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Short Communication

Cyclin Kinase Inhibitor *p21*$^{WAF1/CIP1}$ in Malignant Melanoma

Reduced Expression in Metastatic Lesions

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Immunohistochemical analysis of the expression of the cyclin kinase inhibitor* p21$^{WAF1/CIP1}$ *in a panel of primary and metastatic human melanocytic tumors was performed. It was found that, independent of the* p53 *status, approximately 30% of the primary melanomas and 40% of the metastases completely lacked expression of this cell cycle inhibitor. Some tumors were also analyzed by Northern blotting, and in most of the cases a consistant correlation between mRNA and protein expression was observed. In four benign nevi studied,* WAF1/CIP1 *mRNA was expressed whereas the protein was not detected, suggesting a post-transcriptional regulation of the inhibitor in these cases. In superficial spreading melanomas, a significant correlation between protein expression and tumor thickness was found, with thin lesions showing low protein levels. Interestingly, by comparing primary and metastatic specimens obtained from the same patient, a reduction in* p21$^{WAF1/CIP1}$ *antibody staining was observed in the latter, probably reflecting a more aggressive phenotype of the metastases. In conclusion, our results demonstrate the complexity in the relationship between* p21$^{WAF1/CIP1}$ *expression and tumor phenotype and furthermore suggest that aberrant expression of the cyclin-dependent kinase inhibitor may be of importance in the development and progression of sporadic malignant melanoma. (Am J Pathol 1996, 149:1813–1822)

The orderly progression of cells through the cell cycle depends on a finely tuned balance between the levels of activated cyclins and cyclin-dependent kinases (CDKs) that provide positive growth signals and kinase inhibitors that suppress these effects. In recent years several CDK inhibitor proteins have been discovered.[1,2] The first to be described was the *p21*$^{WAF1/CIP1}$ protein, originally identified as a component of a quarternary complex composed of cyclin D, *cdk4*, and proliferating cell nuclear antigen (PCNA).[3,4] Later, however, it was shown that *p21*$^{WAF1/CIP1}$ inhibits the catalytic activity of all CDKs, although with variable efficiencies.[5,6] Moreover, *p21*$^{WAF1/CIP1}$ can inhibit DNA replication by blocking the ability of PCNA to activate DNA polymerase-δ.[7,8] Recently, it was revealed that *p21*$^{WAF1/CIP1}$ exists in both active and inactive CDK complexes depending on the stoichiometry; whereas multiple molecules are needed for kinase inactivation, one single *p21*$^{WAF1/CIP1}$ molecule may function as an assembling factor for the active CDK complex.[9,10]

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Thus, in human cancer cells, overexpression of full-length *WAF1/CIP1* cDNA has resulted in growth suppression in transfected cells.[11–13]

p21$^{WAF1/CIP1}$ was found to be transcriptionally regulated by wild-type *p53* and can be induced by DNA-damaging agents, suggesting a role in *p53*-mediated growth suppression, DNA repair, and apoptosis.[11,14] However, *p21*$^{WAF1/CIP1}$ has also been suggested to be involved in senescence,[15] terminal differentiation,[16,17] and apoptosis[18] through *p53*-independent mechanisms.

The *WAF1/CIP1* gene is located on chromosome fragment 6p21.2.[11] This region is frequently shown to harbor genetic alterations in human malignant melanomas,[19] and the presence of a melanoma suppressor gene mapped to chromosome 6 has been implicated.[20] Notably, mutations in *WAF1/CIP1* do not seem to be of importance in any cancer type examined so far, including melanomas.[21] Interestingly, the *WAF1/CIP1* gene was recently cloned as a melanoma differentiation-associated gene, *mda6*, the expression of which was up-regulated during melanoma differentiation.[22] A decreased *mda6* mRNA expression in cell lines derived from advanced-stage primary melanomas and metastases as compared with cell lines derived from melanocytes and early-stage primary tumors was revealed, suggesting that aberrant expression of *p21*$^{WAF1/CIP1}$ may play a role in the tumorigenicity of this type of cancer.[22]

On the basis of these observations, we wanted to study whether altered expression of *p21*$^{WAF1/CIP1}$ could also be detected in tumor material obtained from patients and, furthermore, examine whether the protein expression could be used as a prognostic indicator for melanoma development and progression. Moreover, we also wanted to examine the relationship between *p21*$^{WAF1/CIP1}$ and *p53* expression as we recently reported accumulation of a presumably wild-type *p53* protein in advanced-stage primary melanomas and metastases.[23,24] By immunohistochemistry, we screened a panel of primary melanomas from different stages of the disease, as well as melanoma metastases and a number of benign nevi, for expression of *p21*$^{WAF1/CIP1}$. Interestingly, and in contrast to what was expected, we found a significantly higher *p21*$^{WAF1/CIP1}$ expression in advanced primaries as compared with benign nevi and thin malignant lesions. In some of the cases in which samples both of the primary tumor and of a metastasis obtained from the same patient were examined, a substantial fraction of the metastases demonstrated loss of, or reduced, *p21*$^{WAF1/CIP1}$ protein expression as compared with the corresponding primary lesion.

