Progression in Cutaneous Malignant Melanoma Is Associated with Distinct Expression Profiles

A Tissue Microarray-Based Study

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Cutaneous malignant melanoma remains the leading cause of skin cancer death in industrialized countries. Clinical and histological variables that predict survival, such as Breslow's index, tumor size, ulceration, or vascular invasion have been identified in malignant melanoma. Nevertheless, the potential relevance of biological variables still awaits an in-depth exploration. Using tissue microarrays (TMAs), we retrospectively analyzed 165 malignant melanoma samples from 88 patients corresponding to distinct histological progression phases, radial, vertical, and metastases. A panel of 39 different antibodies for cell cycle, apoptosis, melanoma antigens, transcription factors, DNA mismatch repair, and other proteins was used. Integrating the information, the study has identified expression profiles distinguishing specific melanoma progression stages. Most of the detected alterations were linked to the control of cell cycle G1/S transition; cyclin D1 was expressed in radial cases 48% (12 of 25) with significant loss of expression in vertical cases 14% (9 of 65), $P = 0.002$; whereas p16^{INK4a} (89% in vertical versus 71% in metastatic cases, $P = 0.009$) and p27^{KIP1} (76% in radial versus 45% in vertical cases, $P = 0.010$) were diminished in advanced stages. The study also defines a combination of biological markers associated with shorter overall survival in patients with vertical growth phase melanoma, that provided a predictor model with four antibodies (Ki67, p16^{INK4a}, p21^{CIP1}, and Bcl-6). This predictor model was validated using an independent series of 72 vertical growth phase melanoma patients. (*Am J Pathol* 2004, 164:193–203)

Cutaneous malignant melanoma, the most aggressive common skin tumor, is characterized by a multi-factorial etiology. Sun exposure and genetic susceptibility have been proposed as major etiological and predisposing factors that somehow explain the reported increased incidence.^{1,2} Metastatic melanoma tumors have a poor response to any therapy, with a 5-year survival probability ranging from 0 to 30%.^{3–5}

Cutaneous malignant melanoma develops in three different stages, from radial to vertical growth phases and metastatic disease. The underlying molecular events that explain malignant melanoma genesis have been only partially characterized, and only a small number of genes have been identified as playing key roles in melanoma initiation and progression. Among these, some cell cycle regulators, apoptotic, signal transduction, cell adhesion, and matrix digestion genes have been demonstrated to be deregulated in this neoplasm.^{6–11}

The variability of clinical behavior in patients with malignant melanoma is only partially explained by clinical and histological data, and we need to identify biological variables of use in assigning patients to specific risk groups. Although this aim has been achieved for single markers, malignant melanoma has benefited little from the use of massive molecular analytical techniques. The use of tissue microarrays (TMAs) is a technique suitable for malignant melanoma progression study, since it is a powerful tool that allows hundreds of specimens to be interrogated simultaneously with a panel of antibodies or probes directed against selected targets. Information obtained with TMAs not only contributes to the identification of key molecular events, but also facilitates the development of outcome predictor models of unprecedented accuracy.^{12–14}

In this study, we have collected a large series of human malignant melanomas of different stages, and factor, signal-transduction, transcription-factor, differentia-

Supported by grants from the Ministerio de Sanidad y Consumo Fondo de Investigaciones Sanitarias (FIS 01/0037–03) and the Ministerio de Ciencia y Tecnología (MCYT) (Bio2000 CO275/01 and 02), Spain. S. A. is supported by a grant from the Ministerio de Sanidad y Consumo (FIS 01/9269) and the Centro Nacional de Investigaciones Oncológicas (CNIO).

Accepted for publication September 25, 2003.

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tion, and melanoma-specific antigens. The study identifies a set of stage-specific markers, and allows a predictor model to be developed that identifies groups of patients with increased risk of death.

Materials and Methods

Malignant Melanoma Samples

A total of 175 tissue samples from common nevi ($n = 10$), primary radial growth phase malignant melanoma ($n = 28$), primary vertical growth phase malignant melanoma ($n = 66$), skin ($n = 34$), and nodal ($n = 37$) melanoma metastases were examined. Primary (radial and vertical phases) and metastatic tumors used to construct the TMAs were all taken from a series of 88 patients consecutively diagnosed at the 12 Octubre University Hospital, Madrid, between 1995 and 2000. In 2 of 88 cases, biopsies from both vertical and radial phases were included. In 30 of 88 patients, samples corresponding to primary (vertical phase in all cases) and metastatic disease were included. In 11 of 88 patients it was possible to include biopsies from both cutaneous and lymph node metastases. Additional metastases (8 of 88 cases) from patients from whom primary tumors could not be obtained were also included in the study. There was no history of familial occurrence in the selected patients.

All patients were treated according to standard criteria: surgical resection in all primary cases and surgical resection (when possible) plus chemotherapy, if indicated, in cases with demonstrated metastases. For survival analysis, all included samples corresponded to the initial diagnostic specimens before any further treatment.

Paraffin blocks were selected on the basis of the availability of suitable formalin-fixed, paraffin-embedded tissue (at least 1-mm thick). Hematoxylin and eosin-stained slides were available for review in all cases measured as Breslow's index, and histological progression phase were defined according to standard criteria^{15,16} (also see additional information at <http://ajp.amjpathol.org>). Approval for this study was obtained from the 12 Octubre Hospital Institutional Review Board. Informed consent, when necessary, was obtained following the guidelines of the Helsinki Declaration.

