American Journal of Pathology, Vol. 153, No. 1, July 1998
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Protein Expression of the Cell-Cycle Inhibitor $p27^{Kip1}$ in Malignant Melanoma

Inverse Correlation with Disease-Free Survival

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In the present study we analyzed, by immunohistochemistry, a panel of human melanomas for protein expression of the cyclin-dependent kinase (cdk) inhibitor $p27^{Kip1}$ and evaluated whether deregulated expression correlates with clinical outcome for this type of cancer. We found that $p27^{Kip1}$ was strongly expressed by normal melanocytes and benign nevi, whereas in malignant melanoma, a heterogeneous expression pattern was observed. In the case of nodular melanomas, the level of $p27^{Kip1}$ was found to correlate significantly with the thickness of the tumor, with less protein expressed in thicker lesions. We also found that patients having tumors with fewer than 5% $p27^{Kip1}$-staining cells had a significantly higher risk of early relapse of their disease compared with those expressing moderate or high levels. In contrast, the level of $p27^{Kip1}$ did not correlate with tumor thickness or disease-free survival in patients with superficial spreading melanomas, suggesting that $p27^{Kip1}$ may play different roles in these two major pathological subgroups of malignant melanoma. Furthermore, $p27^{Kip1}$ did not appear to have an influence on overall survival for either subgroup. When we examined the combined effect of $p21^{WAF1/CIP1}$ (another cdk inhibitor) and $p27^{Kip1}$ on clinical outcome, we found that analysis of these two cdk inhibitors together may have greater prognostic potential than either alone. In conclusion, our results suggest that virtually complete loss of $p27^{Kip1}$ protein expression has potential importance as a prognostic indicator of early relapse in patients with nodular melanoma. The results, furthermore, underscore the value of analyzing multiple cell cycle regulatory proteins to obtain the most reliable indication of prognosis. ***(Am J Pathol 1998, 153:305–312)***

Normal cell cycle progression is governed by a family of cyclin-dependent kinases (cdks), the activity of which is regulated by association with positive effectors, the cyclins, by phosphorylation and dephosphorylation of the cdk subunit, and by association with small inhibitor molecules, of which $p15^{CDKN2b}$ and $p16^{CDKN2a}$ of the INK4 family and $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ of the KIP family are the best characterized.[1–3]

$p21^{WAF1/CIP1}$ was the first mammalian cdk inhibitor to be identified and was found to be induced by wild-type p53 in response to DNA damage, thereby suggesting a role in the coordination of p53-mediated growth arrest and DNA repair.[4,5] However, $p21^{WAF1/CIP1}$ has also been demonstrated to be involved in cellular senescence, terminal differentiation, and apoptosis through p53-independent mechanisms.[6–9] Notably, the gene encoding $p21^{WAF1/CIP1}$ has been recently cloned and identified as a melanoma differentiating antigen (mda6), the expression of which is up-regulated in more differentiated melanoma cell lines and in melanocytes grown *in vitro*.[10] In contrast, decreased $p21^{WAF1/CIP1}$ mRNA and protein levels have been detected in cell lines derived from advanced melanomas and in tumor specimens obtained from melanoma metastases, as compared with the levels in early-stage malignant lesions, suggesting that loss of $p21^{WAF1/CIP1}$ may contribute to the progression of this type of cancer.[10,11]

$p27^{Kip1}$ was originally identified as a cdk inhibitor, the activity of which is up-regulated *in vitro* by transforming growth factor (TGF)-β, by contact inhibition, or by serum depletion.[12–15] Furthermore, $p27^{Kip1}$ levels are increased during differentiation of cultured cells.[16,17] $p27^{Kip1}$ has been demonstrated to play an important role in regulating progression through G1 and entrance into the S phase of

Supported by stipends from The Norwegian Research Council (V. A. Flørenes) and Lillemor Grobstocks Legacy (V. A. Flørenes and G. M. Mælandsmo), by funding from the Norwegian Cancer Society (J. M. Nesland and R. Holm), and by grants from the National Institutes of Health (CA41223 to R. S. Kerbel) and the National Cancer Institute of Canada (to J. M. Slingerland). R. S. Kerbel is a Terry Fox Research Scientist of the National Cancer Institute of Canada. V. A. Flørenes was supported by a Postdoctoral Fellowship from the Medical Research Council of Canada.

Accepted for publication April 1, 1998.