Materials and Methods

Specimens

Formalin-fixed, paraffin-embedded tissue sections were obtained from 126 primary melanomas, 46 melanoma metastases, and 4 benign nevi. From 34 patients, both primary and metastatic material was collected. Of the primary tumors, 85 were classified as superficial and 36 as nodular, 3 belonged to other histological subgroups (2 lentigo and 1 desmoplastic), and in 2 cases the morphological subtype could not be determined. For Northern blot analysis, fresh tumor tissue was obtained from distant metastases of 24 patients, for which the corresponding paraffin-embedded tissue sections were immunoanalyzed, and also from 26 other patients with metastatic malignant melanoma. In the Northern analysis 4 benign nevi were included as controls. Immediately upon surgery, the tumor tissue was frozen in liquid nitrogen and subsequently stored at −135°C. In addition, the analysis included 17 human melanoma cell lines. A number of these were established by Herlyn et al,[25,26] of which 6 originate from primary lesions. Three of these (WM35, WM1341B, and WM902B) were from radial growth phase or early-stage vertical growth phase melanomas, and 3 (WM115, WM122, and WM983A) were from advanced-stage vertical growth phase tumors, whereas 5 cell lines were from metastatic lesions (WM239, WM9, WM45.1, SK-mel 28, and MeWo). Of the remaining 6 cell lines, 5 (FEMX, HHMSX, LOX, SESX, and THX) were established from tumor tissue obtained from patients treated for metastatic malignant melanoma at the Norwegian Radium Hospital and one (M14) was kindly provided from Dr. R. A. Reisfeld. The Scripps Research Institute (La Jolla, CA).

Immunohistochemical Analysis

Sections of formalin-fixed, paraffin-embedded tissue were immunostained using the avidin-peroxidase complex method described by Hsu et al.[27] The paraffin-embedded sections were deparaffinized, treated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase, and microwaved in 10 mmol/L citrate buffer (pH 6.0) to unmask epitopes.[28] Subsequently, the sections were incubated with normal horse serum to eliminate nonspecific staining. The sections were then incubated 18 to 22 hours at 4°C with a monoclonal *p21*$^{WAF1/CIP1}$ an-

tibody (Ab-1, Oncogene Science, Uniondale, NY) diluted 1:30 (3.3 μg IgG_1/ml), followed by sequential incubations with biotin-labeled secondary antibody and avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). The reaction was finally developed using diaminobenzidine as chromogen. All series included positive and negative controls, the latter obtained by replacement of the monoclonal antibody with mouse myeloma protein of the same subclass and concentration. Four semiquantitative categories were used to describe the number of positively stained cells: −, none; +, <5%; ++, 5 to 50%; +++, >50%.

Western Blot Analysis

Cells were lysed in ice-cold lysis buffer (20 mmol/L Tris/HCl, pH 7.5, 137 mmol/L NaCl, 100 mmol/L NaF, 10% glycerol, 1% Nonidet P-40, 1 mmol/L Na_2VO_3, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.02 mg/ml each of aprotinin, leupeptin, and pepstatin). Subsequently, the lysates were sonicated and clarified by centrifugation and protein quantitated by Bradford analysis. From each sample, 30 μg of total protein was separated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis[29] and transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA). As a loading and transfer control, the membranes were stained with 0.1% naphthol blue-black (Sigma Chemical Co., St. Louis, MO). The membranes were blocked by TBST (20 mmol/L Tris/HCl, pH 7.5, 0.5 mol/L NaCl, 0.25% Tween) containing 10% dry milk and incubated for 1 hour at room temperature with a rabbit polyclonal $p21^{WAF1/CIP1}$ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 in TBST containing 5% dry milk. After washing in TBST, the immunoreactive proteins were visualized using horseradish-peroxidase-conjugated anti-rabbit IgG (Promega Corp., Madison, WI) diluted 1:5000 and the ECL Western blotting detection system (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Northern Blot Analysis