Generation of Tissue Arrays

For characterization of immunohistochemical protein expression, we constructed six different tissue arrays containing the distinctive histological stages of the tumor. Each tissue array was assembled as previously described.¹² Briefly, two 1.5-mm-diameter cylinders of tissue were taken from representative areas of each archival paraffin block and arrayed into a new recipient paraffin block with a custom-built precision instrument (Beecher Instruments, Silver Spring, MD) according to the previously described method.¹²

These tissues had been fixed in 4% formalin (Panreac, Barcelona, Spain) and paraffin-embedded according to routine procedures. Areas chosen for the cylinder core

had high tumor cellularity. To evaluate the most adequate part of each primary tumor we selected the invasive border of the tumor in large lesions and all tumor cells in smaller samples. In addition, normal tissues (skin, tonsil, and reactive lymphoid tissue) and three different cell lines with known cell cycle alterations were placed adjacent to tumoral tissues to serve as internal controls and to ensure the quality of staining slides. Initial sections were stained for hematoxylin and eosin to verify the histopathological findings.

Immunohistochemistry

Three-micrometer tissue sections from the TMA blocks were sectioned and applied to special immunohistochemistry coated slides (DAKO, Glostrup, Denmark). These slides were baked overnight in a 56°C oven. Sections were deparaffinized in xylene for 20 minutes, rehydrated through a graded ethanol series and washed with phosphate-buffered saline. Antigen retrieval was achieved by a 2-minute heat treatment in a pressure cooker, containing 1 liter of 10 mmol/L sodium citrate buffer, (pH 6.5, DAKO) that had been previously brought to the boil. Before staining, endogenous peroxidase activity was quenched with 1.5% hydrogen peroxide (DAKO) in methanol for 10 minutes.

Immunohistochemical staining was performed on these sections using 39 different antibodies, (source and concentration are listed in Table 1). Antibodies used in this study were selected for reporting on key aspects of cell cycle, apoptosis, signal transduction, and melanoma differentiation. We have also considered the status of the markers described in previous studies in this tumor type. After incubation, in the case of nuclear markers, immunodetection was performed with the LSAB Visualization System (DAKO) using 3,3'-diaminobenzidine chromogen as substrate, according to the manufacturer's instructions. In addition, whenever possible, cytoplasmic markers were visualized with the APAAP System (alkaline phosphatase anti-alkaline phosphatase, DAKO), using neo-fuchsin chromogen as substrate to rule out the possibility of a role of endogenous melanin in the observed reactivity. All sections were counter-stained with hematoxylin (DAKO). Negative controls were obtained by omitting the primary antibody.

Scoring Systems

Immunostaining intensity was evaluated by two different pathologists (S.R.A.G. and J.L.R.P.) and scored using uniform and clear cut-off criteria, to maintain the reproducibility of the method. Discrepancies were resolved by simultaneous re-evaluation. Briefly, the result of immunostaining was recorded as negative or positive, and low *versus* high expression, taking into account the expression in tumoral cells and the specific cut-off for each marker (see Table 1 for definition of positive phenotype). As a general criterion, the cut-offs were selected to facilitate reproducibility, and when possible, to translate biological events. Selected sample replicates were highly

Table 1. Parameters Measured and Antibodies Tested in the Study, Indicating Source, Dilution, Visualization Method, Pattern of Reactivity, Threshold and Positive Control (Evaluated in Skin, Tonsil, and Reactive Lymph Node Tissue)

