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Loss of Proapoptotic Bcl-2-Related Multidomain Proteins in Primary Melanomas Is Associated with Poor Prognosis

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Prognosis of primary melanoma is presently based on morphological parameters, mainly tumor thickness. However, more reliable prognostic markers are needed that allow a better stratification of patients, especially with regard to therapeutic options. Here, a retrospective study was performed on patients with primary superficial-spreading melanoma (SSM, $n=44$) or nodular melanoma ($n=16$) of 1.5–4 mm thickness. Thirty patients had survived the follow-up of 10 years, whereas the other 30 patients developed metastases. Tumor sections were analyzed by immunohistochemistry for the expression of regulators of the cell cycle (p21; retinoblastoma protein (pRb)), of the intrinsic or extrinsic proapoptotic pathways (p53; murine double minute gene 2 protein; tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-R1/DR4; TRAIL-R2/DR5) and of Bcl-2-related proteins (Bcl-2, Mcl-1, Bax, Bak, Bok), which regulate the common mitochondrial apoptotic pathway. In SSM, decrease of Bax and Bak was significantly correlated with a poor prognosis: high Bax was associated with 10-year survival rates of 68%, whereas low Bax resulted in only 26% survival, and high Bak was associated with 10-year survival rates of 62%, whereas low Bak resulted in only 10% survival. Regulators of apoptosis may therefore candidate for independent prognostic markers for primary melanomas. The study underlines the particular role of the mitochondrial apoptosis pathway and of proapoptotic Bcl-2-related proteins for melanoma progression.

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INTRODUCTION

Malignant melanoma of the skin results in mortality rates higher than for all other skin cancers together (Garbe and Blum, 2001). A strong issue of melanoma malignancy is the pronounced resistance to conventional chemotherapies, which may result from diverse defects in signaling cascades leading to programmed cell death/apoptosis (Soengas and Lowe, 2003). Melanoma prognosis is based mainly on morphological parameters such as tumor thickness and tumor spread, leaving a 10-year survival expectancy of less than 3% for patients with distant metastases. Melanomas of 1.5–4 mm have a metastatic potential of about 30% (Eigentler *et al.*, 2004), thus challenging for new prognostic markers, which may allow early assessment of the disease, especially with regard to therapeutic options.

Apoptosis is a genetically regulated death program with high impact on growth of neoplasms. A large number of apoptosis-regulating factors have been described, which may also be of prognostic value. Two main death pathways (extrinsic and intrinsic) originate from death receptor stimulation and from intracellular damage signals, respectively. The extrinsic pathway is initiated by binding of death ligands, such as tumor necrosis factor-α, CD95L/Fas ligand or tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), to their respective death receptors, whereas p53 is the master regulator of the intrinsic pathway and triggers cell cycle arrest as well as apoptosis upon cellular stress and DNA damage. Among its characteristic targets are cell cycle inhibitors such as $p21^{CIP1/WAF1}$ and proapoptotic Bcl-2-related proteins such as Bax (Vousden, 2002). P53 itself is negatively controlled by the ubiquitin ligase MDM2.

Intrinsic and extrinsic proapoptotic pathways may meet in the mitochondrial amplification loop, which therefore becomes of particular interest also for anticancer therapies (Cory and Adams, 2002). The characteristic release of proapoptotic mitochondrial factors, such as cytochrome *c*, is controlled by pro- and antiapoptotic Bcl-2-related proteins, which are characterized by the presence of one to four Bcl-2

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Abbreviations: BH, Bcl-2 homology domain; pRB, retinoblastoma protein; SSM, superficial-spreading melanoma; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand

