16, DAPK, MGMT, and Rb*) in four cases (three primaries, one metastatic case) with two to six samples per case (asterisk: DNA not evaluable). The case numbers correspond to Tab. 2. MSP was performed with methylation-specific primers (m) or unmethylation-specific primers (u) and resolved on 2% agarose gels with a 100 bp ladder marker (M). Pos., positive control (in vitro methylated DNA); neg., negative control (DNA isolated of human fibroblasts); c, core location of the sample within the tumour; e, edge (marginal) location of the sample within the tumour.

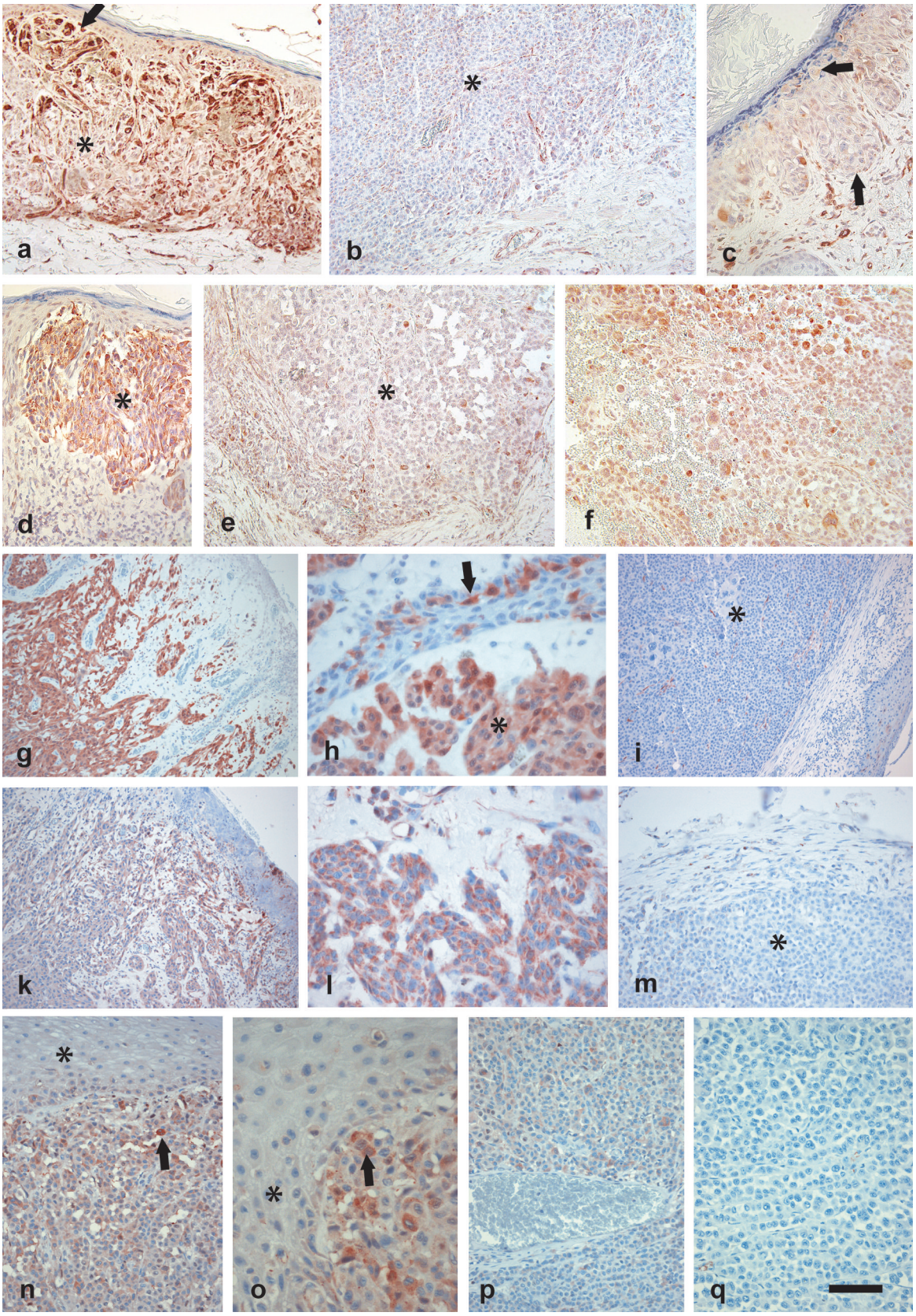

Fig. 2. Microphotographs of protein expression analysis in adjacent sections of the material used for promoter methylation assays (**a-f**: RASSF1A; **g-i**: p16; **k-m**: Rb; **n-q**: DAPK) **a.** SSM (case 3) with inhomogeneous RASSF1A protein expression. While superficial intradermal nests are RASSF1A positive (arrow), deeper melanoma cells do not express RASSF1A (asterisk). **b, c.** RASSF1A negative partial nodular SSM (case 13) shows RASSF1A expression neither in the nodular part (**b**, asterisk) nor in the peripheral intradermal melanoma nests. **c,** arrows; **d.** SSM (case 4) with homogeneous RASSF1A expression (asterisk). **e, f.** Melanoma metastasis (case 21) with inhomogeneous expression: While most of the tumour cells are RASSF1A negative (**e**, asterisk), a small proportion of the metastasis cells shows RASSF1A expression (**f**). **g.** Ulcerated melanoma (case 10) with considerable p16 protein expression (red). **h.** Cytoplasmic and - in some cells - nuclear p16 expression in solid melanoma cell conglomerates (asterisk) or epidermal melanocytes (case 11). **i.** p16-negative melanoma (case 14) (asterisk: melanoma cells). **k.** Ulcerated melanoma (case 13) with Retinoblastoma (Rb) protein positive melanoma cells (red). **l.** Higher enlargement revealed inhomogeneous cytoplasmic distribution of the Rb protein. **m.** Rb-negative melanoma metastasis (case 17) (asterisk: melanoma cells). **n.** Melanoma with widely positive but inhomogeneous staining of death-associated protein kinase (DAPK) (asterisk: epidermis; arrow: highly positive tumour cell) (case 9). **o.