Protein	Clone	Source	Dilution	Visualization system	Reactivity	Threshold	Internal control
Bcl2	124	DAKO	1:25	LSAB/DAB	pos/neg	>10% positive cells	Small lymphocytes
Bcl6	PG-B6p	DAKO	1:10	LSAB/DAB	pos/neg	Any nuclear expressi	CG B-cells in lymph node
Bclx-L	2H12	ZYMED	1:10	APAAP/NF	pos/neg	>10% positive cells	Cell lines
Bmi 1	POLY	Santa Cruz	1:100	ENVI/DAB	pos/neg	>10% positive cells	Mantle cells, normal epithelium
Caveolin	2297	Transduction	1:25	APAAP/NF	high/low	≥50% positive cells	Endothelial cells
Cyclin A	6E6	Novocastra	1:100	LSAB/DAB	pos/neg	≥5% positive cells	Proliferating cells (G2/M)
Cyclin B1	7A9	Novocastra	1:25	LSAB/DAB	pos/neg	≥5% positive cells	Proliferating cells (G2/M)
Cyclin D1	DCS-6	DAKO	1:100	LSAB/DAB	high/low	≥30% positive cells	Endothelial cells and macrophages
Cyclin D3	DCS-22	Novocastra	1:10	LSAB/DAB	pos/neg	≥5% positive cells	Proliferating cells
Cyclin E	13A3	Novocastra	1:10	LSAB/DAB	pos/neg	≥10% positive cells	Proliferating cells
CDK1	1	Transduction	1:1500	LSAB/DAB	pos/neg	≥5% positive cells	Proliferating cells, basal cells in skin
CDK2	8D4	NeoMarkers	1:500	LSAB/DAB	pos/neg	≥5% positive cells	Proliferating cells
CDK6	K6.83	Chemicon	1:10	LSAB/DAB	pos/neg	≥5% positive cells	TMA controls
C-kit	POLYC	DAKO	1:50	APAAP/NF	high/low	≥50% positive cells membranous express	Melanocytes in normal skin
Hdm2	IF2	Oncogen	1:10	LSAB/DAB	pos/neg	Any nuclear expres.	Macrophages, endothelial cells
HMB 45	HMB 45	BIOMENEX	1:10	APAAP/NF	pos/neg	≥50% positive cells	Melanocytes in normal skin
HLA* DP, DQ, DR	J576	CNIO	1:150	LSAB/DAB	pos/neg	Any nuclear expres.	GC
Ki67	MIB1	DAKO	1:100	LSAB/DAB	high/low	≥20% positive cells	Proliferating cells
MELAN A	A103	DAKO	1:10	APAAP/NF	pos/neg	≥50% positive cells	Melanocytes in normal skin
MEL 18	POLY	Santa Cruz	1:100	LSAB/DAB	high/low	≥50% positive cells	Mantle cells, plasma cells
MLH 1	G168-15	PharMingen	1:100	APAAP/NF	high/low	≥50% positive cells	Normal skin
MSH 2	FE11	ONCOGEN	1:100	APAAP/NF	high/low	≥70% positive cells	Normal skin
MUM-1	POLYCL	Santa Cruz	1:200	LSAB/DAB	high/low	≥30% positive cells	Plasma cells, GC
NFKappaB	F-6	Santa Cruz	1:200	LSAB/DAB	pos/neg	Any nuclear expres	Cell lines
P16	F12	Santa Cruz	1:50	LSAB/DAB	high/low	≥50% positive cells	Epithelial cells in normal skin
P21	EA10	Oncogene	1:50	LSAB/DAB	pos/neg	≥10% positive cells	Scattered GC cells
P27	57	Transduction Lab	1:1000	LSAB/DAB	pos/neg	≥10% positive cells	Resting lymphoid cells, upper epidermis in normal skin
P53	DO-7	Novocastra	1:50	LSAB/DAB	pos/neg	≥10% positive cells	Scattered GC cells
PH1**	CIB	CIB	1:2	LSAB/DAB	pos/neg	Any nuclear expres	
PKCβ	28	Serotec	1:500	APAAP/NF	pos/neg	≥10% positive cells	Endothelial cells, adipocytes, macrophages
RING 1B**	3RING1	CIB	1	LSAB/DAB	pos/neg	Any nuclear expres	Mantle cells
Rb	G3-245	BD	1:250	LS	pos/neg	≥10% positive cells	Proliferating cells
Rb P	POLYCL	PharMingen Santa Cruz	1:500	AB/DAB LSAB/DAB	pos/neg	≥10% positive cells	Proliferating cells
RYBP**	POLYCL	CIB	1:25	LSAB/DAB	pos/neg	Any nuclear express	Endothelial cells, plasma cells
S-100	POLYCL	DAKO	1:500	APAAP/NF	pos/neg	≥30% positive cells	Normal melanocytes, nerves
Survivin	POLYCL ONAL	RD Systems	1:1500	APAAP/NF	pos/neg	≥2% positive cells (nuclear expression)	Scattered GC cells
SKP2	1G12E9	ZYMED	1:10	LSAB/DAB	pos/neg	>5% positive cells	Proliferating cells
STAT1	C-136	Santa Cruz	1:50	LSAB/DAB	pos/neg	Any nuclear expression	Reactive lymphocytes macrophages
Topo-isomerase	Ki-S1	DAKO	1:400	LSAB/DAB	pos/neg	≥10% positive cells	Proliferating cells

APAAP, alkaline phosphatase anti-alkaline phosphatase; NF, neo-fuchsin; pos/neg, positive/negative.

BD: BD Biosciences/Transduction Labs (San Diego, California), DAKO Corp (Carpenteria, California), Zymed (South San Francisco, California). Antibodies provided by the Monoclonal Antibodies Unit* (CNIO) and by Dr. Miguel Angel Vidal** (CIB-CSIC, Madrid, Spain).

reproducible in our series (96%), as other authors have found.^{9,14} STAT1-positive expression was considered only with respect to the nuclear signal that translates the active form of this protein.

Statistics

Clinical data and immunohistochemistry scoring were performed blind, and data were compiled only after all analyses were completed. Fisher's exact test was used to test whether a specific protein-expression profile was associated with a particular histological stage of melanoma progression. Significance was concluded for values of $P < 0.05$. All statistical tests were two-sided. To avoid the influence of multiple testing, adjusted P values

were also computed using the method proposed by Hochberg and Benjamini¹⁷ to minimize the false discovery rate.

Complete follow-up survival data for 6 years or more were available in 60 vertical malignant melanoma cases. The overall survival (OS) predictive value of the studied markers these cases was explored using Kaplan-Meier, log-rank test, and Cox regression analysis. A multivariate model was developed by backwards elimination, starting with all markers with a P value lower than 0.10 in the univariate analysis. This statistical significance level was selected to allow for optimal discovery in the initial phases of this analysis. From the final model for each patient a score was computed taking the linear predictor from the multivariate model.

Validation Set

Given the exploratory nature of the study, an independent series of 72 vertical growth phase melanoma cases was included to validate the prognostic value of the set of prognostic markers. This validation set was similar to the training set and included patients with vertical growth phase melanoma diagnosed from 1986 through 1998.

Validation was carried out in two ways: first, the previously mentioned score was computed using the linear predictor from the multivariate model in the original sample and the statistical significance of this score was tested. Differences in survival according to this score are presented graphically for both the original and the validation set of patients. For this representation, score values were collapsed into three risk groups, dividing the whole range into three equally spaced categories. Second, the same multivariate model was fitted in the validation sample to test whether the hazard ratios (HRs) obtained in this set of patients were different from those in the original model. All statistical analyses were carried out using the Stata Statistical Program (StataCorp 2001, Release 7.0).

Results

Clinical Data

The series comprises 88 patients diagnosed with cutaneous malignant melanoma (41 males and 47 females). Their ages at the time of diagnosis ranged from 21 to 91 years (mean, 58 years). The average ages of patients with radial and vertical growth phases were 28 and 61 years and the percentage metastases were 0% and 55%, respectively. The survival analysis was restricted to vertical growth melanomas patients with follow-up information ($n = 60$) as, in this series, only these cases developed metastases and some of them died. In the patients with vertical growth phase melanomas the median follow-up was 61 months (range, 2 to 186 months). During the 6 years considered in the survival analysis, 4 were lost to follow-up, 19 patients died of melanoma, 4 patients died of other causes, and 33 patients remained alive.