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the cell cycle by binding to and preventing premature activation of cdk4/cyclin D and cdk2/cyclin E complexes.[14,18] In addition, constitutive overexpression of $p27^{Kip1}$ causes cell cycle arrest in the G1 phase. Recent studies have demonstrated that transgenic $p27^{Kip1}$ knockout mice develop multi-organ hyperplasia and are larger than their normal littermates.[19–21] This finding supports an important role for $p27^{Kip1}$ in the negative regulation of normal cellular proliferation.

In contrast to the INK4 inhibitors, members of the KIP family, $p21^{WAF1/CIP1}$ and $p27^{Kip1}$, are rarely mutated in human cancer.[22,23] The level of $p27^{Kip1}$ has been shown to be regulated primarily at the post-transcriptional level through the ubiquitin-proteasome-mediated pathway.[24] In colorectal carcinomas, decreased levels of $p27^{Kip1}$ are associated with increased ubiquitin-mediated degradation of $p27^{Kip1}$ and with a less favorable prognosis.[25] Furthermore, low levels of $p27^{Kip1}$ have also been associated with decreased survival of patients with breast,[26–28] gastric,[29] and non-small-cell lung cancer.[30] These findings led us to study whether altered expression of $p27^{Kip1}$ could also be detected in tumor samples obtained from patients with malignant melanoma and, if so, whether such alterations in $p27^{Kip1}$ levels may have value as a prognostic marker. Recently, our panel of primary and metastatic melanomas has been used to analyze protein expression of $p21^{WAF1/CIP1}$. In the aforementioned study, we observed reduced $p21^{WAF1/CIP1}$ protein expression in advanced melanomas; however, no correlation between $p21^{WAF1/CIP1}$ levels and clinical outcome was detected. In another recent study, by Porter et al,[27] $p27^{Kip1}$ and cyclin E proteins were both analyzed in a panel of breast carcinomas. The finding that low $p27^{Kip1}$ and high cyclin E levels appeared to have a powerful synergistic effect as prognostic indicators spurred us to address whether analysis of $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ levels together may be of greater prognostic value for melanoma than either alone.

Materials and Methods

Specimens

Formalin-fixed, paraffin-embedded tissue sections were obtained from 113 primary malignant melanomas, 45 melanoma metastases of which 36 had distant location, and 4 benign nevi. From 32 patients, both primary and metastatic material were collected. Of the primary tumors, 79 were classified as superficial and 34 as nodular. Clinical follow-up was available for 109 patients, and for 104 of these the combined value of $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ could be examined.

Immunohistochemical Analysis

Sections of formalin-fixed, paraffin-embedded tissue were immunostained using the biotin-streptavidin-peroxidase method (Supersensitive Immunodetection System, LP000-UL, Biogenex, San Ramon, CA) and the Optimax Plus Automated cell staining System (Biogenex). Deparaffinized sections were microwaved in 10 mmol/L citrate buffer (pH 6.0) for 20 minutes (four times for 5 minutes each) to unmask epitopes and treated with 1% hydrogen peroxidase (H_2O_2) for 10 minutes to block endogenous peroxidase. The sections were subsequently incubated with monoclonal $p27^{Kip1}$ antibody (K25020, Transduction Laboratories, Lexington, KY) diluted 1:200 (1.25 μg of IgG1/ml) for 30 minutes at room temperature and incubated thereafter with biotin-labeled secondary antibody (1:40) and streptavidin-peroxidase (1:40) for 20 minutes each. Tissue was stained for 5 minutes with 0.05% 3,3′-diaminobenzidine tetrahydrochloride freshly prepared in 0.05 mol/L Tris/HCl buffer, pH 7.6, containing 0.024% H_2O_2 and then counterstained with hematoxylin, dehydrated, and mounted in Diatex. All the dilutions of antibody, biotin-labeled secondary antibody, and streptavidin-peroxidase were made with phosphate-buffered saline, pH 7.4, containing 5% bovine serum albumin. All series included normal skin as positive controls. Negative controls included substitution of the monoclonal antibody with mouse myeloma protein of the same subclass and concentration as the monoclonal antibody. All controls gave satisfactory results. Four semiquantitative classes were used to describe the number of positive stained cells: −, none; +, <5%; ++, 5 to 50%; +++, >50%. The $p27^{Kip1}$ staining was evaluated by one and, in cases that were not obvious, by two observers. In the latter cases a good concordance was always achieved. Based on previous studies, $p27^{Kip1}$ protein expression was classified as high when more than 50% of the tumor cells stained positive[25,26] or, alternatively, when more than 5% were $p27^{Kip1}$ immunoreactive.