Total cellular RNA was prepared and Northern blot analysis performed as previously described.[30] The filters were hybridized with a *WAF1/CIP1* cDNA probe kindly provided by Dr. Bert Vogelstein, Molecular Genetics Laboratory, The Johns Hopkins Oncology Center, Baltimore, MD.[11] The hybridizations were carried out in 0.5 mol/L sodium phosphate (pH 7.2), 7% SDS, and 1 mmol/L sodium EDTA at 65°C overnight as described by Church and Gilbert.[31] For multiple hybridizations, the bound probe was removed by incubating the filters twice for 5 minutes in 0.1× standard saline citrate (SSC), 0.1% SDS at 95 to 100°C. To correct for uneven amount of RNA loaded in each lane, the filters were rehybridized with a kinase-labeled oligonucleotide probe complementary to human 18S rRNA.[30] The mRNA expression levels were classified as follows: −/+, undetectable/low expression; ++ and +++, moderate or high expression.

Statistical Analysis

Comparison between $p21^{WAF1/CIP1}$ and *p53* expression levels were performed using the Fisher's exact test. The relationship between the expression of $p21^{WAF1/CIP1}$ and the median tumor thickness or relapse-free period were evaluated nonparametrically using the Mann-Whitney two-sample test. Kaplan-Meier survival analysis was used to statistically evaluate the survival data. A value of $P < 0.05$ was considered significant.

Results

Expression of $p21^{WAF1/CIP1}$ *in Primary and Metastatic Melanoma Cell Lines*

A good correlation between $p21^{WAF1/CIP1}$ mRNA and protein expression was observed in 17 human melanoma cell lines (Figure 1A), 6 of which were derived from primary lesions at different stages of disease progression, whereas 11 of the *in vitro* cultures were established from metastatic material. Interestingly, and in agreement with previous studies,[22] a remarkably high expression of $p21^{WAF1/CIP1}$ mRNA and protein were detected in the cell lines derived from primary lesions as compared with the cultures established from metastases. Thus, 5 of the 6 (83%) primary *in vitro* cultures expressed high levels (++ or +++) of mRNA and protein, whereas only 5 of 11 (45%) metastatic cell lines showed high expression of the gene.

Expression of $p21^{WAF1/CIP1}$ *in Primary and Metastatic Melanoma Lesions*

Formalin-fixed, paraffin-embedded tissue sections from 126 primary and 46 metastatic lesions were immunohistochemically analyzed for $p21^{WAF1/CIP1}$ expression. Different levels of nuclear staining were found in 69% (87 of 126) of the primaries and 57% (26 of 46) of the metastatic lesions (Table 1). A higher fraction of the primary (35%) compared with

