

# Levels of Cyclin D1 and D3 in Malignant Melanoma: Deregulated Cyclin D3 Expression Is Associated with Poor Clinical Outcome in Superficial Melanoma<sup>1</sup>

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## ABSTRACT

We examined 172 primary (110 superficial and 62 nodular) and 73 metastatic melanomas, as well as 10 benign nevi, for protein expression of cyclin D1 and cyclin D3 and evaluated the relationship between deregulated protein levels and clinical outcome. For both proteins, a heterogeneous nuclear staining pattern was observed. Cyclin D3 was expressed by 96% of primary and 97% of metastatic melanomas. The corresponding percentages for cyclin D1 were 62% and 29%, respectively. In benign nevi, only rare cyclin D3-positive cells and no cyclin D1-positive cells were observed. High levels of cyclin D3 (>5% of the cells stained) were detected in 26 of 62 (42%) nodular melanomas and in 22 of 110 (20%) superficial tumors, whereas no such difference was observed with respect to cyclin D1. In superficial melanomas, a significant concordant staining pattern was observed between cyclin D1 and cyclin D3 ( $P = 0.0009$ ), cyclin D1 and Ki-67 ( $P = 0.0001$ ), cyclin D1 and cyclin A ( $P = 0.02$ ), cyclin D3 and Ki-67 ( $P < 0.00001$ ), and cyclin D3 and cyclin A ( $P = 0.002$ ). Kaplan-Meier analysis revealed that high levels of cyclin D3 were an indicator of early relapse and decreased overall survival for patients with superficial ( $P = 0.001$  and  $P = 0.009$ , respectively) but not nodular ( $P = 0.64$  and  $P = 0.23$ ) melanoma. Cyclin D1 did not have any impact on disease-free and overall survival for either of the subtypes. In conclusion, our results suggest that deregulation of cyclin D3 expression leading to increased proliferation may be a prognostic factor for superficial melanoma, whereas deregulated cell cycle machinery seems to have little impact on disease progression of nodular melanoma.

Received 3/28/00; revised 6/1/00; accepted 6/2/00.

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<sup>1</sup> Supported by the Norwegian Cancer Society.

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## INTRODUCTION

The orderly progression of cells through the cell cycle depends on a fine-tuned balance between the levels of activated cyclins and CDKs<sup>3</sup> that provide positive growth signals and the kinase inhibitors that suppress these effects. The D-type cyclins (D1, D2, and D3) are the first cyclins to be expressed in the G<sub>1</sub> phase and, bound to their kinase partners CDK4 and CDK6, they are likely to play a major role in phosphorylating the retinoblastoma protein, thereby orchestrating progression through the G<sub>1</sub> restriction point (1, 2). The three D-type cyclins are expressed combinatorially in a cell lineage manner in all proliferating cells. However, it is not yet clear to what extent they carry out overlapping or distinct functions.

Based on genetic analysis and experiments with gene transfer and transgenic mice, cyclin D1 and cyclin D2 have been classified as proto-oncogenes. Thus, cyclin D1 has been shown to be activated by chromosomal inversions involving chromosome fragment 11q13 in parathyroid adenomas and by a t(11;14)(q13;32) translocation in B-cell lymphomas (3). Increased cyclin D1 protein levels attributable to gene amplification or abrogated mRNA expression have been observed in a number of different malignancies including cancers of the breast, esophagus, lung, bladder, and liver (4). In a limited study in melanomas, increased cyclin D1 protein expression, as compared with the level in adjacent tissue, was observed in 12 of 37 cases (33%) (5). Furthermore, high levels of cyclin D1 mRNA without concomitant gene amplification have been observed in 63% of metastases from malignant melanoma (6). In line with these observations, elevated cyclin D1 levels in tumors, as compared with the levels in corresponding normal cells, have been shown to be an important prognostic indicator of poor clinical outcome for patients with non-small cell lung cancer (7) as well as with ovarian (8) and esophageal carcinomas (9). In other tumor forms, such as gastric (10), breast (11), and bladder (12) carcinomas, no association with clinical outcome has been documented.

Transcriptional activation of cyclin D2 is one of the earliest events during immortalization of primary B cells by EBV, and aberrant protein expression occurs early in human male germ cell tumorigenicity (13). In gastric cancer, overexpression of cyclin D2 correlates with progression and prognosis (14), and occasional amplification of the cyclin D2 gene has been detected in colorectal tumors (15). Notably, cyclin D2 protein was not detected in a panel of human melanoma cell lines (16).