Statistical Analysis

The relationship between the expression of $p27^{Kip1}$ and the median tumor thickness was evaluated nonparametrically using the Mann-Whitney two-sample test. Kaplan-Meier survival analysis was used to evaluate the relapse-free and overall survival data. A value of $P < 0.05$ was considered significant.

Results

$p27^{Kip1}$ Levels in Primary and Metastatic Melanoma Lesions

Formalin-fixed, paraffin-embedded tissue sections from 113 primary and 45 metastatic malignant melanomas were immunohistochemically analyzed for $p27^{Kip1}$ protein expression. Nuclear $p27^{Kip1}$ staining was detected in 87% (98 of 113) of the primary malignant melanomas (Figure 1A) and in 67% (30 of 45) of the metastases (Figure 1B; Table 1). Furthermore, a higher fraction of primary tumors as compared with metastatic lesions showed $p27^{Kip1}$ immunoreactivity in more than 5% of the cells (54% *versus* 42%).

In 29 cases, both primary and metastatic tumor samples from the same patient could be examined. A decrease in the number of $p27^{Kip1}$-positive cells was ob-

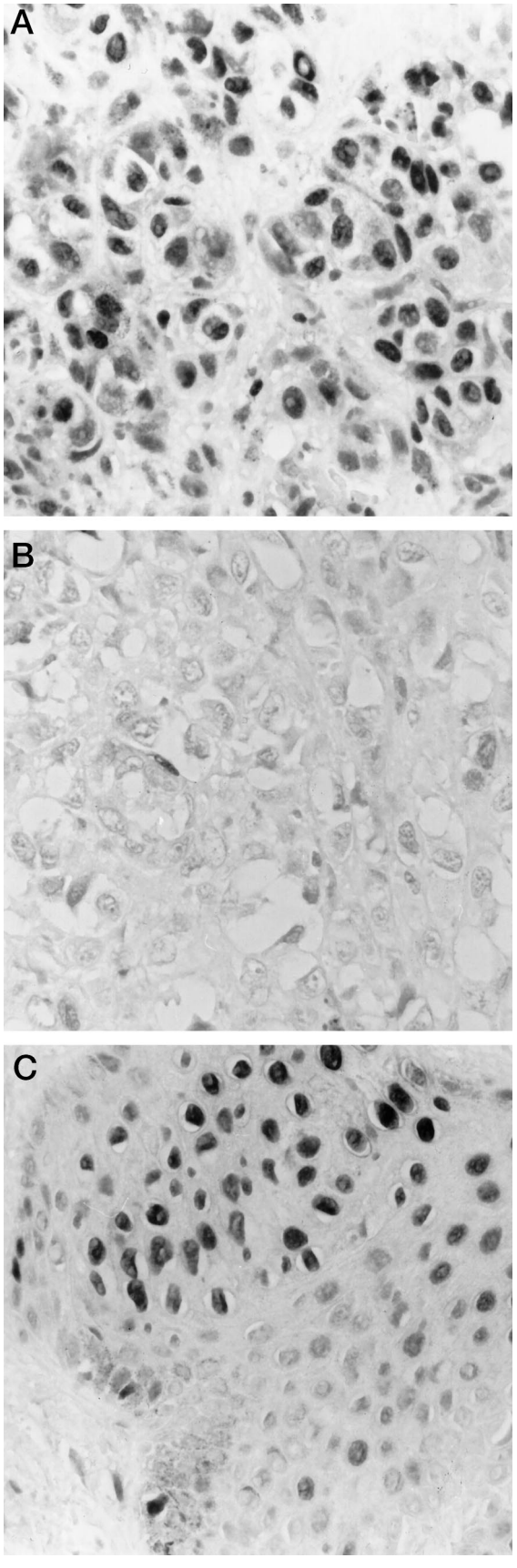

Figure 1. Immunohistochemical analysis showing the level of $p27^{Kip1}$. A: Primary melanoma. B: Metastasis from the same patient as shown in A. C: Benign nevi, including normal skin.

served in 14 of 29 (45%) of the metastatic tumors when compared with the corresponding primary lesion (Figure 1, A and B). In another eight patients, with the same proportion of cells stained in the primary and metastatic tumor, a lower intensity of $p27^{Kip1}$ staining was found in four of the latter. Combining these results, altogether 18 of 29 (62%) of the metastases expressed lower levels of $p27^{Kip1}$ than the corresponding primary tumor (Table 2). In normal skin, distinct protein staining was present in all nucleus-containing layers of the squamous epidermis and underlying adnexal structures. A high $p27^{Kip1}$ level was also observed in the four benign nevi examined (Figure 1C).

