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Advances in Brief

The Transcriptional Repressor of *p16/Ink4a*, Id1, Is Up-Regulated in Early Melanomas[1]

David Polsky,[2] Alison Zuyung Young, Klaus J. Busam, and Rhoda M. Alani[3]

Oncology Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21231-1000 [R. M. A.], and Cell Biology and Genetics Program [A. Z. Y.], and Department of Pathology [K. J. B., D. P.], Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Abstract

The helix-loop-helix transcription factor Id1 coordinates cell growth and differentiation pathways within mammalian cells and has been implicated in regulating G_1-S phase cell cycle transitions. Recently Id1 has been shown to repress Ets- and E-protein-mediated transactivation of *p16/Ink4a*. Because the p16/Ink4a protein has been demonstrated to be inactivated in subsets of familial and sporadic melanomas, we sought to determine whether Id1 regulation of p16/Ink4a expression might be involved in the development of this human tumor. Here we evaluate 21 melanocytic lesions at various stages of malignant progression from common melanocytic nevi to metastatic melanomas and examine these lesions for Id1 and p16/Ink4a expression. We demonstrate that Id1 expression correlates with loss of p16/Ink4a expression in melanoma *in situ*; however, more advanced stages of melanoma do not express Id1 except within perivascular regions, despite overall decreased p16/Ink4a expression in these lesions. Microdissected lesions were evaluated for *p16/Ink4a* sequence, and invasive melanomas that did not express Id1 were found to have sustained inactivating *p16/ink4a* mutations. These data suggest a role for Id1 in regulating p16/Ink4a expression in early melanomas and demonstrate that later genetic changes may provide for irreversible loss of p16 expression in advanced stages of this tumor.

Introduction

Basic HLH[4] DNA binding proteins regulate tissue-specific transcription within multiple cell lineages (1). The Id family of HLH proteins does not possess a basic DNA binding domain and inhibits lineage commitment within multiple cell types through sequestration of basic HLH transcription factors (reviewed in Ref. 2). The Id family member Id1 has been implicated in regulating cellular life span, and we and others have reported delayed senescence of primary human keratinocytes that constitutively express Id1 (3, 4). Because recent studies have illustrated the importance of the pRB tumor suppressor pathway and telomerase activity in regulating primary mammalian cell growth and senescence (5–7), it has been postulated that Id1 regulation of cellular growth and senescence may function through direct regulation of these pathways. Previous studies have demonstrated Id1 reactivation of DNA synthesis in senescent human fibroblasts in cooperation with a mutant SV40 T antigen that is unable to bind pRb, which suggests that Id1 can antagonize the functions of pRb or other members of this tumor-suppressor pathway, including *p16/Ink4a* (8). More recently, Id1 has been demonstrated to oppose Ets-mediated activation of p16/Ink4a via Ras-Raf-MEK signaling (9), and Id1-null mouse embryo fibroblasts have been shown to senesce prematurely because of increased expression of p16/Ink4a (10).

The p16/pRB pathway has been shown to be deregulated in a large majority of human tumors, either through loss of p16 or pRb function or through deregulated expression of cyclin D or cdk4 (reviewed in Ref. 11). Several mechanisms of inactivation of the p16/pRB pathway have been noted in various tumors and transformed cell lines. Among the most common ways in which these gene products are dysregulated in tumors is through gene deletion; inactivating mutations; epigenetic changes such as promoter methylation, protein sequestration, and inactivation (*e.g.*, viral oncoproteins); and posttranslational modification (*e.g.*, inactivating phosphorylation events; reviewed in Ref. 12). To date, little is known about the direct transcriptional regulation of genes within the p16/pRb pathway and their role in the development of human malignancies. Here we investigate whether the HLH protein Id1 may play a role in regulating p16/Ink4a expression during the development of melanocytic tumors. We demonstrate that Id1 expression is elevated within the *in situ* component of malignant melanomas but is not elevated significantly in invasive lesions. Furthermore, we demonstrate that p16/Ink4a expression progressively decreases in association with advancing stages of melanomas and that these invasive lesions have frequently sustained mutations of *p16/Ink4a*.