Cyclin D3 shares considerable homology with cyclins D1 and D2. Although its role in human tumorigenicity has not been fully clarified, in fibroblasts cyclin D3 has been demonstrated to

<sup>3</sup> The abbreviation used is: CDK, cyclin-dependent kinase.

Table 1 Number (percentage) of melanocytic tumors expressing different levels of cyclin D1 and D3

Tumor type	No. of tumors examined	Cyclin D1 <sup>a</sup>				Cyclin D3 <sup>a</sup>			
		-	+	++	+++	-	+	++	+++
Primary melanomas	172	65 (38)	91 (53)	13 (7)	3 (2)	7 (4)	117 (68)	35 (20)	13 (8)
Superficial	110	36 (33)	64 (58)	8 (7)	2 (2)	6 (5)	82 (75)	20 (18)	2 (2)
Nodular	62	29 (47)	27 (43)	5 (8)	1 (2)	1 (2)	35 (56)	15 (24)	11 (18)
Metastatic melanomas	73	52 (71)	18 (25)	2 (3)	1 (1)	2 (3)	32 (44)	22 (30)	16 (22)
Benign nevi	10	10 (100)	0 (-)	0 (-)	0 (-)	0 (-)	10 (100)	0 (-)	0 (-)

<sup>a</sup> Expression of cyclins D1 and D3 was measured as described in "Materials and Methods." -, none; +, <5%; ++, 5-50%; and +++, >50%.

be rate-limiting for G<sub>1</sub> to S-phase transition (17). Most studies have focused on the role of cyclin Ds in the mitogenic stimulation of cell proliferation, although recent data suggest that cyclin D3 and probably also cyclin D2 have a function in differentiation and growth arrest in certain cell types (16, 18). To our knowledge, no studies thus far have focused on evaluating the importance of cyclin D3 on clinical outcome for cancer patients. Based on our previous observation suggesting that cyclin D1 mRNA is more abundant in melanoma metastases than in benign nevi (6) and the fact that cyclin D3 is also expressed in melanomas (5, 19), we wanted to examine the protein levels of these two G<sub>1</sub> cyclins in a panel of human melanomas representative of different stages of the disease. The aim was to evaluate the relationship between tumor levels of cyclin D1 and cyclin D3 and proliferative capacity estimated by Ki-67- and cyclin A-positive immunoreactivity and, furthermore, to examine the influence of deregulated cyclins D1 and D3 on clinical outcome.

## MATERIALS AND METHODS

**Specimens.** Formalin-fixed, paraffin-embedded tissue sections were obtained from 172 primary malignant melanomas, 73 metastases, and 10 benign nevi. Both primary and metastatic material were collected from 47 patients. Of the primary tumors, 110 were classified as superficial, and 62 were classified as nodular. Clinical follow-up was available for all patients.

**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 1 mM EDTA (pH 8.0) for 4 × 5 min to unmask epitopes. After treatment with 1% hydrogen peroxidase for 10 min to block endogenous peroxidase, the sections were subsequently incubated with monoclonal cyclin D1 antibody (ccl2, clone DCS-6; 1:200; Oncogene Research Products, Cambridge, MA) or monoclonal cyclin D3 antibody (M7156, clone DCS-22; 1:25; Dako A/S, Glostrup, Denmark) for 30 min at room temperature. The sections were then incubated with biotin-labeled secondary antibody (1:30) and streptavidin-peroxidase (1:30) for 20 min each. Tissue was stained for 5 min with 0.05% 3,3'-diaminobenzidine tetrahydrochloride freshly prepared in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.024% hydrogen peroxidase and then counterstained with hematoxylin, dehydrated, and mounted in Diatex. All of the dilutions of antibody, biotin-labeled secondary antibody, and

streptavidin-peroxidase were made with PBS (pH 7.4) containing 5% BSA. All series included positive controls. Negative controls included substitution of the primary 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 stained cells: (a) -, none; (b) +, <5%; (c) ++, 5-50%; and (d) +++, >50%. Only nuclear staining was scored as positive. The staining was evaluated by one observer and, in cases where staining was not obvious, by two observers. In the latter cases, a good concordance was always achieved.