Expression Profiling at Each Stage of Malignant Melanoma Progression

Immunohistochemical analysis of protein expression was carried out on 28 radial and 66 vertical growth phase malignant melanomas, 71 metastatic malignant melanoma, and 10 nevus specimens. The results of expression profiling at each stage are detailed in Table 2 and illustrated in Figure 1 and Figure 2, where each phase in the growth of malignant melanoma is distinguished by a specific constellation of markers. Nevertheless, some of the most significant features are worth comment: S-100 was positive in all but one case (the exception being a metastasis). HMB45 and melan A (specific markers of melanocytic origin) were constitutively expressed in the majority of primary malignant melanomas [95.6% (88 of

92) for HMB45 and 92.4% (85 of 92) for melan A] and in most metastatic cases, [81% (55 of 68) and 75% (50 of 67), for HMB45 and melan A, respectively; see Table 2]. Ki-67, a proliferation marker, was significantly higher in vertical growth phase melanoma and metastases than in nevi and radial growth phase tumors.

High levels of expression of specific cyclins (cyclin A, cyclin D1, and cyclin D3) and CDKs (CDK1 and CDK 2) were found when comparing nevus to malignant melanoma. Particularly striking was cyclin D1, which was negative in all nevi and was markedly expressed in radial growth phase malignant melanoma (12 of 25, 48%) and metastases (21 of 65, 32%). Surprisingly, the expression decreased significantly in the deepest component of the primary vertical growth phase tumor (see Figure 1). In the case of cyclin D3, high levels of expression were found only in metastatic stages. We found no significant change in cyclin E protein expression in any melanocytic lesions; however, CDK 2, the normal cyclin E partner, was more strongly expressed in radial growth phase melanoma (27 of 28, 86%) than in nevus (0%) and its expression progressively diminished in the subsequent steps (46 of 66, 48% in vertical cases and 34 of 68, 34% in metastases, respectively).

In the group of cell cycle suppressors, loss of the p16^{INK4a} protein was observed to be associated with advanced stages, with significantly lower expression levels in metastatic cases (48 of 68, 71%) compared with primary tumors (82 of 92, 89.13%) and benign lesions (9 of 9, 100%). The loss of p27^{KIP1} protein expression with melanoma progression was significant in vertical growth phase (30 of 66, 45%) compared with radial growth phase (19 of 25, 76%, $P = 0.010$).

Nuclear expression of the apoptotic inhibitor survivin was negative in all nevi (10 cases), and commonly expressed in malignant melanoma (97 of 159 cases, 61%), showing notably increased expression with melanoma progression. Regarding other proteins, caveolin, a membrane-scaffolding protein, experienced significant losses in primary malignant melanomas compared with nevi. MUM-1/IRF4, a known transcription factor detected in most primary melanocytic lesions (5 of 10, 50% benign nevi and 58 of 92, 70% of primary melanomas in our series, respectively), was progressively lost with tumoral advance and had a very low level of expression in metastatic cases (only 5 of 70, constituting 7% of positive cases).

Simultaneous Analysis of Vertical versus Metastatic Melanoma in the Same Patients

The existence of changes in the expression of a set of relevant molecules, when comparing vertical growth phase with metastatic melanoma, was confirmed in a restricted series of 30 patients, in which simultaneous analysis of samples representative from both stages was performed (Table 3).

Table 2. Contingency Analysis (Fisher's Exact Test) Comparing the Level of Protein Expression in Each Progression Step of Cutaneous Malignant Melanoma