Materials and Methods

Immunohistochemistry. Sections (6–7 μm) were blocked with 10% normal horse serum (Vector) in PBS-TX and incubated with monoclonal anti-p16 (Santa Cruz Biotechnology) antibody diluted 1:250 or with polyclonal anti-p19 (AEC40; gift of N. Sharpless/R. DePinho, Dana Farber Cancer Institute, Boston, MA) antibody diluted 1:200 in PBS-TX with 1% normal horse serum. Sections were then incubated with the biotinylated horse antimouse secondary antibody (Vector) diluted 1:200 in PBS with 1.5% normal horse serum. The Vectastain Elite ABC kit (Vector) was used for horseradish peroxidase staining of sections.

***In Situ* Hybridization.** Sections (6–7 μm) were processed for *in situ* hybridization with [α-^{33}P]UTP-labeled cRNA probes, as described previously (13). The Id1 probe template was generated by Y. Jen of Memorial Sloan-Kettering Cancer Center, New York, NY (14).

***p16/Ink4a* Gene Analysis of Primary Melanomas.** Five-μm sections were placed on uncharged slides, stained with H&E, and allowed to air dry. Tumor cells were separated from normal cells by LCM using an Arcturus Pix Cell instrument. Isolated tumor cells were digested overnight with proteinase K at 37°C according to the NIH protocol. DNA was amplified using Qiagen HotStar Taq, 55 cycles, in a multiplex PCR reaction containing primers for both an internal control gene (either a portion of the *GAPDH* gene or exon 4 or exon 5 of the *p53* gene) and exons 1 or 2 of the *p16* gene, as described previously (15). Amplified bands were gel purified, and both forward and reverse sequences were obtained in the Sloan-Kettering DNA sequencing facility. Deletions were determined if the internal control band amplified, but the p16 band did not. All of the reactions were performed a minimum of three times.

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[2] Current address: Department of Dermatology, NYU Medical Center, 550 First Avenue, New York, NY 10016.

[3] To whom requests for reprints should be addressed, at Oncology Center, Johns Hopkins University School of Medicine, Bunting-Blaustein Cancer Research Building, 1650 Orleans Street, Room 336, Baltimore, MD 21231-1000. Phone: (410) 614-6204; Fax: (410) 614-5015; E-mail: ralani@jhmi.edu).

[4] The abbreviations used are: HLH, helix-loop-helix; pRB, retinoblastoma protein; LCM, laser capture microdissection.