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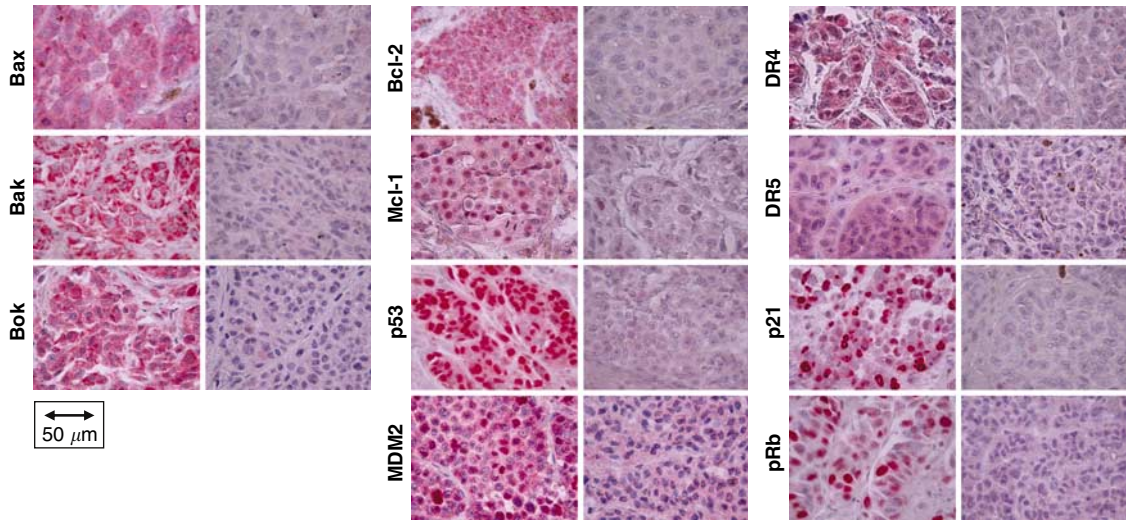

Figure 1. Variant expression of apoptosis and cell cycle markers in primary melanomas. Expression of indicated proteins was investigated by immunohistochemistry in a series of 24 SSM (1.5–4 mm). For each antigen, a positive and a negative tumor is shown. The magnification scale is indicated below the figure.

homology domains (BH1–BH4). Antiapoptotic Bcl-2 proteins such as Bcl-2 or Mcl-1 share all four domains, whereas proapoptotic proteins lack one or several of the BH domains. Proapoptotic Bcl-2 proteins further subdivide in BH3-only proteins such as Bad, Bid, Nbk/Bik, and multidomain proteins such as Bax, Bak, and Bok (Daniel *et al.*, 2001).

In previous work, we have shown that apoptosis resistance of melanoma cells can be overcome by triggering the expression of proapoptotic proteins (Eberle *et al.*, 2003; Hossini *et al.*, 2003; Fecker *et al.*, 2005; Oppermann *et al.*, 2005). In the present retrospective study, we investigated the expression of anti- and proapoptotic mediators of apoptosis pathways in melanoma. As the main result, loss of proapoptotic Bcl-2-related multidomain proteins Bax and Bak in primary melanoma turned out as predictors for an unfavorable prognosis.

RESULTS

Varying expression of apoptosis and cell cycle regulators in primary melanomas

For a first screening, a retrospective study was performed with sections of 24 superficial-spreading melanomas (SSM, 1.5–4 mm tumor thickness). Twelve patients had survived the 10-year follow-up period without tumor progression, whereas the other 12 patients developed metastases and died within the 10-year period (median survival: 31 months). Both subgroups were characterized by comparable median values for age and tumor thickness. By immunohistology, expression of regulators of the cell cycle (p21; retinoblastoma protein (pRb)), of the intrinsic and extrinsic proapoptotic pathway (p53; murine double minute gene 2 protein (MDM2); TRAIL-R1/DR4 and TRAIL-R2/DR5) and of the proapoptotic mitochondrial amplification loop (Bcl-2, Mcl-1, Bax, Bak, Bok) were investigated.

Variable numbers of positive and negative tumors (cutoff for negative tumors: 2%) were seen for each marker (Figure 1). Only 29% of SSM stained positive for p53, the key regulator of the intrinsic apoptosis pathway, whereas 83% were positive for its negative regulator MDM2. Also, the median number of stained cells in positive tumors was lower for p53 (20%) than for MDM2 (50%). Both antigens were restricted to nuclei. Of the death receptor family, both agonistic TRAIL receptors, TRAIL-R1/DR4 and TRAIL-R2/DR5, were expressed in over 90% of the SSM investigated. Also, the median number of stained cells was high: 50 and 70% for DR4 and DR5, respectively. Two characteristic negative regulators of the cell cycle, p21 and pRb, showed nuclear staining in about 60% of the tumors; however, the range of positivity was low for both markers with at maximum 30% positive cells in individual tumors resulting in median values of 5 and 10%, respectively. Of the large Bcl-2 family, critical regulators of the mitochondrial pathway, antiapoptotic Bcl-2 and Mcl-1 as well as proapoptotic Bax, Bak, and Bok, were investigated. Bcl-2-related proteins were found in 75–91% of the tumors. The median number of stained cells in positive tumors ranged between 47 and 65% (Table 2).

