

Reduced β -Catenin Expression in the Cytoplasm of Advanced-Stage Superficial Spreading Malignant Melanoma¹

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ABSTRACT

Purpose: The purpose of the present work was to analyze the expression of β -catenin in a panel of superficial and nodular spreading primary and metastatic melanomas, and to correlate the level of immunohistochemical staining to clinicopathological parameters.

Experimental Design: Expression of β -catenin was examined by immunohistochemistry in 106 superficial and 58 nodular spreading primary melanomas, as well as in 66 metastatic lesions.

Results: Membrane-associated staining was detected in nearly all of the cases, and no association to clinical parameters could be revealed. When cytoplasmic localization of the protein was recorded, a significant higher fraction of the superficial than the nodular spreading primary lesions expressed the protein in the majority of the cells ($P < 0.0001$). Interestingly, metastatic lesions from superficial melanomas demonstrated down-regulated expression of the protein, and in agreement with this a significant inverse correlation between protein expression and the vertical thickness of the primary lesion was detected ($P = 0.012$). Furthermore, a significant correlation between cytoplasmic localization and disease-free survival ($P = 0.0006$) was revealed, but β -catenin did not have any significant impact on overall survival for this group of patients ($P = 0.0824$). No association was detected between β -catenin expression and clinicopathological parameters in the nodular subgroup of melanomas, indicating that the protein may play different roles in the malignant progression of the two main types of melanomas.

Conclusion: In summary, we hypothesize that cytoplasmic β -catenin has a protective role in early melanoma development.

INTRODUCTION

β -Catenin was isolated originally as a protein associated with the cytoplasmic tail of E-cadherin, a transmembrane glycoprotein involved in homotypic cell-cell interactions (1), and plays an important role linking E-cadherin to the cytoskeleton. In addition to the well-defined role in cellular adhesion, β -catenin also function in the Wnt signaling pathway (2, 3). Consistent with this, two different pools of β -catenin exist in the cells: a cadherin and cell membrane-associated pool, and a cytoplasmic/nuclear pool involved in Wnt signaling and subsequent gene transcription. In the cytoplasm, β -catenin is bound in a complex together with AXIN, APC,³ and GSK3 β . In the absence of a Wnt signal, the GSK3 β protein phosphorylates β -catenin and labels it for degradation by the ubiquitin/proteasome pathway. On the other hand, when a Wnt signal is present, the GSK3 β activity is inhibited, thereby resulting in stabilization and accumulation of β -catenin in the cytoplasm (2–4). Subsequently, β -catenin forms complexes with members of the Tcf/Lef family of transcription factors (5), and translocates to the nucleus where it activates transcription of target genes, several of which are known to be of importance for human tumorigenesis such as c-MYC (6), cyclin D1 (7), and MMP7 (8).

Molecular changes increasing the stability of β -catenin in the cytoplasm can presumably enhance the Tcf/Lef transcriptional activity. Thus, deregulation of the Wnt pathway has been shown to occur by several different mutational mechanisms in human cancer (2, 9). Inactivating mutations in the APC tumor suppressor protein, and also mutations in AXIN 1 and 2 (10), appear to inhibit formation of a functional GSK3 β /APC/AXIN/ β -catenin complex (9). Hence, APC mutations have been found in 70–80% of human colorectal carcinomas and also in a subset of other cancers (11, 12), including melanoma cell lines (13). APC mutations have been observed in melanocytic lesions *in vivo*, but the frequency seems to be very low (14). In addition, mutations in the NH₂-terminal part of the β -catenin gene, encoding the phosphorylation sites for GSK3 β , making the protein resistant for phosphorylation-induced proteasomal degradation, have been reported in several types of cancer (2–4). Such mutations have also been found in melanoma cell lines but are rarely observed in melanocytic lesions (15, 16).