**Statistical Analysis.** The relationship between the expression of cyclin D1, cyclin D3, and mean tumor thickness was evaluated nonparametrically using the Mann-Whitney two-sample test. A comparison between the expression of cyclin D1, cyclin D3, and other markers of cell cycle progression was performed using the  $\chi^2$  test. Kaplan-Meier estimates and the log-rank test were used to evaluate the survival data statistically.  $P < 0.05$  was considered statistically significant. The Cox proportional hazards model was used to determine independent prognostic variables for disease-free and overall survival. Covariates giving  $P < 0.05$  were included in the final Cox model.

## RESULTS

**Expression of Cyclin D1 and Cyclin D3 in Primary and Metastatic Melanoma Lesions.** Formalin-fixed, paraffin-embedded tissue sections from 172 primary and 73 metastatic melanomas were analyzed by immunohistochemistry for protein expression of cyclin D1 and cyclin D3. The results are summarized in Table 1. For both proteins, a heterogeneous nuclear staining pattern was observed, and, in most cases, diffuse cytoplasmic staining was present as well (Fig. 1). Cyclin D3 was the most abundant G<sub>1</sub> cyclin expressed by both primary and metastatic melanomas. Thus, whereas 62% (107 of 172) of the primary tumors and 29% (21 of 73) of the metastases expressed detectable levels of cyclin D1 in the tumor cell nuclei, cyclin D3 protein was observed in 96% (165 of 172) and 97% (71 of 73) of the cases, respectively. Furthermore, a higher fraction of metastatic lesions showed cyclin D3 as compared with cyclin D1 immunoreactivity in more than 50% of the nuclei (22% versus 1%). In 47 cases, both primary and metastatic tumors from the same patient could be analyzed. In 27 of the 47 cases (57%), the same number of cyclin D1-positive cells was detected in primary and metastatic tumors, whereas the corresponding percentage for cyclin D3 was 54% (25 of 47). No cyclin D1 immunoreactivity was observed in the 10 benign nevi

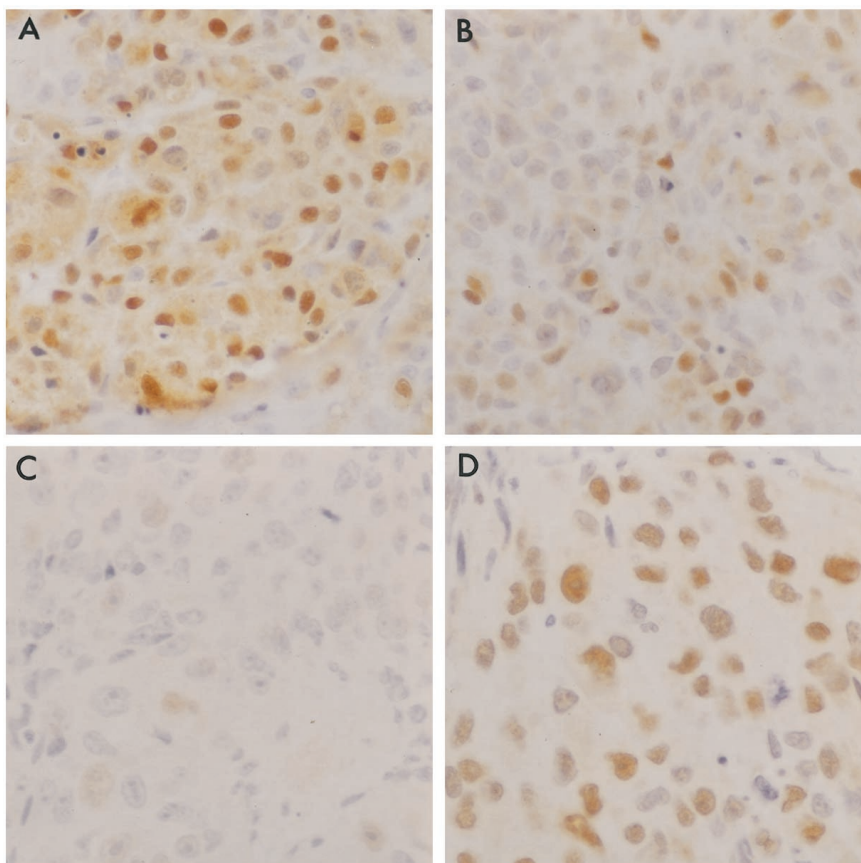


Fig. 1 Immunohistochemical analysis showing examples of cyclin D1 and D3 immunoreactivity in melanocytic lesions. Only nuclear staining was evaluated as positive. Cyclin D1 (A) and cyclin D3 (B) expression in a primary melanoma. Cyclin D1 (C) and cyclin D3 (D) expression in metastasis from the same patient as in A and B.