For the estimation of the prognostic value of the respective markers, their expression was compared in SSM patients with and without tumor progression. As detailed by box-plot analysis (Figure 2), no significant change was seen for markers p21 ($P>0.1$), pRB ($P>0.7$), DR4 ($P>0.6$), DR5 ($P>0.4$), p53 ($P>0.4$), and MDM2 ($P>0.2$; Mann–Whitney *U*-test).

Loss of Bax and Bak in SSM with unfavorable prognosis

According to the first screening with 24 SSM patients, the strongest differences were seen for the proapoptotic Bcl-2-related proteins Bax and Bak. Therefore, immunohistological stainings for Bcl-2-related proteins Bax, Bak, Bok, Mcl-1, and Bcl-2 were performed for a second series of SSM patients (10 with tumor progression and 10 without). Furthermore, Bax, Bak, Bok, and Mcl-1 were investigated in a series of 16

patients with nodular melanoma. The respective patient collectives were characterized by highly similar median values for age and tumor thickness (Table 1).

Immunohistological evaluation of 44 SSM patients revealed statistically significant downregulation of Bax ($P<0.02$) and of Bak ($P<0.05$; Mann–Whitney *U*-test) in patients with tumor progression, whereas Bok, Bcl-2, and Mcl-1 did not reveal significant changes (Figure 3a, Table 2). Double-negative tumors for Bax and Bak were exclusively found in patients with tumor progression (8/22, 36%). Double-positive tumors for Bax and Bak were found in 7/22 (32%) in patients with tumor progression as compared to 16/22 (73%) in patients without tumor progression. In contrast, no downregulation of Bax was seen in nodular melanomas, and the tendency of Bak downregulation was weaker than seen in SSM (data not shown).

For further comparison, 12 melanoma lymph node metastases were investigated for the expression of Bcl-2-related proteins. Only S100-positive cells were evaluated. In these samples, strong downregulation of both pro- and antiapoptotic Bcl-2 proteins was found (Bax, 75%; Bak, 83%; Bok, 75%; Bcl-2, 58%; Mcl-1, 67%; cutoff level: 20% positive tumor cells). These findings may be indicative for a special situation of apoptosis regulators in melanoma metastases independent from their pro- or antiapoptotic function, possibly related to the different growth situation.

With respect to patient survival, Bax and Bak turned out as highly selective. For analysis, patients were subdivided for each marker in two groups corresponding to high or low Bax or Bak expression (cutoff level: 20% positive tumor cells). Only one of 10 SSM patients with low Bak survived 10 years (10%), in contrast to 21 of 34 survivors with high Bak (62%). Similarly, of 19 SSM patients with low Bax, only five survived 10 years (26%), in contrast to 17 of 25 survivors with high Bax (68%). Kaplan–Meier analysis clearly demonstrated the significant correlation between low Bax or Bak expression and reduced SSM patient survival (log-rank test: Bax, $P<0.01$; Bak, $P<0.005$; Figure 3b).

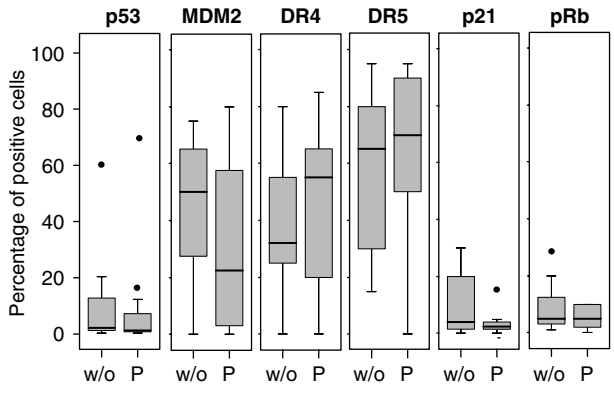

Figure 2. Variant expression of different apoptosis and cell cycle marker proteins. Box-plot diagrams indicate the median percentages and the variation of positive tumor cells in primary melanomas for regulatory proteins of the intrinsic apoptosis pathway (p53, MDM2) of the extrinsic apoptotis pathway (DR4, DR5) and of cell cycle regulators (p21, pRb), as determined by immunohistochemistry. Twelve SSM patients with tumor progression (P) were compared to 12 patients without tumor progression (w/o).