Expression of N-, P-, and E-cadherin, as well as β - and γ -catenin has been examined previously by immunohistochemistry in a small cohort of melanocytic nevi, and primary and metastatic malignant melanomas (17). The most striking finding was loss of membranous P-cadherin and β -catenin in the metastatic compared with the primary lesions. In agreement with

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³ The abbreviations used are: APC, adenomatous polyposis coli; GSK3 β , glycogen synthase kinase 3 β ; CBP, CREB-binding protein; CREB, cAMP-response element binding protein; Lef, lymphoid enhancer factor; Tcf, T-cell factor.

this, Kageshita *et al.* (18) compared the protein expression of β -catenin in melanocytic lesions and reported loss or down-regulation of the protein in the more advanced tumors. In both of these studies a change in the intracellular localization of β -catenin was observed. In the former study, the subgroup of the included primary melanomas ($n = 40$) was not indicated, whereas the latter cohort mostly consisted of acral lentiginous melanomas (58%), a quite common skin disease in the Japanese population but rare in the white-skinned Caucasians. As stated by Osborne (19), it is, therefore, of great interest to examine whether the same association between β -catenin expression and disease progression could be detected in other types of primary melanomas.

MATERIALS AND METHODS

Specimens. Formalin-fixed, paraffin-embedded tumor tissue from 164 primary malignant melanoma, 66 metastasis, and 7 benign nevi were randomly collected from the archives of The Norwegian Radium Hospital and regional hospitals. From 34 patients, both primary (18 originating from superficial and 16 from nodular lesions) and metastatic material was collected. Of the primary tumors, 106 were classified as superficial spreading and 58 as nodular spreading tumors. The median depth of the superficial and nodular spreading tumors was 1.2 mm (range, 0.11–12 mm) and 4.2 mm (range, 0.95–15 mm), respectively. The median age of the whole group of patients was 54 years (range, 19–88 years) and the median follow-up of patients still alive ($n = 97$) was 151 months (range, 26–361 months). Of the metastatic lesions, 32 and 34 were developed from superficial and nodular spreading melanomas, respectively. Treatment of the patients was according to WHO standard.

Immunohistochemical Staining. Sections of formalin-fixed, paraffin-embedded tissue were immunostained using the streptavidin-peroxidase method (Supersensitive Immunodetection System, LP000UL; 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 2×5 min at 800W to unmask epitopes. After treatment with 1% hydrogen peroxidase for 10 min to block endogenous peroxidase, the sections were incubated subsequently with a 1:2000 dilution of monoclonal β -catenin antibody (Transduction Laboratories, Lexington, KY) for 18–22 h at room temperature. The sections were thereafter 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 tetrachloride 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 dilutions of antibody, biotin-labeled secondary antibody, and the streptavidin-peroxidase were made with PBS (pH 7.4). Positive and negative controls were included, the latter obtained by replacement of the monoclonal antibody with mouse myeloma protein of the same subclass and concentration as the monoclonal antibody. All the controls gave satisfactory results. Four semiquantitative categories were used to describe the number of positively stained cells: (–), none; (+), <5%; (++) , 5–50%; and (+++) , >50%. Mem-

branous, cytoplasmic, and nuclear staining were recorded individually.

Statistical Analysis. The relationship between tumor thickness and the expression of β -catenin was evaluated non-parametrically using the Mann-Whitney test, whereas comparisons between β -catenin and cell cycle factors were performed using Pearson χ^2 test. Kaplan-Meier survival estimates and log-rank test were used to evaluate the survival data. A value of $P < 0.05$ was considered as statistically significant. The Cox proportional hazards model was used to determine independent prognostic variables for disease-free survival. Tumor depth, type, and localization of the primary tumor, sex, and age, in addition to β -catenin expression level, were included in the final Cox model. All of the statistical evaluations were performed according to the SPSS statistical package.