Expression of $p27^{Kip1}$ Protein in Relation to Clinical Parameters of Malignancy

We found that there was no significant difference in the distribution of low and high $p27^{Kip1}$ staining between nodular and superficial melanomas using either a 50% or a 5% cutoff to describe high and low $p27^{Kip1}$ levels (Table 1 and results not shown). Interestingly, however, within the nodular subtype, there was a significant variation in $p27^{Kip1}$ staining as a function of the vertical growth of the tumors, with lower levels in thicker lesions ($P = 0.05$, 5% cutoff; Table 3). Using a 50% cutoff to discriminate between high and low $p27^{Kip1}$ levels did not change the results ($P = 0.05$, data not shown).

Clinical follow-up data were available for 109 patients (superficial, $n = 76$; nodular, $n = 33$), of which 35 developed distant metastases. For the total group of patients, there was no correlation between the level of $p27^{Kip1}$ using a 50% cutoff and relapse-free or overall survival ($P = 0.59$ and $P = 0.76$, respectively; Figure 2, A and B). Changing the cutoff (low $p27^{Kip1}$ when $<5\%$ cells stained positive) did not alter the outcome of the evaluation (data not shown). When the superficial and the nodular spreading melanomas were examined separately, there was a trend toward increased disease-free survival in patients whose nodular tumor expressed higher levels of $p27^{Kip1}$ than those having less than 50% of the cells stained positive for the inhibitor ($P = 0.15$; Figure 2C). Interestingly, when the cutoff between high and low $p27^{Kip1}$ protein expression was changed (high when $>5\%$ of the cells were $p27^{Kip1}$ positive), the disease-free survival was clearly reduced for those patients expressing $p27^{Kip1}$ in less than 5% of the tumor cells ($P = 0.0002$; Figure 2D). Notably, the level of $p27^{Kip1}$ in the primary tumor did not influence overall survival for patients with nodular melanoma ($P = 0.68$ with 50% cutoff *versus* $P = 0.41$ with 5% cutoff; data not shown). $p27^{Kip1}$ was not found to have any impact on either disease-free or overall survival for patients with the superficial spreading subtype (Figure 2E and data not shown), and changing the cutoff did not influence the outcome of the evaluation (Figure 2F and data not shown), possibly due to the long duration of follow-up required for the demonstration of survival benefit for this subtype of melanoma. Altogether, these results suggest that $p27^{Kip1}$ may play different roles in the malignant progression of the two major subgroups of

Table 1. Number (Percentage) of Melanocytic Tumors Expressing $p27^{Kip1}$

Tumor type	Number examined	$p27^{Kip1}$ protein level*			
		−	+	++	+++
Primary melanomas	113	15 (13%)	38 (34%)	23 (20%)	37 (33%)
Superficial	79	10 (13%)	31 (39%)	14 (18%)	24 (30%)
Nodular	34	5 (15%)	7 (21%)	9 (26%)	13 (38%)
Metastatic melanoma	45	15 (33%)	11 (25%)	13 (29%)	6 (13%)
Benign nevi	4	–	–	–	4 (100%)

*Measured as described in Materials and Methods.

melanoma, and furthermore, nodular melanomas, which express virtually no $p27^{Kip1}$ in the tumor cell nuclei, have the highest risk of early recurrence.

Comparison between $p27^{Kip1}$ and $p21^{WAF1/CIP1}$ Protein Expression

A total of 104 primary tumors and 31 metastases could be examined for expression of both $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ proteins. Only one primary tumor showed positive immunoreactivity for both proteins in more than 50% of the cells. In contrast, 36 tumors (31%) showed both $p27^{Kip1}$ and $p21^{WAF1/CIP1}$ staining in fewer than 5% of the cells. High (>50% cells stained) staining of both proteins was never seen in metastatic samples, and indeed, 13 (42%) showed almost complete loss (<5% cells stained) of both $p27^{Kip1}$ and $p21^{WAF1/CIP1}$ staining.