DISCUSSION

Prognosis of primary melanoma has been shown to be associated with tumor thickness, ulceration, and lymphangiogenesis (Clark Jr *et al.*, 1969; Breslow, 1970; Dadras *et al.*, 2005). For identifying additional prognostic markers, several immunohistological studies had applied a comparison of primary melanoma and metastases. However, the different growth situation may by itself result in changes of gene expression, which may be even independent from the grade of malignancy, as we found here downregulation of both pro- and antiapoptotic Bcl-2 proteins in lymph node metastases. Other complications may arise from variant gene expression in melanoma subtypes such as nodular melanoma or SSM. The present retrospective study was performed with a tightly defined collective of melanoma patients (SSM, 1.5–4 mm), and patients with good *versus* bad outcome with respect to 10-year survival were compared.

Several previous studies have focused on the expression of characteristic cell cycle regulators; however, deviating data have been published with regard to their prognostic value (Straume *et al.*, 2000; Georgieva *et al.*, 2001; Korabiowska *et al.*, 2001; Bachmann *et al.*, 2004). Our analysis of p21 and pRb expression revealed overall weak expression levels of these proteins in primary melanomas, but no significant change with respect to prognosis.

Apoptosis deficiency represents a characteristic feature of malignancies and may also be related to chemotherapy resistance. Whereas the intrinsic pathway is controlled by

Table 1. Clinical data of melanoma patients

Type[1]	*n*[2]	Progression	Age (years)[3]	Sex (females/males)	Tumor thickness (mm)[3]	Survival (months)[3]
SSM	22	No	55 (56±13)	13/9	2.4 (2.5±0.7)	120
SSM	22	Yes	57 (57±15)	11/11	2.3 (2.4±0.6)	36 (38±23)
NM	8	No	61 (59±10)	6/2	2.6 (2.7±0.7)	120
NM	8	Yes	65 (62±14)	3/5	2.8 (2.9±0.8)	34 (31±18)

[1]Histological type (SSM: superficial-spreading melanoma; NM: nodular melanoma).
[2]Number of patients investigated.
[3]Median values for age and tumor thickness at the time of surgery and patient survival after excision of the primary tumor; mean values and SED are given within parenthesis.

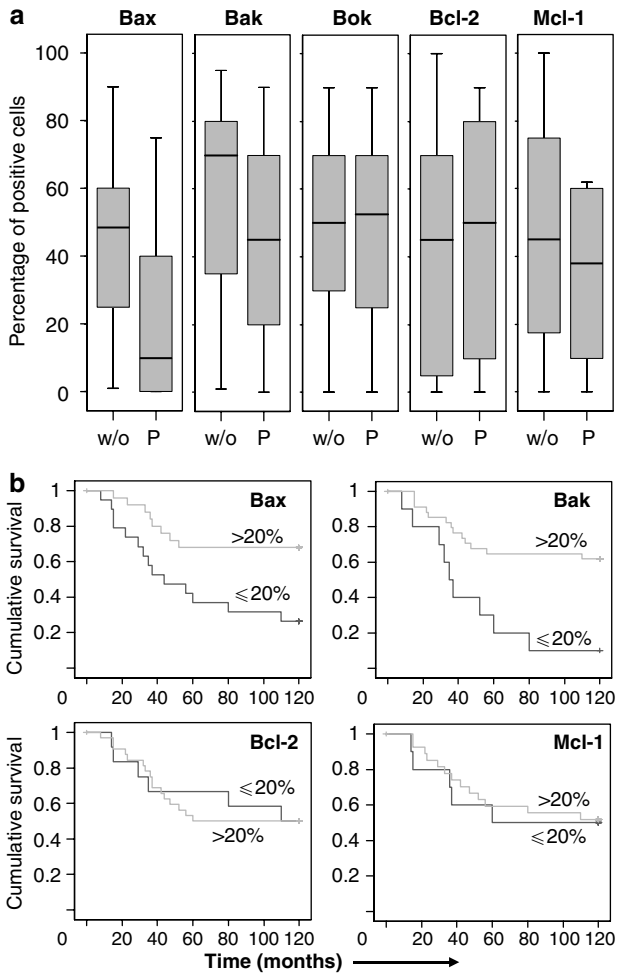

Figure 3. Weak expression of Bax and Bak correlates with reduced survival. (**a**) Box-plot diagrams indicate expression levels of pro- and antiapoptotic proteins of the Bcl-2 family (Bax, Bak, Bok, Bcl-2, and Mcl-1) in a series of 44 SSM from patients with tumor progression (P; $n=22$) and patients without tumor progression (w/o; $n=22$). (**b**) Reduced survival of SSM patients with low expression of Bax and Bak is shown by Kaplan–Meier analyses. For comparison, survival graphs for Bcl-2 and Mcl-1 are also given. High and low expression was defined as more than 20% positive tumor cells, respectively, as up to 20% positive cells.