RESULTS

Protein Expression of β -Catenin in Primary and Metastatic Melanomas. Formalin-fixed, paraffin-embedded tissue from 164 primary (106 superficial and 58 nodular) and 66 metastatic (32 superficial and 34 nodular) melanomas were analyzed by immunohistochemistry for protein expression of β -catenin. Membrane-associated, cytoplasmic, and nuclear staining were scored individually. Because nuclear localization of the protein was found only in 13 biopsies, and the level of nuclear staining always was in agreement with the corresponding staining in the cytoplasm, only this latter parameter was evaluated. Membrane-associated localization of β -catenin was observed in the majority of the examined cases, as >95% of both primary and metastatic lesions of both the main subtypes of cutaneous melanoma demonstrated different levels of positive immunostaining (Fig. 1; Table 1). No clear difference between the subgroups of melanomas was seen when recording the number of patients demonstrating different levels of positive membrane staining. In contrast, a significant higher fraction of superficial than nodular primary melanomas demonstrated cytoplasmic localization of the protein in >50% of the cells (71% versus 38%; $P < 0.0001$). Interestingly, in the superficial subgroup, cytoplasmic expression of β -catenin was lost or down-regulated in the more advanced tumors, as only 25% of the metastatic compared with 71% of the primary biopsies demonstrated cytoplasmic localization of the protein in the majority of the cells (Fig. 1; Table 1). In addition, the protein was totally absent in 25% of the metastases compared with 5% of the primary lesions. In 18 cases, primary and metastatic tumor samples from the same patient could be examined. In agreement with the results obtained when examining the whole panel of primary and metastatic lesions originating from superficial spreading melanomas, a decrease in the number of β -catenin-positive cells was observed in 13 of 18 (72%) metastases as compared with the corresponding primary lesion.

In the nodular subgroup, a similar decrease in cytoplasmic staining in the metastatic compared with the corresponding primary lesion was observed in 7 of 16 (43%) cases. In the whole panel of primary and metastatic lesions originating from nodular spreading melanomas, this tendency was detected as an increase in the number of metastatic biopsies that did not express the protein (30% of the metastases as compared with 16%

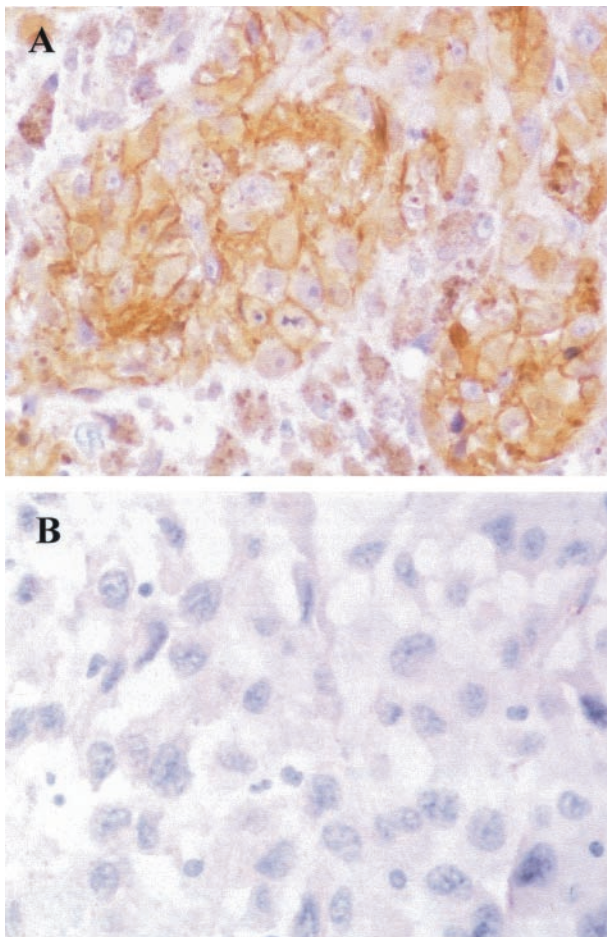


Fig. 1 Representative immunohistochemical analysis of (A) primary superficial melanoma showing membrane-associated and cytoplasmic expression of β -catenin, and (B) a metastasis showing no expression of β -catenin.

of the primary lesions). However, no significant difference was observed between primary and metastatic biopsies originating from nodular tumors in the percentage of cases demonstrating cytoplasmic localization in $>50\%$ of the cells (Table 1). Altogether, this suggests that β -catenin may play different roles in the malignant progression of the two major subgroups of melanoma.