Table 2. Relationship between the Level of $p27^{Kip1}$ Protein in Primary and Metastatic Melanoma from the Same Patient

Metastasis	$p27^{Kip1}$ protein level*			
	Primary tumor†			
	−	+	++	+++
−	2‡	5	2	3
+	2	2	0	1
++	1	2	3	3
+++	1	0	1	1

*Measured as described in Materials and Methods.
†In 29 cases, primary and metastatic tumor from the same patient could be analyzed.
‡In eight cases with the same proportion of cells stained in the primary and metastatic tumor, a lower staining intensity was found in four of the metastases.

Table 3. Relationship between $p27^{Kip1}$ Levels and Depth of Tumor Growth of Primary Melanomas

Tumor type	Immunohisto-chemistry*	Number of patients	Depth of growth† (mm)
Superficial	High	24	1.15 $P = 0.56$
	Low	55	1.15
Nodular	High	13	2.30 $P = 0.05$
	Low	21	5.20

*Scored as described in Materials and Methods: <50% cells stained, low expression; >50% cells stained, high expression. Changing the cutoff to high when more than 50% of the cells were p27 positive did not alter the outcome. A total of 113 tumors were analyzed.
†Measured as the median thickness in each group.

When assessing the effect of reduced levels of both $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ proteins on clinical outcome for the whole group of patients, we observed that moderate to high levels of both proteins in the primary tumors did not correlate with increased disease-free and overall survival (data not shown). When pathological subgroups of melanoma were analyzed separately, however, we found that, in nodular melanoma, low levels of $p21^{WAF1/CIP1}$ staining in cases that also showed reduced $p27^{Kip1}$ (<50% cells stained positive) was a stronger predictor of relapse of disease ($P = 0.01$) than low $p27^{Kip1}$ alone ($P = 0.15$; Figures 3A and 2C). However, with a 5% cutoff between high and low $p27^{Kip1}$ expression, less than 5% $p27^{Kip1}$-positive cells was still the strongest indicator of early relapse ($P = 0.0002$), and $p21^{WAF1/CIP1}$ did not add any additional power to the evaluation ($P = 0.001$; Figures 2D and 3C).

Recently, in primary superficial melanomas, we observed a significant correlation between $p21^{WAF1/CIP1}$ protein expression and tumor thickness, with thin lesions showing the lowest protein levels ($P = 0.005$).[11] Neither $p21^{WAF1/CIP1}$ nor $p27^{Kip1}$ levels examined alone were found to have any impact on the clinical outcome for this group of patients. However, when $p27^{Kip1}$ and $p21^{WAF1/CIP1}$ were assayed together, we found that low levels of $p27^{Kip1}$ (<5% cells stained) together with high levels of $p21^{WAF1/CIP1}$ (>5% cells stained) was a stronger predictor of early recurrence than $p27^{Kip1}$ alone ($P = 0.09$ *versus* $P = 0.95$; Figures 3D and 2F).

In conclusion, we have shown that almost complete loss of $p27^{Kip1}$ protein has prognostic value for relapse of disease for patients with nodular melanoma, but not for those with superficial malignant melanoma. $p27^{Kip1}$ was not predictive of overall survival in either group. Furthermore, a combined analysis of $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ proteins may have a greater prognostic potential for relapse-free survival in both of these pathological subgroups.

Discussion

In the present study we examined the protein level of $p27^{Kip1}$ in a panel of primary and metastatic human melanoma specimens and evaluated whether deregulated expression of this cdk inhibitor was correlated with clinical outcome. Our panel of melanoma specimens has been previously analyzed for the protein expression of $p21^{WAF1/CIP1}$, another member of the KIP family of cdk