Table 2 Relationship between cyclin D1, cyclin D3, and depth of tumor growth of primary melanomas

Tumor type	Marker	Immunohistochemistry		Depth of growth <sup>c</sup> (mm)	P
		Expression level <sup>a</sup>	No. of patients <sup>b</sup>		
Superficial	Cyclin D1	Low	97	1.82	0.59
		High	10	1.51	
	Cyclin D3	Low	85	1.51	
		High	22	2.89	
Nodular	Cyclin D1	Low	55	4.85	0.03
		High	6	2.51	
	Cyclin D3	Low	35	4.27	
		High	26	5.09	

<sup>a</sup> Expression level was scored as described in “Materials and Methods:” -/+, low expression; ++/+++, high expression.

<sup>b</sup> Tumor thickness was available for a total of 168 patients (107 superficial and 61 nodular melanomas).

<sup>c</sup> Measured as the mean thickness in each group.

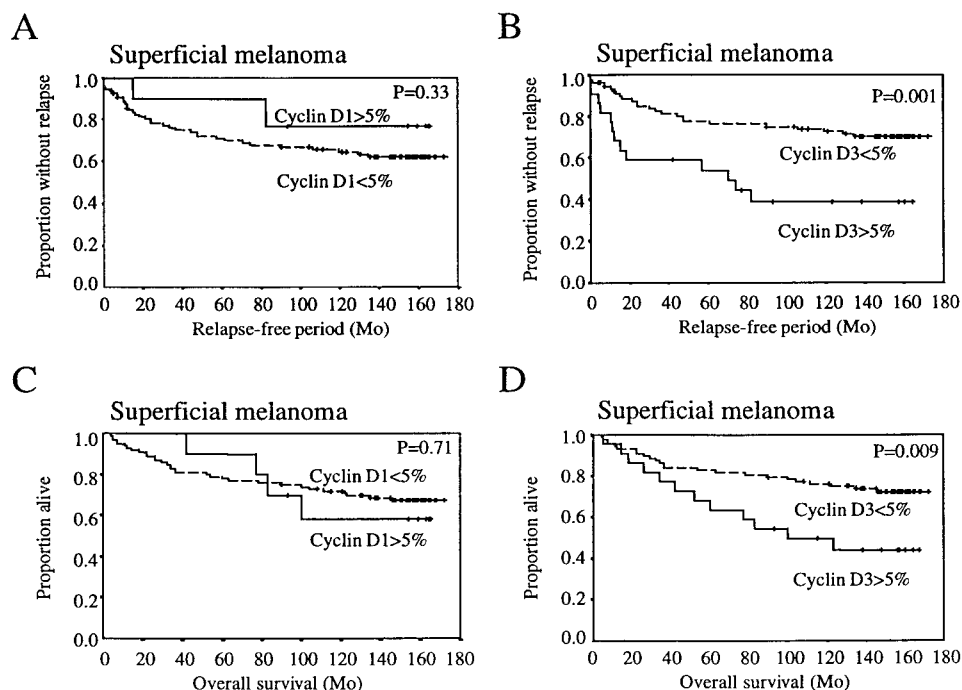
examined, whereas low levels (less than 5% of the cells stained) of cyclin D3 were detected in 100% of the cases.

**Expression of Cyclin D1 and Cyclin D3 in Relation to Clinical Parameters.** Because very few tumors expressed any of the examined proteins in more than 50% of the cells, in the following analysis a 5% cutoff will be used to describe high and low protein levels. When examining the number of superficial

and nodular melanomas expressing high cyclin D1 levels, no difference could be observed; 10 of 110 (9%) of the superficial melanomas expressed high levels of cyclin D1, and the corresponding numbers for nodular melanomas were 6 of 62 (9%). In contrast, high levels of cyclin D3 were detected in 26 of 62 nodular melanomas (42%), whereas only 22 of 110 (20%) superficial tumors expressed cyclin D3 in more than 5% of the cells (Table 2). Furthermore, the cyclin D3 expression varied significantly with the thickness of superficial melanomas (Table 2), with lower expression in thinner lesions ( $P = 0.03$ ). In contrast, in nodular melanomas, high cyclin D1 levels correlated with thinner lesions ( $P = 0.03$ ).