	Nevus		Radial/Nevus		Radial		Vertical/Radial		Vertical		Met/Vertical		Metastasis		Met/Nevus	
	c/to	%(+)N	p	p*	c/to	%(+)R	p	p*	c/to	%(+)V	p	p*	c/to	%(+)M	p	p*
Melanoma specific proteins																
<i>HMB45</i>	10/10	100%	1.000	1.000	26/26	100%	0.574	0.831	62/66	94%	0.036	0.108	55/68	81%	0.199	0.310
<i>Melan A</i>	2/10	20%	0.000	0.000	25/26	96%	0.668	0.905	60/66	91%	0.021	0.084	50/67	75%	0.001	0.007
<i>S-100</i>	10/10	100%	1.000	1.000	26/26	100%	1.000	1.000	66/66	100%	1.000	1.000	68/69	99%	1.000	1.000
Cell Cycle																
<i>Cyclin A</i>	0/10	0%	0.003	0.016	14/26	54%	0.347	0.607	43/66	65%	0.344	0.602	49/66	74%	0.000	0.000
<i>Cyclin B1</i>	0/10	0%	0.303	0.489	4/25	16%	0.410	0.662	16/63	25%	1.000	1.000	15/58	26%	0.102	0.209
<i>Cyclin D1</i>	0/10	0%	0.007	0.028	12/25	48%	0.002	0.015	9/65	14%	0.021	0.084	21/65	32%	0.053	0.131
<i>Cyclin D3</i>	0/9	0%	0.303	0.489	6/27	22%	0.073	0.279	5/66	8%	0.009	0.050	17/66	26%	0.109	0.199
<i>Cyclin E</i>	0/10	0%	1.000	1.000	2/25	8%	0.335	0.704	12/66	18%	0.467	0.676	8/65	12%	0.587	0.770
<i>CDK1</i>	0/10	0%	0.002	0.012	15/27	56%	0.364	0.612	29/66	44%	0.154	0.323	21/67	31%	0.054	0.126
<i>CDK2</i>	1/10	10%	0.000	0.000	27/28	86%	0.001	0.012	46/66	48%	0.114	0.266	34/68	34%	0.162	0.284
<i>CDK6</i>	0/9	0%	0.303	0.489	6/27	22%	0.227	0.561	24/66	36%	0.005	0.035	10/67	15%	0.597	0.737
<i>p16</i>	9/9	100%	0.553	0.774	23/26	88%	1.000	1.000	59/66	89%	0.009	0.050	48/68	71%	0.102	0.209
<i>p21</i>	0/10	0%	0.073	0.192	13/25	32%	0.795	1.000	28/66	27%	0.100	0.263	42/66	42%	0.011	0.040
<i>p27</i>	7/10	70%	0.694	0.911	19/25	76%	0.010	0.053	30/66	45%	0.380	0.570	25/68	37%	0.082	0.181
<i>SKP2</i>	1/9	11%	0.391	0.587	8/26	31%	0.346	0.632	28/65	43%	0.491	0.687	33/67	49%	0.037	0.104
<i>p53</i>	7/9	78%	0.620	0.840	24/28	86%	0.770	1.000	53/66	80%	0.074	0.207	61/66	92%	0.196	0.317
<i>Rb</i>	2/10	20%	0.005	0.023	20/26	77%	1.000	1.000	49/66	74%	0.353	0.593	46/69	67%	0.011	0.040
<i>pRb</i>	0/10	0%	0.007	0.028	12/25	48%	0.345	0.659	40/66	61%	0.602	0.766	38/69	55%	0.001	0.007
<i>HDM 2</i>	1/10	10%	0.012	0.042	16/28	57%	0.232	0.541	47/66	71%	0.551	0.723	51/66	77%	0.000	0.000
<i>BCL-6</i>	0/10	0%	1.000	1.000	1/24	4%	1.000	1.000	5/64	8%	0.115	0.254	1/65	2%	1.000	1.000
Apoptosis																
<i>BCL-2</i>	10/10	100%	0.157	0.300	20/26	77%	0.336	0.672	55/63	87%	0.000	0.000	27/60	45%	0.001	0.007
<i>BCLX-L</i>	0/10	0%	0.145	0.305	6/23	26%	0.070	0.294	6/66	9%	0.204	0.389	12/66	18%	0.348	0.487
<i>SURVIVIN</i>	0/10	0%	0.038	0.106	10/28	36%	0.002	0.015	46/65	71%	0.356	0.575	41/66	62%	0.000	0.000
Transcription factors																
<i>MUM-1</i>	5/10	50%	0.079	0.175	22/26	85%	0.008	0.048	36/66	55%	0.000	0.000	5/70	7%	0.002	0.011
<i>PKCB</i>	0/10	0%	0.078	0.182	7/24	29%	0.032	0.149	37/66	56%	0.000	0.000	12/68	18%	0.346	0.501
<i>STAT-1</i>	0/10	0%	0.034	0.102	10/25	40%	0.815	0.978	29/66	44%	0.029	0.094	17/68	25%	0.107	0.204
DNA repair																
<i>MLH-1</i>	9/9	100%	0.077	0.190	15/22	68%	0.001	0.012	64/66	97%	0.000	0.000	40/67	60%	0.023	0.074
<i>MSH-2</i>	9/10	90%	1.000	1.000	24/26	92%	0.079	0.277	65/65	100%	0.028	0.098	60/66	91%	1.000	1.000
Membrane receptor																
<i>CAVEOLIN</i>	8/10	80%	0.001	0.007	4/25	16%	0.283	0.626	19/66	29%	0.104	0.257	29/67	43%	0.042	0.110
<i>C-KIT</i>	1/10	10%	0.000	0.000	22/25	88%	0.000	0.000	17/66	26%	0.542	0.734	14/68	21%	0.677	0.812
Transcription regulators: Polycomb																
<i>BMI 1</i>	10/10	100%	0.016	0.052	15/26	58%	0.170	0.476	26/63	41%	0.860	1.000	27/69	39%	0.000	0.000
<i>PH 1</i>	0/10	0%	0.552	0.799	3/28	11%	0.543	0.815	11/66	17%	0.181	0.362	5/66	8%	1.000	1.000
<i>RING 1B</i>	8/10	80%	1.000	1.000	22/26	85%	0.575	0.805	61/65	78%	0.002	0.017	34/66	52%	0.170	0.286
<i>RYBP 2</i>	0/10	0%	0.152	0.304	6/25	24%	0.092	0.297	6/63	10%	1.000	1.000	6/69	9%	1.000	1.000
<i>MEL 18</i>	7/9	78%	0.164	0.299	24/26	96%	0.435	0.677	58/65	89%	1.000	1.000	61/68	90%	0.282	0.423
Proliferation																
<i>Ki 67</i>	0/10	0%	1.000	1.000	1/28	4%	0.000	0.000	26/66	39%	0.294	0.537	32/65	49%	0.004	0.019
Others																
<i>TOPOISO</i> [†]	1/10	10%	0.000	0.000	20/26	77%	1.000	1.077	52/66	79%	0.015	0.070	38/66	58%	0.006	0.025
<i>HLA</i> [‡]	0/10	0%	1.000	1.000	0/24	0%	0.183	0.480	7/66	11%	0.792	0.978	9/69	13%	0.594	0.756

Each level is compared with the less aggressive stage (radial growth phase versus nevus, vertical versus radial and metastatic versus vertical). *P* values comparing nevus versus melanoma are also included (right-hand columns). Data reflect the number of positive cases of the total (c/to), the corresponding percentage and significance (*p*-value (*p*) and adjusted *p*-values (*p*^{*})) of the compared phases.