Seven melanocytic nevi were included in the analysis, and five of these demonstrated positive immunostaining both associated with the plasma membrane and also in the cytoplasm in the majority of the cells (Table 1). None of the examined cases expressed the protein in the nucleus.

Immunohistochemical Expression of β -Catenin in Relation to Clinical Parameters. The immunohistochemical staining of β -catenin was examined in relation to clinical parameters of melanoma progression. A high percentage of the examined cells demonstrated extended positive immunostaining; thus, staining in $>50\%$ of the cells has been defined as cases demonstrating high protein expression. When relating the cytoplasmic localization of β -catenin to the thickness of the

primary lesion, a significant correlation between high protein expression and thin lesions was observed in the superficial spreading subgroup (Table 2; $P = 0.012$). No such relationship was found for the nodular spreading melanoma subgroup. The membrane-associated localization of the marker did not show any correlation to the vertical growth of the primary lesions in either of the two subgroups (Table 2).

In agreement with the observed correlation between tumor depth and cytoplasmic localization of β -catenin in superficial spreading melanomas, a significant increase in disease-free survival ($P = 0.0006$; Fig. 2A) was observed for patients having lesions demonstrating cytoplasmic staining in the majority of the cells. The same tendency was revealed when examining overall survival, but cytoplasmic β -catenin staining did not turn out to be a significant predictor for patient demise ($P = 0.08$; Fig. 2B). Membrane-associated localization of β -catenin showed no significant correlation with patient relapse-free or overall survival. For the nodular spreading melanomas, no significant relationships or clear tendencies were observed between β -catenin expression and prognostic factors, neither for cytoplasmic (Fig. 2, C and D) nor membrane-associated localization of the protein (data not shown). In multivariate analysis, using tumor thickness, type (superficial *versus* nodular), and cytoplasmic localization of β -catenin as covariants, β -catenin did not reach statistical significance as independent prognostic factor predicting disease-free survival.

Relationship between the Expression of β -Catenin, Cyclin D1, and p27^{Kip1}. This panel of malignant melanoma biopsies has been analyzed previously for expression of the proliferation marker Ki67 in addition to the cell cycle factors cyclin A, D1, and D3, and the cyclin kinase inhibitors p21^{WAF1/CIP1} and p27^{Kip1} (20–23). Cyclin D1 is one of the targets for the Tcf/Lef transcription factor and, therefore, it was of interest to compare the observed pattern of immunohistochemical staining of β -catenin with the expression of the different cell cycle factors. Surprisingly, a significant inverse correlation was detected between cytoplasmic localization of β -catenin and cyclin D1 in the superficial subgroup of the biopsies ($P = 0.038$). Previous results have indicated that cyclin D1 is closely linked to the proliferation marker Ki67 in this panel, and in agreement with that observation, we also detected a tendency toward an inverse correlation between Ki67 and β -catenin staining in the cytoplasm ($P = 0.086$). Interestingly, membrane-associated localization of β -catenin turned out to be inversely correlated to the cell cycle inhibitor p27^{Kip1} ($P = 0.0025$). No association was found between β -catenin, cyclin A, D3, and p21^{WAF1/CIP1}.

In the nodular spreading subgroup of the biopsies the only association observed was an inverse correlation between cyclin D3 and cytoplasmic β -catenin ($P = 0.034$).