When the total group of patients was analyzed, there was a significant correlation between a high level of cyclin D3 and a decreased relapse-free period ( $P = 0.01$ ). In addition, a trend toward increased overall survival rate was observed for patients with less than 5% of the tumor nuclei positive for cyclin D3 ( $P = 0.11$ ). Interestingly, when superficial and nodular tumors were analyzed separately, a high level of cyclin D3 was a significant predictor of early relapse and decreased overall survival for patients with superficial lesions ( $P = 0.001$  and  $P = 0.009$ , respectively; Fig. 2). In contrast, the level of cyclin D3 did not have any impact on disease-free and overall survival for patients with the nodular subtype ( $P = 0.64$  and  $P = 0.23$ ; Fig. 3). The level of cyclin D1 in tumor cells, on the other hand, did not have any influence on either relapse-free or overall survival for the total group of patients

Fig. 2 Kaplan-Meier curves demonstrating the relationship between the protein expression of cyclin D1 (A), cyclin D3 (B), and relapse-free and overall survival (C and D) for patients with superficial spreading melanoma ( $n = 110$ ).



( $P = 0.34$  and  $P = 0.66$ ), and evaluating the subgroups separately did not change the outcome (Figs. 2 and 3). In multivariate analysis using tumor thickness, type (superficial *versus* nodular), and expression of cyclins D1 and D3 as covariants, neither cyclin D1 nor cyclin D3 reached statistical significance as independent prognostic factors (Table 3).

**Relationship Between the Expression of Cyclin D1 and Cyclin D3 and Other Cell Cycle Markers.** A significant relationship between cyclins D1 and D3 was obtained for the superficial tumors ( $P = 0.0009$ ), but not for the nodular subtype ( $P = 0.19$ ; Table 4) tumors. Because part of our panel of melanoma specimens had been analyzed previously for protein expression of cyclin A and the proliferation marker Ki-67,<sup>4</sup> as well as for the CDK inhibitors p21<sup>WAF1/CIP1</sup> (20) and p27<sup>Kip1</sup> (21), we examined the relationship between cyclins D1 and D3 and these different parameters. For superficial spreading melanomas, a significant covariation was revealed between cyclin D1 and cyclin A ( $P = 0.02$ ) and cyclin D1 and Ki-67 ( $P = 0.0001$ ), as well as between cyclin D3 and cyclin A ( $P = 0.002$ ) and cyclin D3 and Ki-67 ( $P < 0.000001$ ). For the nodular subtype, none of the above-mentioned associations reached statistical significance (Table 4). No relationship between cyclin D1 or D3 and p21<sup>WAF1/CIP1</sup> and p27<sup>Kip1</sup> was detected for any of the subgroups (data not shown).

## DISCUSSION

In the present study, we used immunohistochemistry to examine the level of cyclin D1 and cyclin D3 in a panel of

primary and metastatic human malignant melanomas and evaluate to what extent deregulated protein expression had an impact on clinical outcome. It has been suggested that cyclin D3 is the most widely expressed D-type cyclin, expressed by all proliferating somatic cells with an intact G<sub>1</sub> checkpoint (16). In our panel of melanoma specimens, we found that 96% of the primary tumors and 97% of the metastatic melanomas expressed detectable levels of cyclin D3 in the nuclei. This is in contrast to cyclin D1, which was expressed by 62% of the primary tumors and 29% of the metastatic lesions. Cyclin D1 has been shown to respond to external stimuli, and it may be speculated, therefore, that the higher frequency of cyclin D1 expression in primary as compared with metastatic melanomas may result from interaction with specific growth factors present in the microenvironment, a hypothesis that has also been proposed by Oyama *et al.* (22) for breast cancer.

Coordinated expression of cyclin D1 and D3 was observed in 67% of the primary tumors, a finding in agreement with a study on breast cancer by McIntosh *et al.* (23), who suggested that more than one D-type cyclin may play a role in tumor development and/or progression. Furthermore, it has been suggested that concordant overexpression of both D-type cyclins may reflect a defect in their proteolysis (24). In support of posttranscriptional regulation (25, 26), we did not observe an association between mRNA and protein levels of cyclin D1 in metastatic melanomas (data not shown; Ref. 6), although differences in sensitivity and specificity between the two assays cannot be excluded.