Significant results are indicated in bold. N, nevus; R, radial growth phase; V, vertical growth phase; M, metastases). [†], Topoisomerase II. [‡], HLA DP, DQ, DR.

Protein Expression and Survival Analysis

None of the radial growth phase malignant melanoma developed metastases or cause-related death, thus we have only considered vertical growth cases with follow-up information (*n* = 60) in the survival analysis. The results of this analysis using Kaplan-Meier and Cox regression models are shown in Table 4 (more data con-

cerning the univariate analysis are available in Supplemental Table 1 at <http://ajp.amjpathol.org>). Shorter overall survival (OS) was significantly related to loss of p16^{INK4a}, high Ki-67, and positive expression of cyclin E, p21^{CIP1}, Bcl-6, Rb, and HLA DP.

The Cox multivariate regression model included four biological markers: p16^{INK4a}, Bcl-6, Ki 67, and p21^{CIP1}.

Figure 2. Signature in melanoma progression. Representative proteins differentially expressed in cutaneous malignant melanoma identified by tissue array analysis. Subheadings indicate protein function and **arrows** represent up- or down-regulation in radial growth phase melanoma relative to nevus; vertical growth phase relative to radial growth phase and metastasis *versus* vertical growth phase. If there was no difference in protein expression between one phase and the comparative phase, the name of the marker is not included. See also Table 2 for differences in protein expression. A total of 175 specimens were evaluated, including 10 nevus, 28 radial malignant melanoma, 66 vertical malignant melanoma, and 71 metastases (34 skin metastases and 37 nodal metastases).

The strongest predictors were p16^{INK4a} and Bcl-6. The probability of death was around eight times higher when either p16^{INK4a} was lost or Bcl-6 was positive.

The predictive model was not substantially altered by the inclusion of Breslow's index as a variable, although the effect of Ki-67 was somewhat attenuated, probably reflecting its close relationship with Breslow's index. This fact corroborates the view that the markers included in the prognostic model and Breslow's index each contain independent information. To validate the predictor model, TMAs from an independent series of patients with follow-up information ($n = 72$) were stained and evaluated for the predictive set of markers.

A risk score was computed for each patient taking the linear predictor from the multivariate model. The formula of the score is depicted below, when each marker is considered 0 if negative and 1 if positive:

$$2.09 \cdot \text{bcl-6} + 0.88 \cdot \text{Ki-67} - 2.07 \cdot \text{p16} + 0.86 \cdot \text{p21}$$

When this score was calculated for patients in the validation sample, it was significantly related to the probability of death ($P = 0.001$).

Figure 3 presents the survival curves for the original and the validation groups collapsing the score into three categories, as described in the Materials and Methods, and specifically demonstrates the existence of a subset of tumors with an unfavorable profile, corresponding to

the third risk group. Finally, when the same multivariate model was fitted using the validation sample (see right-hand columns in Table 4) the loss of p16^{INK4a} and the expression of Bcl-6 were again identified as the most significant predictors.

Discussion

The analysis using TMAs allows the integration not only of the capability for detecting multiple proteins in large series of patients, but also the recognition of discrete cell subpopulations, such as intraepidermal melanocytes, which constitute the bulk of the malignant melanoma in the initial phases of melanoma genesis. The current study demonstrates the suitability of this technique in the study of a classical model in human tumor progression: malignant melanoma. To our knowledge, only a few studies based solely on the expression of single proteins^{9,18–20} have previously provided evidence of the feasibility of this powerful tool in malignant melanoma samples.

The present paper reports a comparative analysis of the different stages of the malignant melanoma sequence. Thus, each step in malignant melanoma progression is characterized by the expression of a specific profile: including cyclin D1, cyclin A, CDK1, CDK2, STAT1 in radial growth phase; Ki 67, survivin, and PKC-B

Figure 1. TMA design for melanoma progression. Cylinders corresponding to nevus (A), radial growth phase (B), vertical growth phase (C), and metastases (D). Immunohistochemical patterns. Core samples with positive and negative (or high and low) expression for selected antibodies are shown. All of the cases are representative of nevus-column A, and of each malignant melanoma progression step-columns B, C, and D. Note the high expression of cyclin D1 in radial (B) and metastases (D) when compared with vertical cases (C) and the loss of p16^{INK4a} expression in metastases. Note also the loss of p27^{KIP1} and caveolin with tumor progression and the nuclear survivin expression in melanomas when compared with nevi. Original magnification for A–D, $\times 40$; insets, $\times 600$ and $\times 800$, respectively. HE, hematoxylin and eosin.

Table 3. Comparative Expression of Primary *versus* Metastatic Tumors in the Same Patient (30 Patients)