DISCUSSION

β -Catenin protein expression was examined by immunohistochemistry in a panel consisting of superficial and nodular spreading primary and metastatic melanomas. A significant larger fraction of superficial than nodular spreading primary melanomas demonstrated cytoplasmic localization of β -catenin in the majority of the cells. Interestingly, metastatic lesions from

Table 1 Number (percentage) of melanocytic tumors demonstrating membrane-associated and cytoplasmic localization of β -catenin

Type of melanomas	Lesion	Number examined	Membranous localization ^a				Cytoplasmic localization ^a			
			0	<5%	5–50%	>50%	0	<5%	5–50%	>50%
Superficial	Primary	106	2 (2)	9 (9)	45 (42)	50 (47)	5 (5)	4 (4)	21 (20)	76 (71)
	Metastatic	32	1 (3)	7 (22)	11 (34)	13 (41)	8 (25)	6 (19)	10 (31)	8 (25)
Nodular	Primary	58	1 (2)	8 (14)	21 (36)	28 (48)	9 (16)	5 (8)	22 (38)	22 (38)
	Metastatic	34	1 (3)	4 (12)	9 (26)	20 (59)	10 (30)	1 (3)	9 (26)	14 (41)
Benign nevi		7	—	—	2 (29)	5 (71)	—	—	2 (29)	5 (71)

^a Scored as percentage of positively stained cells.

Table 2 Relationship between immunohistochemical staining of β -catenin and depth of primary tumor growth

Type of lesion	% of tumor cells stained	Membranous localization			Cytoplasmic localization		
		No. examined	Depth of growth (mm)	<i>P</i>	No. examined	Depth of growth (mm)	<i>P</i>
Superficial	>50%	51	1.6	0.33	75	1.5	0.012
	≤50%	52	2.0		28	2.6	
Nodular	>50%	28	4.7	0.70	22	4.3	0.37
	≤50%	30	4.7		36	5.0	

superficial melanomas showed down-regulated expression of the protein, and in agreement with this a significant inverse correlation between cytoplasmic localization of β -catenin and the depth of the primary lesion was observed. Furthermore, cytoplasmic staining of β -catenin was found to be significantly associated with disease-free survival for this group of patients. We also observed down-regulation of cytoplasmic β -catenin expression in metastatic lesions originating from nodular tumors, but in this subtype of melanoma β -catenin expression did not turn out to have prognostic importance.

Our results are in agreement with previous studies demonstrating loss of β -catenin expression associated with melanoma progression (17, 18). However, the three studies can not be compared directly, because the melanoma subgroups were not indicated in one of the investigations (17), and the second study (18) was performed on a panel mostly consisting of acral lentiginous melanomas. In agreement with Sanders *et al.* (17), we did not observe β -catenin staining in the nucleus of the examined benign nevi, even when the sections demonstrated high expression of the protein in the cytoplasm. In contrast with our findings, both the previous studies reported changes in intracellular localization of β -catenin in more advanced tumors, but the sites of expression were not consistent. Whereas in most primary acral lentiginous melanomas the protein was localized only in the cytoplasm (18), the primary lesions examined by Sanders *et al.* (17) demonstrated positive immunostaining at the plasma membrane. In both studies a higher percentage of mixed cytoplasmic and membranous staining were reported in the metastatic lesions, meaning that acral lentiginous melanomas were reported to lose cytoplasmic expression, whereas the melanomas examined by Sanders *et al.* (17) were reported to lose membranous expression of the protein, concomitant with disease progression.

In sharp contrast with both of these previous studies, but in accordance with recent results from Reifenberger *et al.* (14), nearly all of our examined cases, both benign nevi and malig-

nant melanocytic lesions, showed variable β -catenin staining associated with the plasma membrane. The differences in intracellular localization can be explained by the fact that different antibodies were used and also by differences in the category of melanocytic lesions examined. However, altogether the results indicate that down-regulation of cytoplasmic β -catenin during disease progression is a common pattern in acral (18) and superficial spreading melanomas.