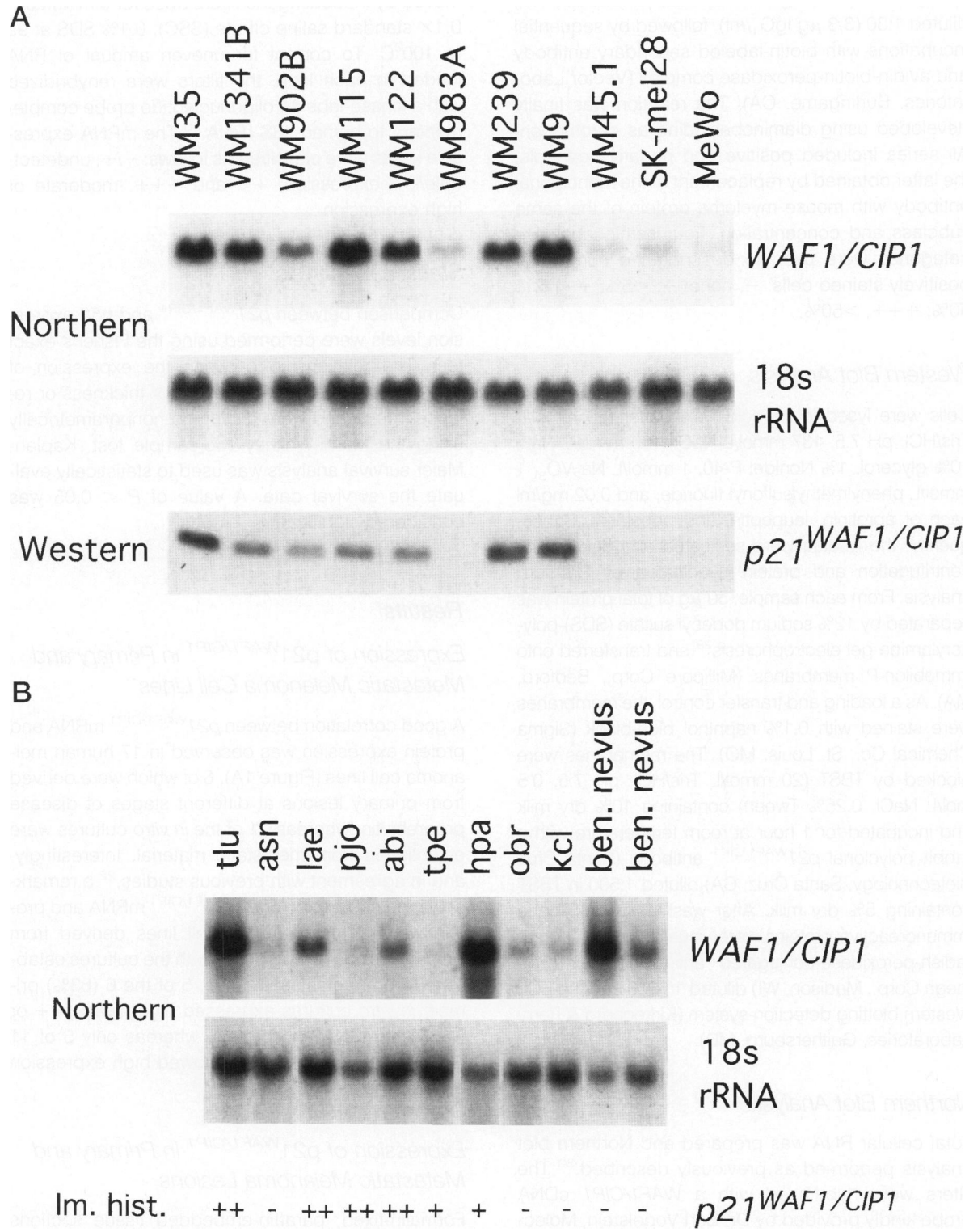

Figure 1. A: *Representative Northern and Western blots showing the expression of* p21$^{WAF1/CIP1}$ *in melanoma cell lines derived from early primary (WM35, WM1341B, and WM902B), advanced primary (WM115, WM122, and WM983A), and metastatic (WM239, WM9, WM45.1, SK-me128, and MeWo) lesions. For the Northern blots, 5 μg of total RNA was loaded in each lane and subsequently hybridized with probes encoding* p21$^{WAF1/CIP1}$ *and 18S rRNA as a control. For the Western blot, optical densitometric readings of the protein concentrations was performed and 30 μg was loaded in each lane. The membrane was subsequently hybridized with a polyclonal* p21$^{WAF1/CIP1}$ *antibody.* B: *Representative Northern blot showing the expression of* p21$^{WAF1/CIP1}$ *in malignant melanoma metastases (tlu, asn, lae, pjo, abi, tpe, hpa, obr, and kcl) and benign nevi. The corresponding* p21$^{WAF1/CIP1}$ *protein expression levels, quantified by immunohistochemistry (Im. hist.), are indicated below the panel.*

the metastatic (22%) lesions showed $p21^{WAF1/CIP1}$ staining in more than 5% of the cells (++/+++). $p21^{WAF1/CIP1}$ protein expression was not detected in normal skin adjacent to the tumors (Figure 2B) with the exception of sebaceous glands, as also reported by others.[32] Fresh tumor material was available from 24 of the metastatic biopsies, and by comparing $p21^{WAF1/CIP1}$ mRNA and immunohistochemically analyzed protein expression, a reasonably good correlation, with some exceptions, was observed (Figure 1B). Notably, among the 4 benign nevi examined, all expressed *WAF1/CIP1* mRNA (Figure 1B) whereas none of them showed any protein staining (Figure 2A).

In 34 of the cases, both the primary and metastatic tumor samples from the same patients were analyzed for $p21^{WAF1/CIP1}$ protein expression, and in 53% of the cases (18 of 34), a decrease in the number of positively stained cells was observed in the metastases as compared with the respective primary tumor (Figure 2, B and C). Furthermore, 8 patients were found to have the same number of positively stained cells in the primary and the metastatic lesion, whereas 4 of these metastases clearly demonstrated lower staining intensity. When these cases were combined, 22 (65%) of the metastases expressed less $p21^{WAF1/CIP1}$ than the corresponding primary tumor.