Marker	Vertical (+) vs. metastases (-)	Vertical (-) vs. metastases (+)	Vertical (+) vs. metastases (+)	Vertical (-) vs. metastases (-)	Missing values*
BCL-2	9/30 (30%)	1/30 (3.3%)	9/30 (30%)	4/30 (13.3%)	7/30 (23.3%)
Cyclin D1	1/30 (3.3%)	4/30 (13.3%)	3/30 (10%)	17/30 (56.6%)	5/30 (16.6%)
Cyclin D3	2/30 (6.6%)	3/30 (10%)	1/30 (3.3%)	21/30 (70%)	3/30 (10%)
CDK 6	10/30 (33.3%)	4/30 (13.3%)	3/30 (10%)	10/30 (33.3%)	3/30 (10%)
HMB 45	3/30 (10%)	1/30 (3.3%)	23/30 (76.6%)	0/30 (0%)	3/30 (10%)
Melan A	7/30 (23.3%)	1/30 (3.3%)	17/30 (56.6%)	1/30 (3.3%)	4/30 (13.3%)
MLH 1	13/30 (43.3%)	0/30 (0%)	13/30 (43.3%)	0/30 (0%)	3/30 (10%)
MSH 2	2/30 (6.6%)	0/30 (0%)	24/30 (80%)	0/30 (0%)	4/30 (13.3%)
MUM 1	11/30 (36.6%)	0/30 (0%)	1/30 (3.3%)	17/30 (56.6%)	1/30 (3.3%)
P16	8/30 (26.6%)	2/30 (6.6%)	17/30 (56.6%)	0/30 (0%)	3/30 (10%)
P21	7/30 (23.3%)	6/30 (20%)	5/30 (16.6%)	9/30 (30%)	3/30 (10%)
PKCB	12/30 (40%)	2/30 (6.6%)	2/30 (6.6%)	11/30 (36.6%)	3/30 (10%)
RING 1B	11/30 (36.6%)	0/30 (0%)	12/30 (40%)	3/30 (10%)	4/30 (13.3%)
STAT 1	7/30 (23.30%)	1/30 (3.3%)	3/30 (10%)	17/30 (56.6%)	2/30 (6.6%)
Topoisomerase	4/30 (13.3%)	3/30 (10%)	4/30 (13.3%)	15/30 (50%)	4/30 (13.3%)

Vertical (+) *versus* metastases (-); number of cases positive in vertical phase, lost in metastasis; vertical (-) *versus* metastases (+), number of cases acquiring the expression of some marker; vertical (+) *versus* metastases (+), number of cases positive in vertical phase and in metastasis; vertical (-) *versus* metastases (-), cases without marker expression; *missing values, non-valuable data.

in vertical growth phase; and cyclin D1, cyclin D3, and loss of p16^{INK4a}, BclII, and MUM1 in metastatic melanoma (Figure 2). The results of this study illustrate how melanoma cells progress, as do other aggressive tumors, through the deregulation of the mechanisms controlling proliferation and escape from programmed cell death.²¹ Strikingly, progression is meticulously choreographed, and each step is distinguished by the expression of a characteristic set of proteins.

Many of the changes observed in this series involve a key cell cycle step: the progression beyond the G1/S stage. Indeed, most of the cell cycle regulatory proteins found in this study to be deregulated are involved in the control of the G1/S transition, including cyclin D1, D3,

cyclin E, CDK2, p16^{INK4a}, p27^{KIP1}, and p21^{CIP1}. Although many of these changes are stage-specific, in general it seems that an increasing degree of expression of cyclins and CDKs, in conjunction with a loss of CDK inhibitors, facilitated the progression to advanced clinical and histological stages. These findings confirm previous observations in single G1/S markers in human malignant melanoma specimens^{6,7,9,22-24} and is supported by experimental observations in cell lines^{25,26} and animal models.²⁷⁻³⁰ Additionally, studies of familial malignant melanoma have demonstrated mutations in genes regulating G1/S transition, including p16^{INK4a} and CDK4.^{8,31,32}

One of the most striking changes observed in this series is related to cyclin D1 protein. Cyclin D1 is nega-

Table 4. Biological Markers Associated with Patient's Outcome

6-year overall survival (from primary vertical growth phase melanoma to death)													
Training set										Validation set			
Univariate analysis					Multivariate analysis					Multivariate including Breslow			
Marker	Total Deaths cases	Hazard <72m	ratio	95% CI	p value	Hazard ratio	95% CI	p value	Hazard ratio	95% CI	p value	Hazard ratio	95% CI
BCL-6 neg	54	14	1.00		0.001	1.00		<0.001	1.00		0.002	1.00	
BCL-6 pos	5	5	5.97	2.07-17.18		8.10	2.56-25.6		6.59	1.92-20.5		6.59	2.03-21.38
Cyclin E neg	50	12	1.00		0.011								
Cyclin E pos	10	7	3.39	1.32-8.70									
HLA neg	55	15	1.00		0.029								
HLA pos	5	4	3.46	1.13-10.59									
Ki 67 low	37	9	1.00		0.047			0.068	1.00		0.248	1.00	
Ki 67 high	23	10	2.50	1.01-6.17		2.41	0.94-6.17		1.76	0.67-4.62		1.40	0.43-4.44
p16 neg	6	5	1.00		0.002	1.00		0.001	1.00		0.001	1.00	
p16 pos	54	14	0.20	0.07-0.57		0.13	0.04-0.41		0.14	0.04-0.45		0.19	0.06-0.61
p21 neg	44	9	1.00		0.010			0.065	1.00		0.106	1.00	
p21 pos	16	10	3.25	1.32-8.02		2.36	0.95-5.86		2.16	0.85-5.52		1.57	0.55-4.46
Rb neg	16	1	1.00		0.045								
Rb pos	44	18	7.86	1.05-58.91									
Breslow									1.00				
									1.68	0.94-2.99	0.079		

Kaplan-Meier, log rank test and Cox regression multivariate analysis of overall survival (OS), measured at time from primary vertical growth phase to death. The follow-up was 6 years. For the training set the significant *p*-values for the univariate analysis and the multivariate analysis before and after introducing Breslow's index are shown. Data from the multivariate analysis in the validation set are also included (right column). (All values from the univariate analysis in the training set are available in Supplemental Table 1 at <http://ajp.amjpathol.org>).