** Higher enlargement showed that melanoma cells of the same regions showed different degree of DAPK expression (case 9). **p.** Comparable inhomogeneous pattern in a melanoma metastasis with low DAPK expression (case 16). **q.** DAPK-negative section of a melanoma metastasis (case 17). Scale bar: a, c, d, f, and l, 30 µm; b, g, l, and k, 100 µm; e, m, n, and p, 50 µm; h and o, 15 µm.

correlation) and as well as that of *p16* and *DAPK* (r=0.303, p=0.035, n=49).

Relations of hypermethylation and clinico-pathological features

Logistic regression identified the epitheloid tumour cell type (versus all other tumour cell types together) as the most powerful independent predictor of promoter hypermethylation in the *RASSF1A* and *p16* CpG islands (Tab. 3). Interestingly, higher mitosis rate and female sex correlated with *RASSF1A* promoter hypermethylation (p=0.013 and p=0.018, respectively; Table 3).

Expressional analysis in histological sections

Protein expressions of RASSF1A, p16, Rb, or DAPK were studied in histological sections of seven, seven, four, or five cases, respectively. We found nuclear and cytoplasmic expression of RASSF1A and p16; while expression of Rb and DAPK was cytoplasmic (Fig. 2). Adjacent samples of these sections were previously investigated for promoter hypermethylation (Table 2). In Fig. 3, promoter methylation state and protein expression date were compared. We found a high inverse correlation between promoter hypermethylation and the number of protein expressing cells for all investigated genes ($r = -0.75$, $p < 0.001$; $r = -0.78$, $p < 0.001$; $r = -0.78$, $p < 0.001$; or $r = -0.66$, $p = 0.008$ for RASSF1A, p16, Rb, or DAPK, respectively; Spearman correlation) (Fig. 3). Intra-sectionally, melanoma cell protein expression was more homogeneous for p16 (74.2% of the investigated samples showed homogeneous p16 protein expression, which was defined by a maximum deviation of not more than 30% between different analyzed microscopic fields), followed by Rb (68.8%). In contrast, RASSF1A and DAPK showed a comparably low degree of homogeneity of protein expression within