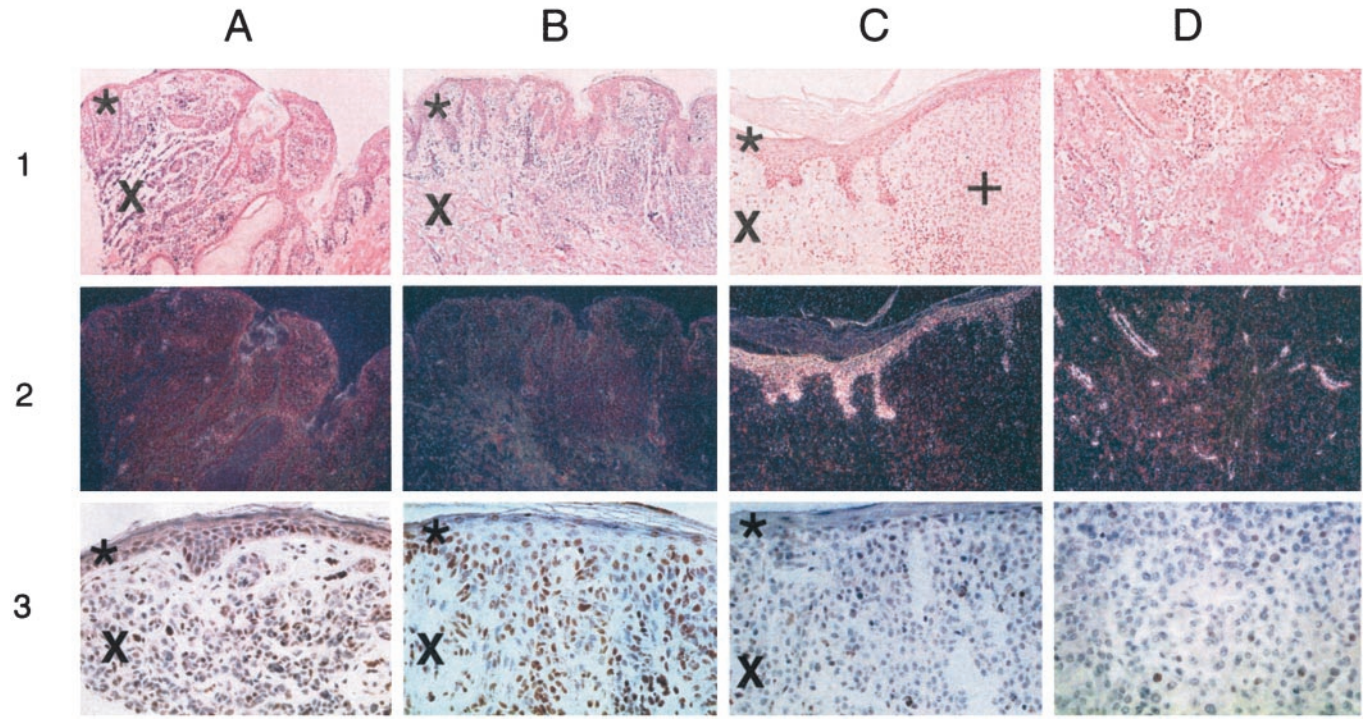

Fig. 1. Id1 and p16 expression patterns in progressive stages of malignant melanoma development. H&E staining (*row 1*), *in situ* hybridization for Id1 expression (*row 2*), and immunohistochemical (IHC) staining for p16/Ink4a (*row 3*) in a normal compound nevus (*column A*), a dysplastic nevus (*column B*), a primary cutaneous invasive melanoma (*column C*), and a metastatic melanoma (*column D*). In lesions with both epidermal and dermal components present: 226, the epidermis; **X**, the dermis. **+**, the invasive melanoma component of the lesion in column C. *Brown stain* on IHC is positive for p16, *blue stain* is counterstain. Staining patterns are representative of all tissue samples examined. Detailed methods will be provided on request.

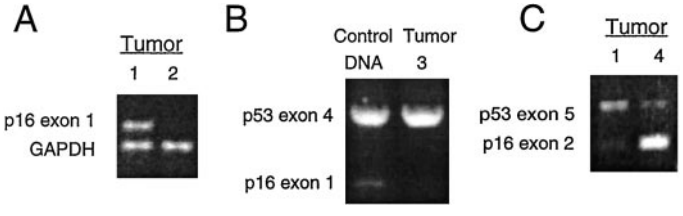

Fig. 2. Genetic analysis of p16Ink4a Exons 1 and 2. *A*, multiplex PCR of exon 1 (204 bp) and a portion of the *GAPDH* gene (139 bp). There is a lack of amplification of exon 1 in tumor 2, consistent with a deletion of this exon. *B*, multiplex PCR yielding the 204-bp exon 1 product and a portion of the *p53* gene that includes exon 4 (340 bp). There is a lack of amplification of exon 1 from tumor 3, consistent with a deletion of this exon. *C*, multiplex PCR of a portion of exon 2 (147 bp) and a portion of the p53 gene that includes exon 5 (209 bp). Exon 2 bands were cut from the gel, purified, and sequenced. The band from tumor 1 contained the mutation described in the Table.