Figure 3. Kaplan-Meier estimation of overall survival according to the assigned probability in the multivariate analysis. A score was built-up according with the multivariate model. The survival curves for the original and the validation groups are presented. Patients are grouped collapsing the score values into three categories (low, intermediate, and high risk patients) obtained by dividing the whole hazard ratio range into three equally spaced categories (groups).

tive in nevi (0 of 10) and expressed in 12 of 25 (48%) radial growth phase malignant melanomas. These findings strongly suggest a role for cyclin D1 in malignant melanoma development. Moreover, this study confirms the previous observation that cyclin D1 is increasingly expressed in malignant melanoma,^{6,9,33} and also indicates that the precise stage where cyclin D1 is increased is the radial growth phase, showing a trend ($P = 0.08$) in the metastatic stage. The diminishing expression toward the deep reticular dermis observed in vertical growth phase melanoma cases is consistent with the findings of Sauter and co-workers,⁹ in which the high level of cyclin D1 expression seemed to be confined to the superficial component of the tumor.

Vertical growth phase malignant melanoma shows a simultaneous increase in proliferation (Ki67) and a decrease in p27^{KIP1} protein expression ($p < 0.001$) comparing with radial growth phase or benign lesions. Loss of p27^{KIP1} is required for the G1/S transition, as has been demonstrated in different experimental models.³⁴ The study of SKP2, a protein involved in the ubiquitination and degradation of p27^{KIP1}, shows a constant increase during the different stages of melanoma, which could explain the parallel increase in proliferation and decrease in p27^{KIP1} expression. A potential role of the extracellular matrix has also been proposed for inducing p27^{KIP1} degradation in melanoma.³⁵

The significant loss of p16^{INK4a} expression in the present series is correlated with advanced stages of malignant melanoma (metastatic disease). The p16^{INK4a} protein exerts an inhibitory function in the cell cycle by acting at the level of the cyclin D/CDK4-Rb pathway. Inactivation of p16^{INK4a} by homozygous deletions, point mutations, and promoter methylation has been involved in the genesis of malignant melanoma in familial and sporadic cases.^{10,23,26}

Malignant melanomas also feature defective regulation of apoptosis in parallel with progression, as shown by the

increasing overexpression of survivin from nevus (0 of 10) to radial (10 of 28, 36%), vertical growth phases (46 of 65, 71%), and metastases (41 of 66, 62%). The frequent expression of survivin in our series is consistent with the results of previous studies,^{11,36} suggesting that its activation is critical in resistance to apoptosis in malignant melanoma. This observation must be added to those already reported concerning APAF 1 in malignant melanoma.³⁷

The transcription factor MUM-1/IRF4 also seems to play a role in melanoma progression, as previously suggested.¹⁸ Interestingly, our results reflect how this expression decreases according to tumor progression, from 85% (22 of 26) in radial and 55% (36 of 66) in vertical growth phase to 7% (5 of 70) in metastases.

Caveolin, a structural membrane-scaffolding protein, is involved in cell-signal transduction and in angiogenesis inhibition.³⁸ It has been proposed that caveolin down-regulation may be associated with loss of apoptotic stimulus induced by caspase-3³⁹ and also with a significant role in neovascularization and, consequently, in development of metastatic disease.^{40–42} In this study, caveolin shows a loss of expression in malignant melanoma compared with nevus, which suggests that down-regulation of caveolin expression may contribute to malignant melanoma development.

This study also develops a predictor model for survival. The proteins included are p16^{INK4a}, Ki67, p21^{CIP1}, and Bcl-6. The data obtained from the overall survival model identify a group of patients with relatively short-term survival (<6 years) that seems to depend on an association between the absence of p16^{INK4a} or the presence of Bcl-6 protein expression in conjunction with either high proliferation (Ki 67 >20) or positive p21^{CIP1}. The absence of p16^{INK4a} expression as a prognostic marker in cutaneous malignant melanoma has previously been noted.¹⁰ On the other hand, no data about the expression and significance of Bcl-6 protein in malignant melanomas

have been reported to date. In this study, only a small number of invasive melanoma cases (5 of 64, 8%) expressed this protein, but it is noteworthy that all positive cases were strongly associated with poor prognosis in the univariate and the multivariate models. The inclusion of Breslow's index in this predictive model does not produce any substantial change, thus showing that this index and the biomarkers included represent independent information. The independent series used to validate the model confirmed the adverse prognostic value of both the loss of p16^{INK4a} and the acquisition of retinoblastoma markers.

In summary, a combination of tissue microarrays and clinical and pathological data can facilitate rapid characterization of candidate biomarkers of the development and regulation of progression in malignant melanoma tumors. The present analysis has highlighted the G1/S checkpoint abnormalities as being a critical step in the genesis and progression of malignant melanoma and has successfully identified new candidate proteins involved in melanoma genesis and progression as possible therapy targets.

Acknowledgments

We thank the Spanish National Tumor Bank Network, CNIO, and are particularly grateful to Alicia Maroto (12 de Octubre Hospital, Madrid), Dr. Pilar Fernández Segoviano (Getafe Hospital), Dr. José L. Sarasa (Fundación Jiménez Díaz, Madrid), Dr. Anastasio Serrano Egea and Dr. Jesús Razquin Murillo (Virgen de la Luz Hospital, Cuenca), and Dr. Teresa Rivera (Móstoles Hospital) for their help in providing specific tissue samples for this study. Dr. Miguel Angel Vidal (Centro de Investigaciones Biológicas-Consejo Superior de Investigaciones Científicas) and Dr. Giovanna Roncador (CNIO) have kindly provided some of the antibodies used in this study. We also thank Gonzalo Herrero Tambo for his expertise and excellent assistance with schematic pictures, Dr. David Rimm and Ana Diez for their valuable guidance with TMA construction, and Dr. Phil Mason for his help with the final version of the manuscript.

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