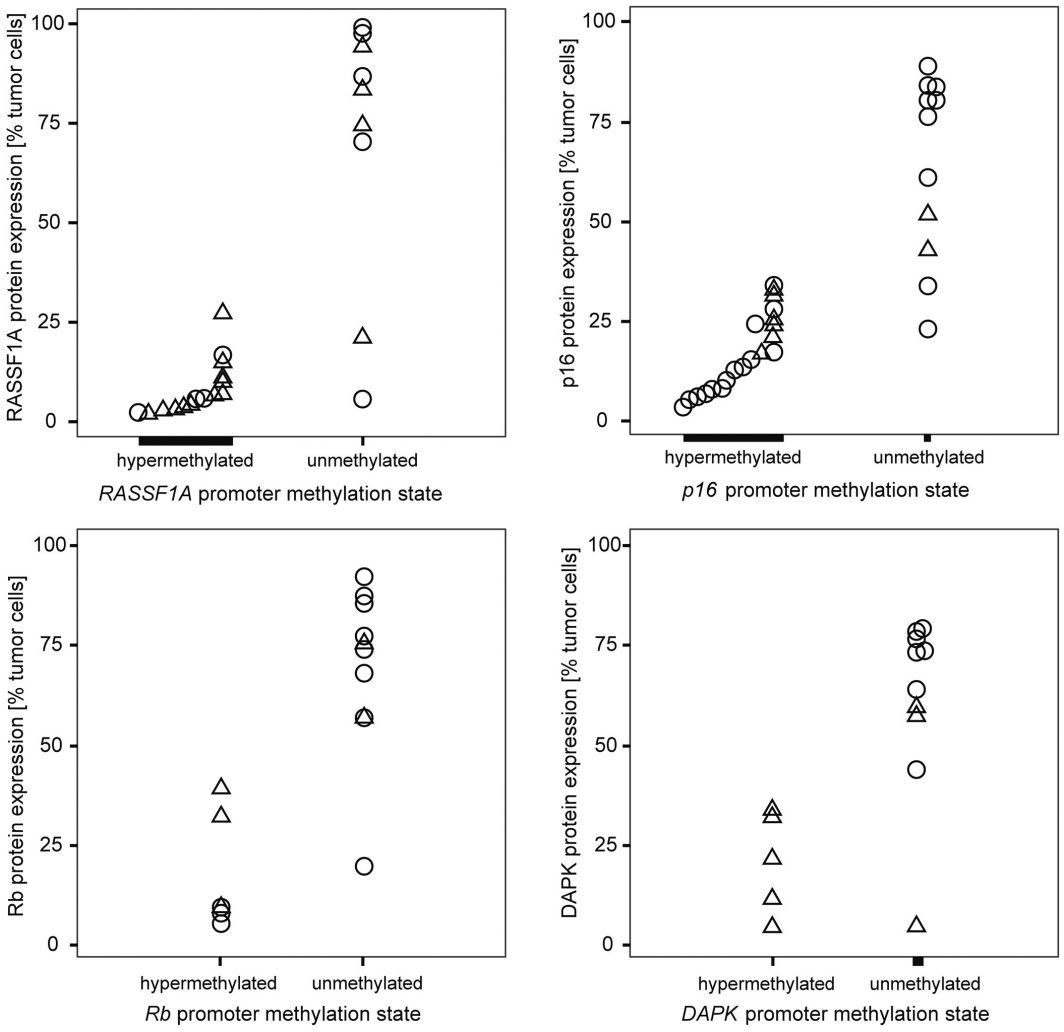

Fig. 3. Comparison of promoter methylation states (hypermethylated versus unmethylated) and protein expression by immunostaining (median % of positive tumour cells in ten microscopic high power field) in selected samples. The symbols O and Δ reflect homogeneous or heterogeneous intra-sample protein expression, respectively. A significant inverse correlation between promoter hypermethylation and the number of protein expressing cells was detected ($r = -0.75$, $p < 0.001$; $r = -0.78$, $p < 0.001$; $r = -0.78$, $p < 0.001$; or $r = -0.66$, $p = 0.008$ for RASSF1A, p16, Rb, or DAPK, respectively; Spearman correlation)

the analyzed samples (37.5% and 46.7%, respectively).