A number of studies have demonstrated the importance of cyclin D1 in regulating progression through the G<sub>1</sub> restriction point by activating CDK4/6 (2). However, an association to classical markers of proliferation such as Ki-67 has not always

<sup>4</sup> V. A. Flørenes, G. M. Mælandsmo, R. Faye, J. M. Nesland, and R. Holm. Cyclin A expression in superficial malignant melanoma correlates with clinical outcome, submitted for publication.



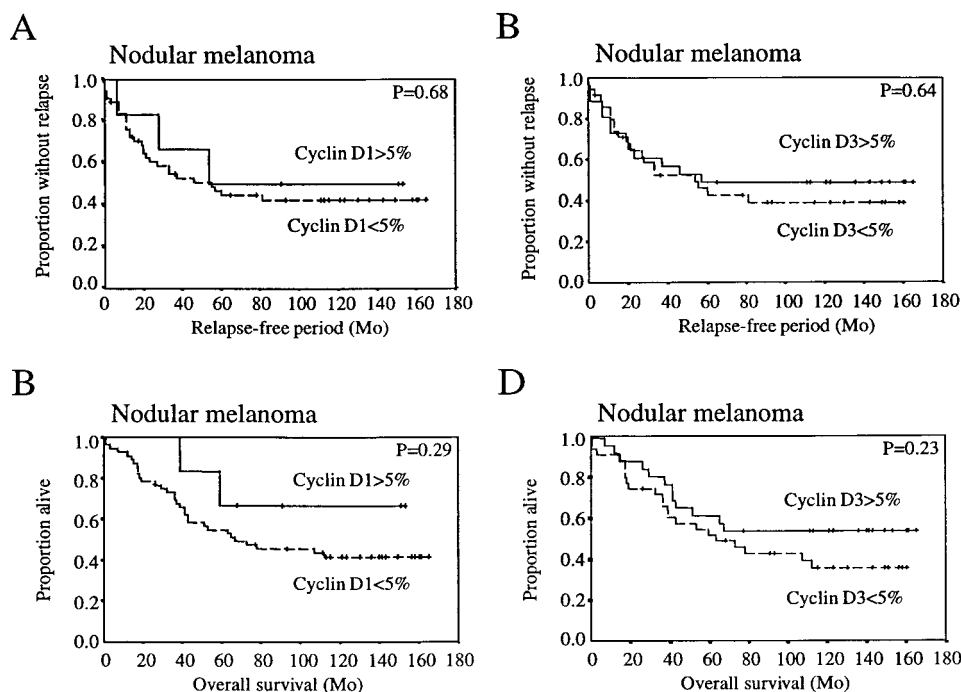


Fig. 3 Kaplan-Meier curves demonstrating the relationship between the protein expression of cyclin D1 (A), cyclin D3 (B), and relapse-free and overall survival (C and D) for patients with nodular spreading melanoma ( $n = 62$ ).

Table 3 Multiple regression of relapse-free and overall survival

Variables	Relapse-free period		Overall survival	
	<i>P</i>	Hazard ratio (95% CI) <sup>a</sup>	<i>P</i>	Hazard ratio (95% CI)
Tumor thickness	<0.0001	1.3 (1.2–1.4)	<0.0001	1.2 (1.1–1.2)
Type	0.43	0.8 (0.4–1.5)	0.31	1.3 (0.8–2.3)
Cyclin D1	0.74	0.9 (0.3–2.2)	0.96	1.0 (0.4–2.3)
Cyclin D3	0.13	1.5 (0.9–2.6)	0.97	1.0 (0.6–1.7)

<sup>a</sup> CI, confidence interval.

been achieved (22, 27). Cyclin D3, on the other hand, has been shown to have a dual function in proliferation as well as differentiation (18). In the present study, we found a strong relationship between cyclin D1, cyclin D3, and the proliferation markers Ki-67 and cyclin A in superficial melanomas, which suggests a coordinated operation of the cell cycle leading to increased proliferation. This is in agreement with Doglioni *et al.* (19), who observed a consistent correlation between cyclin D3 level and proliferation in a very limited number of primary and metastatic melanomas (eight cases each). Interestingly, despite the fact that a higher percentage of nodular as compared with superficial melanomas express cyclin D3 in more than 5% of the cells (42% versus 20%), no correlation between cyclins D1 and D3 and proliferation rate (Ki-67 and cyclin A) was observed for this subtype. Therefore, this finding may suggest that cyclin D1 and cyclin D3 in nodular melanomas have functions other than accelerating cell cycle progression. Accordingly, in lobular breast cancer, cyclin D1 has been shown to activate the estrogen receptor independently of CDK4 activity (28), thereby having the potential to be involved in inducing expression of genes that play a role in restricting cancer cell invasion and motility (29).