Results

Id1 and p16/Ink4a Expression within Melanocytic Lesions. The *p16Ink4a* gene has been demonstrated to be inactivated in familial and sporadic melanomas by a variety of mechanisms (16). To determine whether Id1 may play a role in *p16/Ink4a* regulation during melanomagenesis, we evaluated Id1and p16 expression in melanocytic lesions at various stages of melanoma progression. Archival samples of five common melanocytic nevi, five atypical (dysplastic) nevi, six primary cutaneous melanomas of different thickness and level of invasion, and five metastatic melanomas were evaluated by immunohistochemistry (p16) and *in situ* hybridization (Id1) for expression of p16 and Id1. As has previously been demonstrated (17, 18), we noted decreasing p16 expression as a function of increasing malignant potential in all melanocytic lesions, with 70–90% p16 positivity in compound and dysplastic nevi (Fig. 1, *A-3* and *B-3*), 30–50% positivity in *in situ* and invasive melanomas (Fig. 1*C-3*), and <10% positivity in metastatic lesions (Fig. 1*D-3*). In contrast, *in situ* evaluation of Id1 expression in these lesions demonstrated no Id1 expression in either compound or dysplastic nevi (Fig. 1, *A-2* and *B-2*) but marked Id1 expression in the epidermis in both the intraepidermal (*in situ*) melanoma component and neighboring keratinocytes (Fig. 1*C-2*); however, the invasive components of these melanomas failed to express significantly elevated levels of Id1. Metastatic melanomas demonstrated significantly elevated Id1 expression only around the vasculature within the lesions as has previously been noted in other malignancies (13), which suggests that Id1 may play an important role in early melanomagenesis but is not likely to be necessary for invasion or metastasis of this tumor.

Sequence Analysis of the *p16/Ink4a* Gene in Primary Melanomas. To determine whether the invasive melanomas that failed to express Id1 possessed a wild-type *p16/Ink4a* gene, we isolated invasive melanoma cells from the tissues evaluated using LCM. The *p16/Ink4a* gene was amplified by PCR and sequenced using previ-

Table 1 *Sequence analysis of p16/Ink4a in melanoma samples*

Tumor cells were separated from normal cells by LCM using an Arcturus Pix Cell instrument. Isolated tumor cells were digested overnight with proteinase K. DNA was amplified using QIAGEN HotStar Taq, 55 cycles, in a multiplex PCR reaction containing primers for both an internal control gene (either a portion of the *GAPDH* gene or exon 4 or exon 5 of the *p53* gene) and exons 1 or 2 of the *p16* gene. Amplified bands were gel purified, and both forward and reverse sequences were obtained. Deletions were determined if the internal control band amplified, but the p16 band did not.

Tumor depth (mm)	Exon 1	Exon 2
10	Wild type	His83Tyr[a]
4	Deleted	Wild-type[b]
1.6	Deleted	Wild-type[b]
1.0	Indeterminate[c]	Wild-type[b]
0.25	Indeterminate[c]	Wild-type[b]
Melanoma *in situ*	Not done	Not done

[a] This mutation has been previously described in a melanoma cell line, but not in a melanoma tissue specimen.
[b] Partial sequence.
[c] Indeterminate, unable to amplify the fragment as well as the internal control.

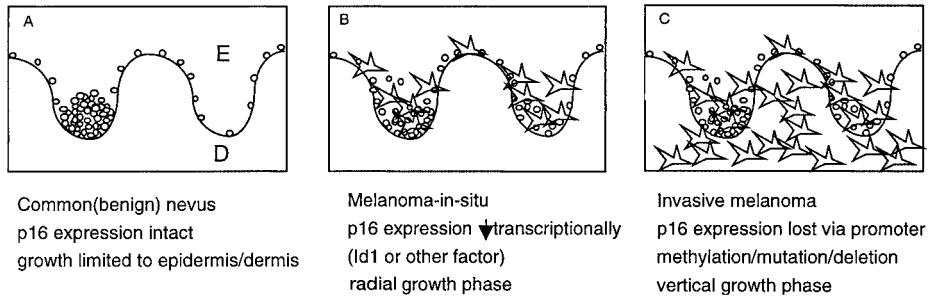

Fig. 3. Model for regulation of p16/Ink4a expression during the evolution of melanocytic tumors. *A*, common (benign) nevus with nest of nevomelanocytes at dermo-epidermal junction possesses intact p16/Ink4a expression and organized growth. *E*, epidermis; *D*, dermis. *B*, melanoma *in situ* is limited to the epidermis with radial growth of malignant cells. p16/Ink4a expression is decreased by transcriptional control (Id1 or other factors). *C*, invasive melanoma possesses horizontal growth phase into the dermis with loss of p16/Ink4a expression because of promoter methylation or sustained genetic mutation/deletion at the p16/Ink4a locus.