Discussion

There are several events which could transform melanocytes into a malignant tumour, however, the genetic mechanism of malignant development is poorly understood. The epigenetic inactivation of tumour suppressor genes seems to be a crucial step in the progression of melanoma (van Doorn et al., 2005). In our study, we detected frequent hypermethylation of all investigated tumour suppressor genes in melanoma. The methylation pattern showed that aberrant methylation is frequently heterogeneous in several tumour samples. This observation may suggest that aberrant methylation is a rather late event in the development and progression of cutaneous melanoma and may represent a random result. Our study is the first report which has investigated the intra-tumoural variation of aberrant methylation of *bona fide* tumour suppressor genes in melanoma. Sigalotti et al. have demonstrated in-vitro that the expression of MAGE-A3 can be heterogeneous in different single melanoma cell lines and corresponds with promoter methylation state (Sigalotti et al., 2004). In contrast, our results show firstly that heterogeneity of promoter methylation of tumour suppressor genes can occur *in-situ*. Previously, we showed that stem-line heterogeneity occurs in superficial spreading melanoma (Helmbold et al., 2005). Other groups have investigated the intra-tumoural variation of melanoma by analyses of microsatellite markers (Boni et al., 1998; Takata et al., 2000; Nakayama et al., 2001; Rao et al., 2003). Based on these analyses, it was shown that primary cutaneous melanomas are able to exhibit morphological and chromosomal heterogeneity intra-tumourally, but single melanoma metastases were regarded as most likely clonal in origin (Nakayama et al., 2001). In contrast, our data suggest that the methylation pattern in distinct metastases of the same case can be different, and therefore these metastases may originate from different tumour cell clones. Recently, a report by Sensi et al. has shown that NRAS and BRAF mutations occur mutually exclusively at a single cell level in the same human melanoma indicating genetic heterogeneity of the tumour (Sensi et al., 2006). As expected, we showed that protein expression is highly down-regulated by gene promoter hypermethylation. In some cases, protein expression may also be inactivated by genetic factors with low phenotypic protein levels and unmethylated gene promoter.

Aberrant methylation of the *RASSF1A* tumour suppressor gene located at 3p21.3 was described previously in different tumour entities (Dammann et al., 2000, 2005) The frequency of *RASSF1A* promoter hypermethylation has been demonstrated between 16% and 57% in malignant melanoma by MSP (Spugnardi et al., 2003; Furuta et al., 2004; Hoon et al., 2004; Reifenberger et al., 2004). Our methylation specific PCR data are consistent with these results. Additionally, we detected that *RASSF1A* methylation can be inhomogeneously distributed within a melanoma tumour. Moreover, we demonstrate for the first time that methylation of the *RASSF1A* promoter leads to lack of expression of RASSF1A in different histological sections. This result quantitatively validates our MSP data on the phenotypic level. RASSF1A encodes a negative Ras effector, which inhibits proliferation of cancer cells and acts through a Ras-dependent pro-apoptotic pathway (Dammann et al., 2000; Vos et al., 2000; Dreijerink et al., 2001; Khokhlatchev et al., 2002; Ortiz-Vega et al., 2002). We showed previously that RASSF1A interacts with microtubules and controls mitotic progression (Liu et al., 2003). The tumour suppressor RASSF1A regulates the stability of mitotic cyclins by inhibiting the anaphase-promoting complex and Cdc20 (Song et al., 2004). Consistent with this aspect of RASSF1A function, we detected in the present study that *RASSF1A* inactivation correlates with a higher mitosis rate. It was reported that RASSF1A induces cell cycle arrest by engaging the *Rb* checkpoint and inhibits cyclin D1 accumulation (Shivakumar et al., 2002). Thus, inactivation of *RASSF1A* may represent a crucial step in the pathogenesis of melanomas. Activation of the proto-oncogene Ras or BRAF and inactivation of *RASSF1A* are mutually exclusive events in the development of certain cancers (van Engeland et al., 2002; Dammann et al., 2003; Xing et al., 2004). Therefore, it would be interesting to analyze in future whether Ras and/or BRAF mutations occur preferentially in melanomas without *RASSF1A* inactivation.