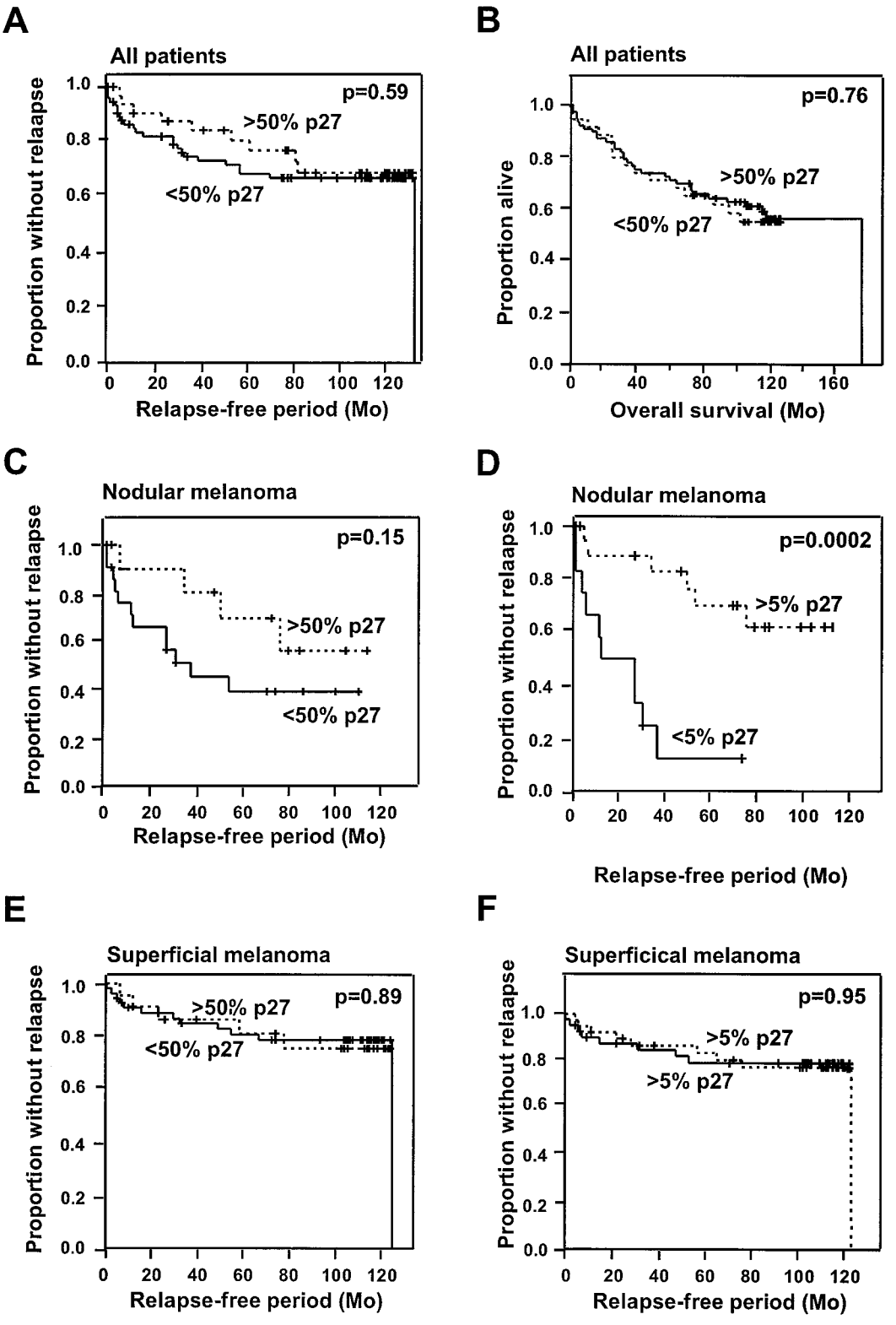

Figure 2. Kaplan-Meier curves demonstrating the relationship between the expression of $p27^{Kip1}$ and relapse-free (A) and overall (B) survival for the entire panel of melanoma patients (n = 109) and relapse-free survival divided by the following subtypes: nodular (C and D; n = 33) and superficial (E and F; n = 76). $p27^{Kip1}$ was considered as high when more than 50% of the tumor cells stained positive (A, B, C, and E) or high when more than 5% showed positive immunoreactivity (D and F).