An association between accumulated β -catenin protein expression and favorable prognosis has been reported also in ovarian (24) and hepatocellular carcinoma (25). In both studies, elevated levels of mutated β -catenin in the nucleus was shown to be a significant predictor of prolonged patient survival. Only a very small proportion of our melanomas demonstrated nuclear localization of the protein, and no attempt has been made to examine whether the *CTNNB1* gene is mutated in the lesions with elevated protein levels. Nevertheless, together these studies argue for the possibility that extended expression of β -catenin in early carcinogenesis might be a favorable parameter.

Highly relevant to our results, showing a link between high levels of β -catenin staining and good prognosis, is a recent paper demonstrating induction of apoptosis following β -catenin transfection into fibroblast, as well as melanoma and colorectal carcinoma cells (26). β -Catenin-induced apoptosis in the transfected cells was independent of Tcf transcriptional activity and cell cycle/apoptosis regulators like p53, E2F1, and Rb, suggesting that overexpressed β -catenin might bind other proteins through a potential death domain (26). Furthermore, it has been shown that overexpression of several members in the Frizzled-Dishevelled cascade, including β -catenin, can induce apoptosis after transfection into COS7 and 293 cells (27). A link between GSK3 β activity, nuclear factor κ B signaling, and cell survival has been published previously (28). Inhibition of GSK3 β activity, causing accumulation of cytoplasmic β -catenin and simultaneous inhibition of nuclear factor κ B survival signals, might,

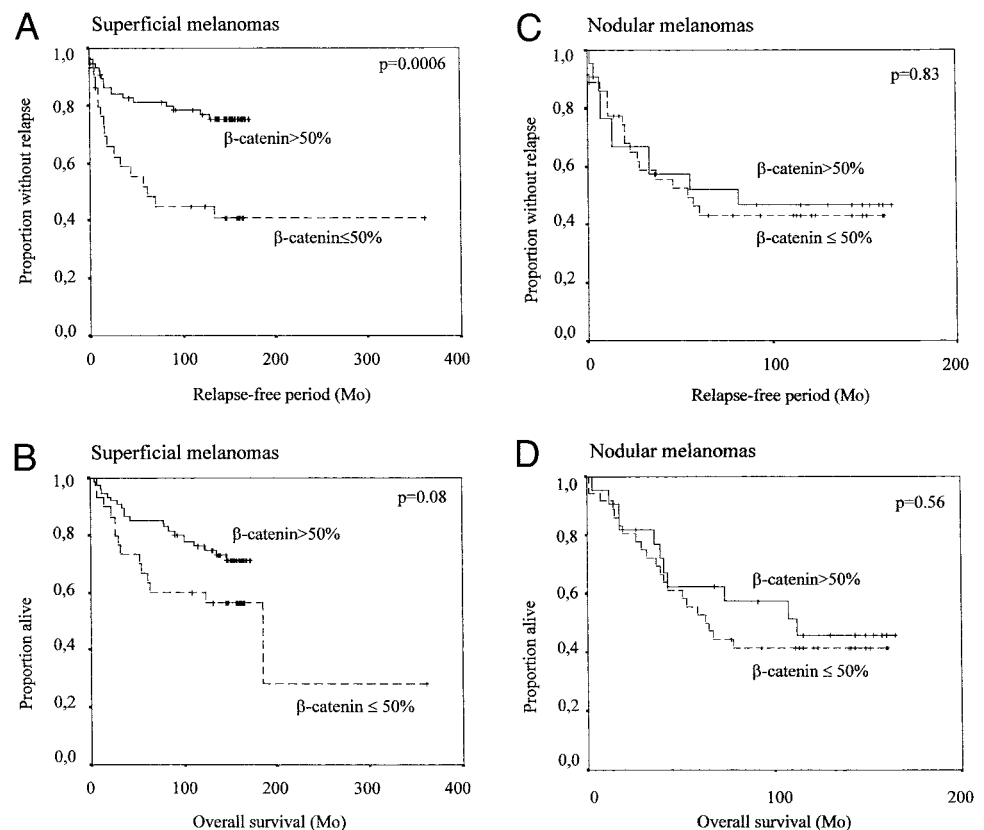


Fig. 2 Kaplan-Meier curves demonstrating the relationship between the expression of β -catenin, and relapse-free (A and C) and overall (B and D) survival for patients with superficial (A and B) and nodular (C and D) spreading melanomas ($n = 106$). Mo, months.

therefore, be an alternative hypothesis explaining the high expression of β -catenin in early melanomas.