ously published primers (15). An internal control gene was simultaneously amplified to assess deletions of *p16*. Sequence analysis of the *p16/Ink4a* gene revealed inactivating deletions or missense mutations in invasive melanomas of Breslow level 1.6 mm or greater which suggested a high likelihood of genetic alteration leading to altered p16/Ink4a expression in invasive lesions that failed to express high levels of Id1 (Fig. 2; Table 1).

Discussion

Inherited mutations of the *p16/Ink4a* gene or the associated cdk4 cyclin-dependent-kinase confer a predisposition to the development of melanoma (19). In sporadic melanomas, however, the frequencies of loss of heterozygosity at the *p16/Ink4a* locus, *p16/Ink4a* intragenic mutations, and promoter methylation are extremely low in thin lesions (<10% in lesions of <4 mm), but loss of p16 expression is associated with progression of disease (17, 20, 21), which suggests that other mechanisms allow for decreased p16 expression in early melanomas. Because the transcriptional regulatory protein Id1 has recently been identified as a repressor of p16/Ink4a transcription (9, 10, 22), we sought to determine whether transcriptional inactivation of *p16/Ink4a* by Id1 might play a role in the initiation or progression of sporadic melanomas. In the present study, we examined the expression patterns of the HLH protein Id1 and the cell cycle regulatory protein p16/Ink4a in a spectrum of melanocytic lesions from benign nevi to metastatic melanomas. As expected, we noted decreasing expression of the *p16/Ink4a* gene with malignant progression of melanocytic lesions; however, Id1 expression was limited to the *in situ* component of invasive melanomas and perivascular regions of metastatic tumors. Sequence analysis of the *p16/Ink4a* gene revealed genetic mutations associated with invasive tumors that did not express Id1. Our data demonstrate that Id1 expression correlates with decreased p16/Ink4a expression in early melanomas that are confined to a radial growth phase, which suggests that Id1 transcriptional repression of p16/Ink4a may represent one of the earliest mechanisms of dysregulation of p16 expression in melanoma initiation. We, therefore, propose a model for p16 inactivation in melanomagenesis that occurs via multiple steps that entail reversible transcriptional inactivation of p16 expression in early (radial growth phase) tumors (via Id1 or other repressors) that allows for bypass of cellular senescence, subsequent acquired epigenetic changes in cells with extended life span (*e.g.*, promotor methylation), and finally acquired irreversible genetic alterations (*e.g.*, mutations, deletions) associated with tumor progression, invasion, and metastasis (Fig. 3). Such a mechanism of p16 repression in tumors represents a novel method of inactivation of this tumor suppressor gene, which has been shown to be critical for the evolution of both familial and sporadic human tumors. Interestingly, Id1 expression has also been noted to be up-regulated in pancreatic tumors, which, like melanomas, frequently demonstrate inactivation of the tumor suppressor *p16/Ink4a* (23).

In conclusion, our data provide preliminary evidence for a role for Id1 in functional inactivation of p16/Ink4a in early melanomas. Because these studies suggest that Id1 expression in melanomas is limited to the earliest stages of this tumor, marker studies for Id1 in melanocytic lesions of uncertain malignant potential may play a critical role in the histopathological classification of melanocytic lesions in the future and may help determine the appropriate treatment and prognosis of melanocytic lesions. Although larger studies will need to be undertaken to confirm these findings, they provide a useful paradigm for understanding the mechanisms of development of non-familial melanomas and the potential role for Id1 regulation of *p16/Ink4a* in the evolution of these tumors.

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