Table 2. Localization and expression of antigens in SSM

Antigen	n[1]	Staining[2]	Positive tumors[3] (%)	Positive cells[4] (%)
Bax	44	Cyto	75	47
Bak	44	Cyto	86	65
Bok	44	Cyto	91	53
Bcl-2	44	Cyto	82	60
Mcl-1	37	Cyto	78	50
p53	24	Nucl	29	20
MDM2	23	Nucl	83	50
DR4	22	Cyto	91	50
DR5	20	Cyto	95	70
p21	24	Nucl	58	5
pRb	24	Nucl	67	10

[1]Number of superficial-spreading melanomas (SSM) investigated by immunohistology.
[2]Cyto, cytoplasmic staining pattern; nucl, nuclear staining pattern.
[3]Percentage of melanoma lesions that stained positive for the respective antigen (cutoff level for positive tumors: 2% stained cells).
[4]Median percentage of stained cells in the positive tumors.

p53 and its negative regulator MDM2, the extrinsic apoptotic pathway is based on the activation of death receptors (Sprick and Walczak, 2004). In the present study, neither significant changes in expression of p53 and MDM2 nor of the death receptors TRAIL-R1/DR4 or TRAIL-R2/DR5 were obvious, possibly indicating that the block at one or the other branch of the apoptosis pathways may be less contributive for melanoma progression.

For many tumors, extrinsic and intrinsic apoptosis pathways merge in the mitochondrial amplification loop, which therefore becomes of particular interest for anticancer approaches (Cory and Adams, 2002). Also for melanoma cells, we had demonstrated a high impact of the mitochondrial pathway for apoptosis control (Raisova *et al.*, 2001; Hossini *et al.*, 2003; Oppermann *et al.*, 2005). Pro- and antiapoptotic Bcl-2-related proteins appear as master regulators of this pathway. Antiapoptotic Bcl-2 or Mcl-1 are integral components of the outer mitochondrial membrane and they prevent mitochondrial damage (Daniel *et al.*, 2001). Their high expression has been attributed to chemoresistance in melanoma (Tang *et al.*, 1998; Jansen *et al.*, 2000), and the ratio of Bax to Bcl-2 appeared as decisive for melanoma cell susceptibility to apoptotic signals (Raisova *et al.*, 2001). Accordingly, antisense strategies targeting Bcl-2 or Mcl-1 are underway (Jansen *et al.*, 2000; Thallinger *et al.*, 2003). In the present study, the majority of primary melanomas revealed Bcl-2 as well as Mcl-1 expression, but no relation to prognosis.

Central players of the mitochondrial apoptosis pathway are the multidomain, proapoptotic Bcl-2-related proteins Bax, Bak, and Bok, which are assumed as antagonists for antiapoptotic Bcl-2 proteins (Daniel *et al.*, 2003). Whereas Bax is found in the cytosol before induction, Bak and Bok already reside in the mitochondrial membrane and in the endoplasmic reticulum (Daniel *et al.*, 2001). Loss of Bax expression as a negative prognostic marker has been reported for breast, ovarian, pancreatic, and esophageal cancer (Krajewski *et al.*, 1995; Friess *et al.*, 1998; Tai *et al.*, 1998; Guner *et al.*, 2003; Daniel *et al.*, 2003). In the present study, we found loss of Bax to be associated with tumor progression in SSM, and 10-year survival rates were dramatically decreased. In previous work, we demonstrated the critical role of the Bax/Bcl-2 ratio for melanoma cell susceptibility to apoptotic stimuli (Raisova *et al.*, 2001). Thus, loss of Bax in melanomas may reflect reduced susceptibility to immune response and chemotherapy.

No immunohistochemical data on Bak or Bok were available for melanoma, but cisplatin-induced apoptosis in

melanoma cells has been shown to require Bak (Mandic *et al.*, 2001). The proapoptotic activity of Bok has been demonstrated in neuronal cells and cervical carcinoma cells (Hsu *et al.*, 1997; Yakovlev *et al.*, 2004). The present study revealed expression of Bak and of Bok in the majority of primary melanomas. Downregulation of Bak was associated with poorer prognosis in SSM, whereas Bok did not reveal any significant changes.