Expression of $p21^{WAF1/CIP1}$ *in Relation to Clinical Parameters of Malignancy*

The expression of $p21^{WAF1/CIP1}$ was examined in relation to clinical parameters of melanoma progression. When we examined the number of superficial and nodular primary melanomas expressing different protein levels, no difference was observed (Table 2). Interestingly, the $p21^{WAF1/CIP1}$ expression varied significantly with the thickness of the superficial melanomas (Table 2), with lower expression in thinner lesions (Mann-Whitney $P = 0.005$). No significant correlation was, however, found between $p21^{WAF1/CIP1}$ expression and survival. This may be explained by the fact that the majority (60%) of the lesions were thinner than 1.5 mm and thus supposed to have a good prognosis. The follow-up period may therefore be too short (mean, 8.5 years) to detect any significant differences. The correlation between tumor thickness and $p21^{WAF1/CIP1}$ expression was not found in the nodular subtype of tumors, probably due to a greater mean thickness in this group. Furthermore, in the cases for which metastatic tumors had developed, no significant relationship between relapse-free period and expression level in the primary tumor was revealed (Mann-Whitney).

Comparison between $p21^{WAF1/CIP1}$ *and* p53 *Protein Expression*

Previously, our panel of primary and metastatic malignant melanomas were immunohistochemically analyzed for accumulation of the *p53* protein,[23,24] and by utilizing these results, it was possible to make a comparison between the *p53* and $p21^{WAF1/CIP1}$ expression levels in the tumors (Table 3). The results strongly suggest that there exists a *p53*-independent regulation of the inhibitor expression in malignant melanomas. This is consistent with other reports of p53-independent $p21^{WAF1/CIP1}$ regulation.[21,33] Altogether, 22 tumors demonstrated positive *p53* protein staining, and 16 of these (73%) simultaneously expressed $p21^{WAF1/CIP1}$. However, a total of 87 tumors showed positive staining for the latter protein, and only 16 of these (18%) expressed detectable levels of *p53*.

Recently, fresh tumor material was collected from 50 patients with sporadic metastatic melanoma, and by Northern blotting the possible involvement of the *pRb/p16*^{INK4}*/cdk4*/cyclin D1 pathway in the tumorigenicity was studied.[30] To examine whether any correlations might exist between this pathway and the expression of $p21^{WAF1/CIP1}$, we compared the previ-

Table 1. *Number (Percentage) of Melanocytic Tumors Expressing* $p21^{WAF1/CIP1}$

Tumor type	Number examined	Expression level*			
		−	+	++	+++
Primary melanomas	126	39 (31)	43 (34)	36 (29)	8 (6)
Superficial	85	26 (31)	29 (34)	25 (29)	5 (6)
Nodular	36	11 (30)	12 (33)	10 (28)	3 (8)
Other†	5	2 (40)	2 (40)	1 (20)	
Metastatic melanoma	46	20 (43)	16 (35)	9 (20)	1 (2)
Benign nevi	4	4 (100)			

*Measured as described in Materials and Methods.
†Two cases of lentigo and one desmoplastic malignant melanoma. In the last two cases, the morphological type of the primary tumor was not determined.