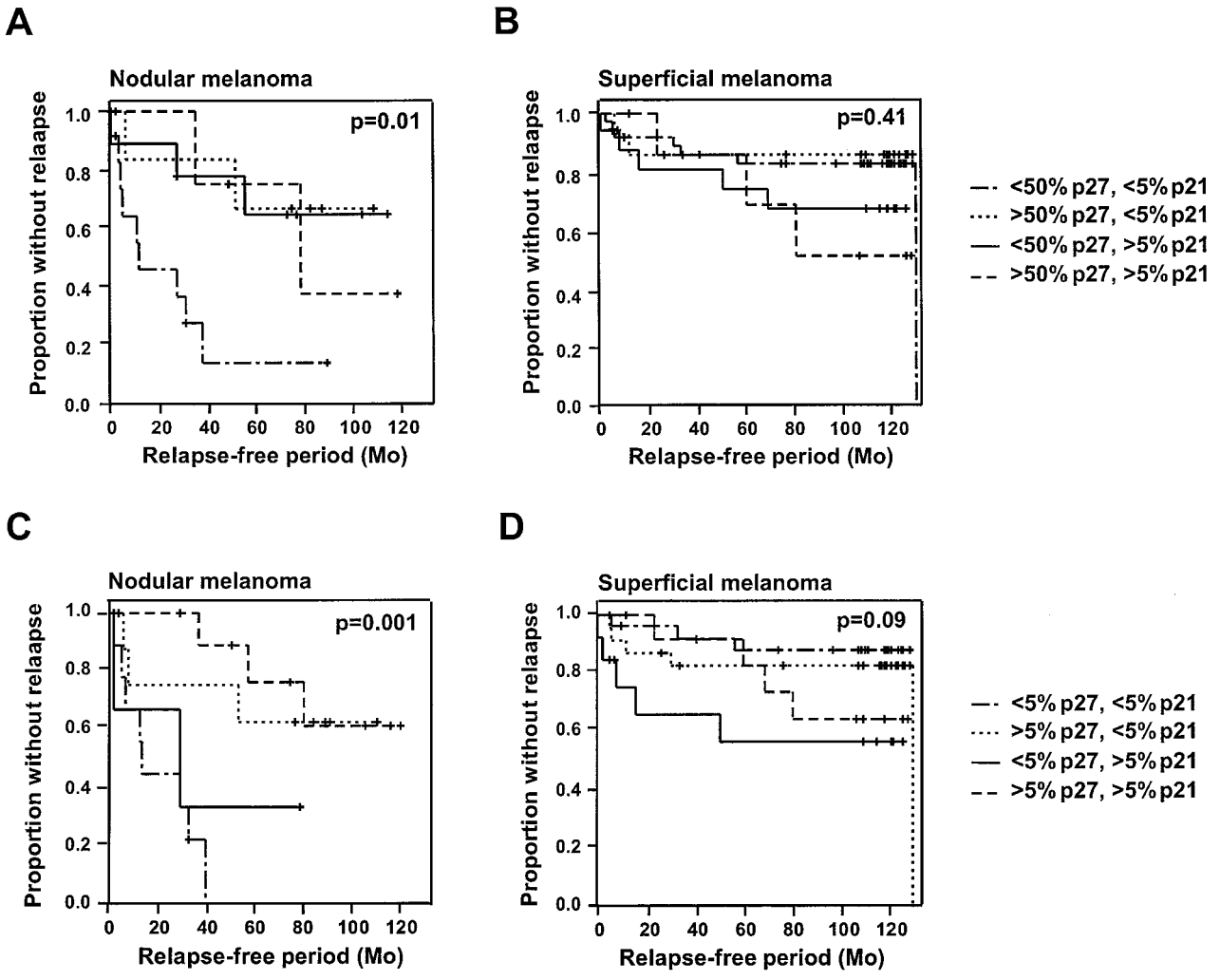

Figure 3. Kaplan-Meier curves demonstrating the association between relapse-free survival and expression of $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ in nodular (A and C) and superficial (B and D) melanoma. In A and B, $p27^{Kip1}$ was considered as high when more than 50% of the tumor cells stained positive, and in B and D, when more than 5% expressed the protein. In all cases, $p21^{WAF1/CIP1}$ was considered as high when more than 5% of the cells showed positive $p27^{Kip1}$ immunoreactivity. The different combinations of high and low $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ are indicated.

inhibitors.[11] We therefore made a comparison between the protein levels of $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ and evaluated their combined prognostic value for this group of patients.

In agreement with previous reports for normal tissue of epithelial origin,[26,30,31] we found that $p27^{Kip1}$ was highly expressed in normal skin, including keratinocytes, melanocytes, and benign nevi, supporting the notion that one important function of $p27^{Kip1}$ may be to regulate quiescence in normal cells.[32–34] This is in contrast to $p21^{WAF1/CIP1}$, the expression of which is not detectable in melanocytes and benign nevi *in vivo*[11,35] and which is also very low in G_0-arrested melanoma cell lines *in vitro*.[32] Thus, these two cdk inhibitors may play different roles in proliferation of cells of the melanocytic linage.