In our cases, the highest frequency of total (55%) and inhomogeneous (41%) methylation was observed for *p16*. The cell cycle inhibitor p16 located at 9p21 is down-regulated in about 50% of primary and metastatic melanomas (Keller-Melchior et al., 1998; Pavey et al., 2002). This expressional analysis fits our methylation data. We observed that the methylation state of *p16* and *Rb* correlates significantly. Both tumour suppressor genes encode prominent regulators of the cell cycle. Inhibition of *p16* results in *Rb* activation by phosphorylation and cell cycle progression. Mutational alterations of *p16* and *Rb* were first described in hereditary melanoma (Hussussian et al., 1994; Kamb et al., 1994; Marx, 1994; Yang et al., 2004). Inactivation of *p16* is frequently found in sporadic melanoma, and this event may be associated with metastasization (Talve et al., 1997). Previously, aberrant methylation of the *p16* promoter was detected in different melanoma entities including uveal melanoma and in the melanoma-related clear cell sarcoma (Rosas et al., 2001; van der Velden et al., 2001; Straume et al., 2002; Tyler et al., 2003; McCloud et al., 2004; Xu et al., 2004). The higher methylation of *p16* observed could be due to different carcinogenesis in distinct geographic regions and/or the very heterogenous pattern of *p16* methylation. An important finding of our study was that the central parts of tumours exhibit a higher methylation level than peripheral sections in the majority of the melanomas.

This fact may be relevant for the lower levels of *p16* methylation observed in other studies that used only a single, probably in most cases peripheral, sample of each tumour.

In our cases, hypermethylation was also frequently detected in the promoters of *Rb*, *DAPK* and *MGMT*. Previously, the methylation status of the *Rb* promoter was not investigated in primary melanoma. The *Rb susceptibility* gene is located at 13q14.2 and often epigenetic inactivated in tumours (Gonzalez-Gomez et al., 2004). It was reported that *Rb* can be up-regulated in primary malignant melanoma, but down-regulated during metastasization (Saenz-Santamaria et al., 1995; Korabiowska et al., 2001). Thus, inactivation of this cell cycle regulator may represent an important event in the pathogenesis of melanoma. Chen et al. have reported that the DNA repair enzyme O6-Methylguanin-DNA methyltransferase (*MGMT*; located at 10q26) is lost in highly malignant tumours (Chen et al., 1992). The death-associated protein kinase (*DAPK*; located at 9q34.1) has a central role in tumourigenesis and is associated with proapoptotic pathways (Feinstein et al., 1995). Epigenetic inactivation of *DAPK* and *MGMT* was detected in lung carcinoma, ovarial and uterine tumours and in malignant melanoma (Toyooka et al., 2003; Bai et al., 2004; Hoon et al., 2004). So far, data on intra-tumoural variability of *DAPK* and *MGMT* promoter hypermethylation in primary tumours have not been available, and here we report that heterogeneity of their hypermethylation can occur frequently in melanoma. Recently, Kohonen-Corish et al. have analysed the methylation of *MGMT* in distant melanoma metastases and reported that there was heterogeneity in the presence of *MGMT* methylation between metastases from the same patient (Kohonen-Corish et al., 2006).

An interesting aspect is that the central parts of the investigated melanomas presented significantly more tumour cells with hypermethylated promoters than the edges. This is in accordance with a previous publication that has demonstrated that central tumour parts often contain a assortment of different tumour cell clones while the edges contain mostly single clones (Helmbold et al., 2005).

In summary, we analyzed 339 assays of 101 different tumour sections of 23 melanoma cases, allowing the following conclusions: 1) hypermethylation of the promoters of tumour suppressor genes is a frequent event in melanoma, 2) promoter methylation can be found habitually intra-tumourally inhomogeneously distributed in primary melanomas and melanoma metastases, 3) promoter hypermethylation is more frequent in the central (thicker) tumour parts than in samples from the tumour edges (peripheral samples), 4) intra-tumoural heterogeneity of promoter hypermethylation allows the interpretation that methylation of certain gene promoters occurs late in melanoma development and is not necessarily associated with early malignant transformation. These findings are additional arguments for clonal instability of malignant melanoma. A further important aspect of our results is that extrapolation from a limited topographical tumour region to the total tumour biological status does not seem to be recommendable. This hypothesis is in accordance with a growing body of evidence on intra-tumoural heterogeneity in other solid tumours, tumoural stroma, or tumoural microvessels (Helmbold et al., 2003; Fathers et al., 2005). Especially in prognosis studies, missing intra-tumoural sample representativeness reasonably results in a reduction of the sensitivities or specificities. Finally, epigenetic heterogeneity of cutaneous melanoma may have profound implications for the choice of specific therapeutic interventions in future.

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