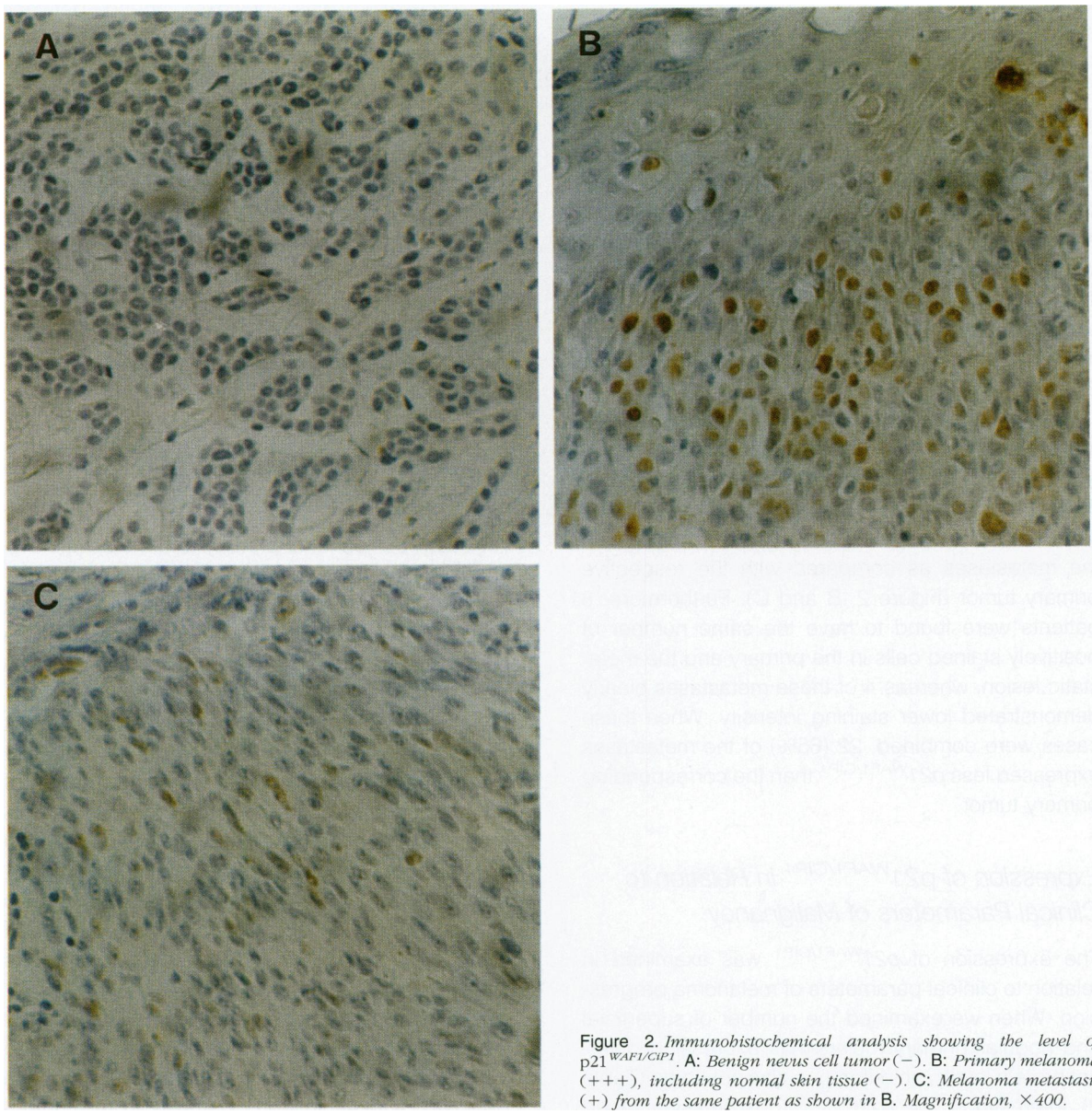

Figure 2. *Immunohistochemical analysis showing the level of* $p21^{WAF1/CIP1}$. A: *Benign nevus cell tumor* (−). B: *Primary melanoma* (+++), *including normal skin tissue* (−). C: *Melanoma metastasis* (+) *from the same patient as shown in* B. *Magnification,* ×400.

ous results with the observed *WAF1/CIP1* mRNA levels. Altogether, 31 of the examined metastases showed expression of the $p21^{WAF1/CIP1}$ transcript, and 27 (87%) of these expressed low or undetectable amounts of *INK4* mRNA, indicating that tumors that have lost or down-regulated *INK4* expression frequently express *WAF1/CIP1*. Furthermore, a substantial number of these metastases demonstrated high levels both of the *cdk4* (63%) and cyclin D1 (54%) transcripts,[30] suggesting that a high level of the *cdk4*/cyclin D1 complex combined with loss, down-regulation, or inactivation of both $p16^{INK4}$ and $p21^{WAF1/CIP1}$ may be an important mechanism to allow an aggressive metastatic phenotype to evolve.

Discussion

In agreement with Jiang et al,[22] we observed a significant inverse correlation between $p21^{WAF1/CIP1}$ expression and the tumorigenicity of human melanoma cell lines in nude mice, suggesting that aberrant regulation of the CDK inhibitor $p21^{WAF1/CIP1}$ may play a role in the development and/or progression of this type of skin cancer.[21,22] The fact that *WAF1/CIP1* (*mda6*) also has been identified as a gene encoding a melanoma differentiation-associated protein[22] prompted us therefore to examine to what extent the phenomenon observed in the cell lines was reflected in melanoma specimens.