In conclusion, our data underline the critical role of the mitochondrial pathway for apoptosis signaling in melanoma. At an early stage of melanoma development, loss of expression of proapoptotic Bcl-2-related proteins may block apoptotic signals, as derived from the immune system or from the intrinsic apoptotic pathway. This loss appears to be characteristic for tumor progression and may thus serve as a negative prognostic marker for primary melanoma.

MATERIALS AND METHODS

Melanoma patients and immunohistology

Sections of primary melanomas resulted from patients with SSM or nodular melanoma (1.5–4 mm tumor thickness). Clinical follow-up for 10 years was available for all surviving patients. Respective patients subgroups were characterized by highly comparable mean age and tumor thickness (Table 1). Sections of 12 melanoma lymph node metastases resulted from a separate patient collective.

Sections of 4–6 μm from formalin-fixed and paraffin-embedded melanoma samples were attached to SuperFrost plus slides (Roth, Karlsruhe, Germany) at 60°C for 4 hours. After xylol treatment, they were re-hydrated and equilibrated in Tris-buffered saline, pH 7.5, followed by pressure cooker boiling for 3 minutes in sodium citrate buffer (10 mM, pH 6) and equilibration in Tris-buffered saline. Subsequent incubation steps were performed in humidity chambers at room temperature, and all incubation steps were followed by washing slides thoroughly in Tris-buffered saline, 0.05% Tween. Sections were treated first at room temperature for 30 minutes with protein blocking solution (DakoCytomation, Hamburg, Germany) and were then incubated with the respective primary mouse monoclonal or rabbit polyclonal antibody for 1 hour (Bok antibody for 14 hours). For mouse monoclonal antibodies, slides were then incubated (1) for 30 minutes with rabbit anti-mouse antibody (DakoCytomation, Z0259) diluted 1:20 in growth medium supplied with human serum (HUSE) (87.5% RPMI medium, 10% heat-inactivated fetal calf serum, 12.5% human serum), and (2) for 30 minutes with the alkaline phosphatase anti-alkaline phosphatase (APAAP) complex (DakoCytomation, D0651) diluted 1:50 in RPMI (10% fetal calf serum). Both incubations were repeated once for 10 minutes. For the detection of rabbit polyclonal antibodies, slides were incubated (1) for 30 minutes with mouse anti-rabbit antibodies (DakoCytomation, M0737) diluted 1:200 in HUSE; (2) for 30 minutes with rabbit anti-mouse antibody solution diluted 1:20 in HUSE, and (3) for 30 minutes with the APAAP complex diluted as described above.

Slides were then stained for 15 minutes with the fuchsine substrate–chromogen system (DakoCytomation, K0624). Counterstaining was performed with Mayer's hematoxylin (Merck, Darmstadt, Germany). Optimal dilutions were established for all antibodies. Negative controls were always run in parallel: mouse IgG1 control antibody (DakoCytomation, X0931; for monoclonal antibodies), or the primary antibody was omitted (polyclonal antibodies).

The following mouse monoclonal and rabbit polyclonal antibodies and antibody dilutions have been applied: Bax (YTH-2D2, Biozol, Eching, Germany, 1:800); Bak (polyclonal, DakoCytomation, 1:175); Bok (polyclonal, New England Biolabs, Frankfurt a. M., Germany, 1:50); Bcl-2 (clone 124, DakoCytomation, 1:100); Mcl-1 (RC13, Acris, Hiddenhausen, Germany, 1:50 or polyclonal, AHP472, Serotec, Düsseldorf, Germany, 1:1500); p53 (DO-7, DakoCytomation, 1:75); MDM2 (SMP14, Dianova, Hamburg, Germany, 1:100); DR4 (polyclonal, Acris, 1:150); DR5 (polyclonal, Acris, 1:100), p21 (6B6, BD Pharmingen, Heidelberg, Germany, 1:400); pRb (G3-245, BD Pharmingen, 1:750); and S100 (polyclonal, DakoCytomation, 1:500).

Evaluation and statistics

For each slide, staining of the complete tumor section was evaluated by microscopy without knowing the patients' clinical details. For each section, the percentage of melanoma cells with strong, medium, weak, or absent staining was determined. Melanoma cells in primary tumors and metastases were confirmed by S100 staining. Only strong and medium cell staining was considered as positive. Statistical significance was calculated by Mann–Whitney *U*-test. Kaplan–Meier survival analysis was performed for patients with low and high expression of markers (cutoff value: 20% positive cells). Significance of differences in survival was determined by means of the log-rank test.

The research committee of the Charité Universitätsmedizin Berlin has approved the described studies. The study was conducted according to the Declaration of Helsinki Principles.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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