In agreement with what has been observed for other types of tumors,[26,27,30,31] $p27^{Kip1}$ was found to be heterogeneously expressed in malignant melanomas, with a trend toward less $p27^{Kip1}$ immunoreactivity in metastatic lesions as compared with the levels seen in the corresponding primary tumors. However, when the panel of malignant melanomas was divided into the two most common pathological subtypes, the superficial and the nodular, important differences were observed. In nodular melanomas, thicker tumors had significantly less $p27^{Kip1}$ than thinner, and potentially less aggressive, lesions. Furthermore, we found that patients whose tumors showed virtually no $p27^{Kip1}$ immunoreactivity had a significantly shorter relapse-free period than those whose tumors expressed higher $p27^{Kip1}$ levels. This finding is in accordance with recent reports indicating that $p27^{Kip1}$ is a prognostic indicator of disease-free survival for patients with breast, colon, gastric, and non-small-cell lung cancers.[25–30] Notably, Ki-67 immunostaining of a limited number of the melanomas revealed no clear correlation between the p27 levels and cell proliferation (data not shown), a finding that has been demonstrated also by others,[25,26,36,37] suggesting that p27 may have additional functions that are not strictly related to cell cycle progression.

The most reliable prognostic marker for patients with melanoma is considered to be the vertical thickness of

the primary tumor.[38] Due to the low number of cases analyzed, we were, however, unable to determine whether the observed impact of p27Kip1 on disease-free survival for patients with nodular melanoma was independent of tumor thickness. Notably, p27Kip1 was not found to have any impact on overall survival for this group of patients. This could be due to the need for longer follow-up or to limitations in the power of the study. p27Kip1 has been suggested to have functions related to cell adhesion[39–41] and may also play a role in tumor invasion.[29] Based on these findings, reduced p27Kip1 levels may correlate with other events that favor metastasis formation by allowing cells to escape from the primary site.

In superficial melanomas, no association between p27Kip1 levels, tumor thickness, and relapse-free period could be detected. In contrast to nodular melanomas, the superficial spreading lesions are characterized by an initial radial growth phase that is generally considered to be significantly less aggressive than the vertical growth phase of superficial spreading melanoma.[38] It may be speculated, therefore, that one reason for the observed differences in growth pattern between these two pathological subtypes may be related to differences in their responsiveness to altered levels of p27Kip1.

We next evaluated the prognostic potential of combined analysis of p21WAF1/CIP1 and p27Kip1. In nodular melanomas, low p21WAF1/CIP1 levels in combination with less than 50% of the cells showing p27Kip1 immunoreactivity was a better indicator of disease failure ($P = 0.01$) than p27Kip1 alone ($P = 0.15$). In this regard, it is of note that Porter et al[27] found that a combined analysis of p27Kip1 and cyclin E had a greater prognostic value than either factor alone. However, the strongest predictor of disease-free survival for patients with nodular melanoma was the virtual complete loss of p27Kip1 protein (<5% tumor cells stained, $P = 0.0002$) as compared with those having higher levels of the cdk inhibitor. Altogether, our results suggest that p27Kip1 is a more important predictor of the aggressiveness of nodular melanomas than p21WAF1/CIP1.

We have shown previously that the protein level of p21WAF1/CIP1 increases with tumor thickness of superficial melanomas.[11] One reason for the observed differences in aggressiveness between superficial spreading and nodular melanomas could be that p21WAF1/CIP1 functions to delay cell cycle progression and thereby tumor growth in the former. However, when evaluating the combined effect of p21WAF1/CIP1 and p27Kip1 in superficial melanomas, we found that moderate to high levels of p21WAF1/CIP1 (>5% cells stained) in combination with a low level of p27Kip1 (<5% cells stained) was a stronger indicator of disease recurrence than either inhibitor alone. Thus, in accordance with reports suggesting that p21WAF1/CIP1 may act as an assembling factor,[42] it might be speculated that p21WAF1/CIP1 in early-stage superficial melanomas plays a role in activating cdk complexes, thereby facilitating cell cycle progression. Given the small sizes of the melanoma subsets studied, the prognostic potential of combined analysis of p21WAF1/CIP1 and p27Kip1 needs, however, to be verified in larger cohorts of both superficial or nodular melanoma subtypes.

In summary, our results suggest that virtually complete loss of p27Kip1 protein may have potential as an indicator of early relapse of disease in patients with nodular melanoma. Studies with larger sample sizes are needed to assess whether p27Kip1 has significant prognostic value independent of tumor thickness.

Acknowledgments

We gratefully thank Ellen Hellesylt and Mette Ingrud for technical assistance and Cassandra Cheng for secretarial help.

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