Table 2. *Relationship between* $p21^{WAF1/CIP1}$ *Expression in Primary Melanomas and Clinical Parameters of Malignancy*

Tumor type	Immunohistochemistry	Number of patients†	Depth of growth (mm)‡		Relapse-free period (months)§			Survival (months)‖	
Superficial	High	30	1.60	$P = 0.005$	21.5	(10)	NS	100	NS
	Low	55	0.87		21.5	(12)		108	
Nodular	High	13	4.80	NS	43.5	(4)	NS	79	NS
	Low	23	3.00		13.0	(11)		66	
Other¶	High	1	ND	ND	30.0	(1)	NS	37	NS
	Low	4	1.95		11.5	(4)		45	
Total	High	44	1.78	NS	29.0	(15)	NS	96	NS
	Low	82	1.66		13.0	(27)		98	

NS, not significant; ND, not determined.
*Scored as described in Materials and Methods. −/+, low expression; ++/+++, high expression.
†A total of 126 patients were analyzed.
‡Measured as the median thickness in each group.
§Measured as the median relapse-free period in each group. The number of patients who had developed metastases is shown in parentheses.
‖Measured as the median survival in each group.
¶Two cases of lentigo and one desmoplastic malignant melanoma. In two of the cases, the primary tumor was not determined.

Similar to the results obtained for normal and malignant brain tissues,[34] $p21^{WAF1/CIP1}$ expression was not detectable by immunohistochemistry in normal skin, including melanocytes, or benign nevi but was heterogeneously expressed in malignant melanoma tumors. Approximately 30% of the primary tumors completely lacked $p21^{WAF1/CIP1}$ expression, and the protein could not be detected in 40% of the metastases, a finding similar to what was observed for metastatic melanoma cell lines.[21] Moreover, in the majority (65%) of cases in which $p21^{WAF1/CIP1}$ expression in primary and metastatic tumors from the same patient could be compared, both the proportion of stained cells and the intensity of staining were higher in the primary lesions, suggesting that down-regulation of this cell cycle inhibitor may be associated with acquisition of a metastatic phenotype.

In most cases, a good correlation between $p21^{WAF1/CIP1}$ mRNA and protein expression was observed, which might suggest, as also reported by others,[21] that the protein expression is largely transcriptionally regulated. Interestingly, however, all four benign nevi examined expressed *WAF1/CIP1* mRNA whereas the protein was not detectable by immunohistochemistry. Similar observations have been reported for melanoma[21] as well as for ovarian carcinoma[35] cell lines, raising the possibility that post-transcriptional regulation of $p21^{WAF1/CIP1}$ may also occur.

The finding that $p21^{WAF1/CIP1}$ was not expressed in normal skin and benign nevi is in agreement with previous reports[32,36] but stand in apparent contrast to the hypothesis that $p21^{WAF1/CIP1}$ might function as a melanoma differentiation-associated protein[22] and with the observation that the inhibitor is highly expressed by melanocytes grown *in vitro*.[21] The discrepancy may in part be explained by the fact that the latter studies have been performed on actively growing cultures of melanocytes, a situation that does not necessarily reflect their proliferative state *in vivo*.[21] In this regard, it has been reported that in human fibroblasts, which normally express low levels of $p21^{WAF1/CIP1}$, the inhibitor can be transiently induced in response to serum and growth factors,[33] suggesting that under such circumstances $p21^{WAF1/CIP1}$ may function as a CDK/cyclin assembling factor, thereby promoting cell cycle progression. In contrast, overexpression of $p21^{WAF1/CIP1}$ in response to cytokines[37–39] and differentiation agents[16,40] may lead to growth

Table 3. *Relationship between Expression of* p53 *and* $p21^{WAF1/CIP1}$

Type of lesion	Expression of *p53**	Number analyzed	Expression of $p21^{WAF1/CIP1}$*		
			Positive	Negative	
Primary (n = 126)	Positive	22	16	6	NS
	Negative	102	70	32	
	ND	2	1	1	
Metastatic (n = 46)	Positive	14	8	6	NS
	Negative	30	17	13	
	ND	2	1	1	

NS, not significant; ND, not determined.
*Measured as described in Materials and Methods. −, negative expression; +/++/+++, positive expression.

arrest. The increased level of $p21^{WAF1/CIP1}$ seen in melanoma cell lines after treatment with differentiation agents is probably responsible for loss of proliferative capacity rather than differentiation. Thus, expression of other yet unknown genes may be of greater importance for induction and maintenance of a differentiated phenotype by the melanocytes.