Interestingly, and in agreement with the close association

between cyclin D3 and proliferation in superficial melanomas, a significant positive association between cyclin D3 and tumor thickness was observed, whereas no such association was observed with respect to cyclin D1. Surprisingly, in nodular melanomas, a high level of cyclin D1 was significantly associated with thinner lesions. Thus, it may be speculated that in the latter case, cyclin D1 may form an inactive complex with CDK2, thereby leading to cell cycle arrest, as has been demonstrated for senescent fibroblasts (30). Furthermore, in neuronal cells, moderate expression of cyclin D1 stimulates cell growth, whereas overexpression results in apoptosis (31).

Most studies have focused on the role of cyclin D1 in tumor progression, and little is known about the contribution of cyclin D3. In the present study we show for the first time, to the best of our knowledge, an association between cyclin D3 and clinical outcome for melanoma patients. Interestingly, Kaplan-Meier analysis revealed a statistically significant association between cyclin D3 and relapse-free and overall survival for patients with superficial spreading melanoma, which suggests that cyclin D3 deregulation provides a selective growth advantage that is related to progression of this type of cancer. These findings are in accordance with our recent observations of a

Table 4 Relationship between expression of cyclin D1, cyclin D3, cyclin A, and Ki-67

Type of lesion	No. of tumors analyzed	Immunohistochemistry		Expression of cyclin D1 <sup>a</sup>			Expression of cyclin D3 <sup>a</sup>		
		Marker	Expression level	Low	High	P	Low	High	P
Superficial	110	Cyclin D1	Low				84	16	0.0009
			High				4	6	
	Cyclin A	Low	67	33	0.02	62	8	0.002	
		High	3	7		26	14		
110	Ki-67	Low	83	17	0.0001	77	11	<0.000001	
		High	3	7		9	13		
Nodular	62	Cyclin D1	Low				31	25	0.19
			High				5	1	
	Cyclin A	Low	14	42	0.65	11	5	0.31	
		High	2	4		25	21		
	62	Ki-67	Low	25	31	0.80	18	18	0.37
			High	3	3		10	16	

<sup>a</sup> Expression of cyclins D1 and D3 was measured as described in "Materials and Methods:" -/+, low expression; +/+ + +, high expression.

significant association between tumor expression of cyclin A and Ki-67 and clinical outcome for patients with superficial melanoma.<sup>4</sup> In contrast, overexpression of cyclin D3 was not a predictor of early relapse for patients with nodular melanomas, a finding in agreement with the observed lack of association between cyclin D3, mitotic activity, and tumor thickness. Notably, no correlation between cyclin D1 protein levels and clinical outcome was observed for either of the two subtypes of melanoma, a finding in agreement with studies on superficial urinary bladder (12), gastric (10), and breast cancers (11). On the other hand, overexpression of cyclin D1 protein has been associated with poorer clinical outcome for patients with carcinomas of the anterior tongue (32), the esophagus (9), the ovary (8), and non-small cell lung cancer (7), among others.

In conclusion, our results indicate that cyclin D3 is the D-type cyclin most frequently expressed by human melanomas, and that the expression level of cyclin D3 may be an important factor in predicting the clinical outcome for patients with superficial spreading melanoma, whereas the level of cyclin D1 expression has no impact on tumor progression. Furthermore, neither cyclin D1 nor cyclin D3 had an impact on clinical outcome for patients with nodular melanoma, thus underscoring the hypothesis that other factors not related to cell cycle progression may play an important role in the aggressiveness of this subtype of melanoma.

## ACKNOWLEDGMENTS

We are grateful to Ellen Hellesylt, Mette Ingrid, and Liv Inger Håseth for excellent technical assistance and to Dr. Eva Skovlund for valuable help with the statistics.

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*Clin Cancer Res* 2000;6:3614-3620.

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