Our data indicate that β -catenin is lost or down-regulated in more advanced tumors. Mutations and unusual splicing of β -catenin mRNA, as well as mutations in the *APC* gene, has been reported in melanoma cell lines and taken as favorable for increased stability of the protein (13). However, the possibility still exist that gene aberrations might have the opposite effect, causing a decrease or complete loss of the encoded protein. Furthermore, cytoplasmic localization of β -catenin is regulated by proteasomal degradation. Thus, all molecular changes enhancing the activity of the GSK3 β /AXIN/APC complex, labeling β -catenin for degradation and increasing the proteasomal activity, will subsequently cause a down-regulation of the protein. Finally, less Wnt-receptor activity, causing decreased downstream signaling, will accelerate β -catenin degradation and cause lower expression of the protein in the cytoplasm. Whether the observed down-regulation of β -catenin in the advanced superficial melanomas can be explained by any of these postulated mechanisms remains to be investigated.

One of the well-known transcriptional targets for Tcf/Lef is cyclin D1 (7). Surprisingly, in the panel of superficial melanoma biopsies a significant inverse correlation between cytoplasmic localization of β -catenin and cyclin D1 was observed. High expression of β -catenin concomitant with low expression of cyclin D1 in early melanomas is indicative of low proliferative activity, which is also manifested by low level of the proliferation marker Ki-67. It has been reported that Tcf can be acety-

lated by CBP, thereby abolishing the ability to interact with β -catenin and to activate transcription (29). In addition, Tcf activity can be inhibited by the transcriptional repressor Groucho (30, 31). Whether CBP or Groucho activity is present in the melanomas, and, thus, may be responsible for the lack of a positive correlation between β -catenin and cyclin D1 expression, is not known. Furthermore, in the nodular subtype of melanoma a significant inverse correlation between cytoplasmic localization of β -catenin and cyclin D3 was revealed. To our knowledge this is the first time an association between these two proteins has been reported, and no attempt has been made to study this further.

An intriguing association is the significant inverse correlation between the membrane-associated localization of β -catenin and the cell cycle inhibitor p27^{Kip1} ($P = 0.0025$) found in the superficial spreading melanomas. Interestingly, transfection of p27^{Kip1} was reported to cause cell-cycle arrest, cell scattering, and induction of cell migration in hepatocellular carcinoma cells (32). The mechanisms by which p27^{Kip1} can induce such phenotypic changes is not known, but it was hypothesized that p27^{Kip1} might target a non-cyclin-dependent kinase complex. β -Catenin plays a dual role in tumor development, both as a transcriptional regulator and also as a component of the adhesion apparatus. When achieving a metastatic and invasive phenotype, tumor cells are supposed to lose adhesive properties; thus, down-regulation of membranous β -catenin are often observed (33). Whether β -catenin can be down-regulated by high expression of p27^{Kip1} in hepatocellular carcinomas, and, thus, responsible for the induced migration, remains to be investigated.

The observed inverse correlation between the staining levels of these two proteins in superficial melanoma support such a speculation.

In conclusion, we have demonstrated that cytoplasmic localization of β -catenin was associated with favorable prognosis for patients with superficial but not nodular spreading melanomas. The mechanisms behind this rather controversial and protective role of β -catenin in superficial melanomas remains to be elucidated, but one explanation might be that β -catenin participates in induction of apoptosis in early lesions and that the expression is lost or down-regulated in the more advanced tumors.

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