Interestingly, and in line with the undetectable level of $p21^{WAF1/CIP1}$ in benign nevi, we found a significant correlation between the protein expression and the thickness of superficial spreading melanomas, with the thinner lesions demonstrating lower $p21^{WAF1/CIP1}$ levels. It is conceivable that, in early melanocytic lesions, $p21^{WAF1/CIP1}$ is not essential for halting cell proliferation because of a redundancy of other functioning CDK inhibitors. In this regard, we have observed that melanoma cell lines arrested in G0 express high levels of the closely related cyclin kinase inhibitor $p27^{KIP1}$.[39] In addition, our analysis revealed an inverse correlation between $p16^{INK4}$ and $p21^{WAF1/CIP1}$ mRNA expression in early primary melanoma cell lines (results not shown). Furthermore, Reed et al[41] reported homogeneous $p16^{INK4}$ protein staining of all cells in atypical nevi and melanoma *in situ*, whereas in biopsied material nearly 50% of the cells in more advanced primary lesions showed reduced $p16^{INK4}$ protein staining. Moreover, the $p16^{INK4}$ gene has been found homozygously deleted or mutated in 70% of the melanoma cell lines examined,[42] and due to the close chromosomal localization, loss of $p16^{INK4}$ might often also involve inactivation of the related inhibitor $p15^{INK4B}$.[43] Thus, it might be that the higher level of $p21^{WAF1/CIP1}$ observed in the more advanced primary melanomas is induced by the loss of other cyclin kinase inhibitors and reflects a feedback mechanism used by the tumor cells in an attempt to halt increased cell proliferation.[34] The elevated level of $p21^{WAF1/CIP1}$ might, however, not be enough to restrict tumor progression, and in line with the elevated level of $p16^{INK4}$ observed in cells lacking functional *pRb*, it might be speculated whether inactivation of other essential components of the cell cycle machinery may trigger the high level of $p21^{WAF1/CIP1}$ observed in the advanced cases.

In agreement with results obtained in cell lines,[21] we found that in a substantial number of cases $p21^{WAF1/CIP1}$ expression was lower in the metastases as compared with the corresponding primary lesion. Recently, we also showed that a majority (59%) of the melanoma metastases lacked detectable $p16^{INK4}$ mRNA expression, whereas both cyclin D1 and *cdk4* messages were up-regulated.[30] According to the stoichiometric regulation of $p21^{WAF1/CIP1}$ activity, it might be speculated whether the cyclin/CDK complexes have reached the threshold level in the metastases, allowing tumor progression, concomitant with a decrease in the $p21^{WAF1/CIP1}$ level. A similar mechanism to escape cell cycle inhibition has been postulated by Nasmyth and Hunt,[44,45] who suggested down-regulation of the inhibitor levels as a result of proteolytic degradation or inactivation triggered by the surplus cyclins. Moreover, inactivation by phosphorylation has been suggested as a likely mechanism to regulate the activity of the cell cycle inhibitors.[45] It is conceivable that such post-transcriptional inactivation may account for the few metastases (22%) demonstrating high $p21^{WAF1/CIP1}$ protein staining. Alternatively, inactivation of other essential cell cycle components, as also suggested for the advanced primary lesions, may result in the high expression of $p21^{WAF1/CIP1}$.

The G1 checkpoint has been regarded to consist mainly of two regulating pathways, each organized by the tumor suppressors *p53* and *pRb*. A complex relationship exists between these pathways, which communicate through several points of intersection. One way to communicate is through the cell cycle inhibitor $p21^{WAF1/CIP1}$, transcriptionally activated by *p53* and affecting *pRb*-mediated cell cycle arrest. In this paper, we suggest that the expression level of $p21^{WAF1/CIP1}$ is regulated mainly by *p53*-independent mechanisms in sporadic melanomas. In addition, our results lend weight to the hypothesis that $p21^{WAF1/CIP1}$ may function to titrate the CDK/cyclin complexes and help in determining the threshold kinase activity required for cell cycle progression. Furthermore, the results suggest that down-regulation of $p21^{WAF1/CIP1}$ expression is associated with development of a metastatic phenotype. Thus, although gene aberrations involving *TP53* and *RB1* have not been found to play a dominant role in the etiology of sporadic melanomas, the present results, in addition to the previously observed up-regulation of *cdk4* and cyclin D1 concomitant with low levels of $p16^{INK4}$,[30] provide support for the involvement of these growth-regulating pathways in the tumorigenicity of sporadic malignant melanoma.

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