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Advances in Brief

Overexpression of *p53* Is a Late Event in the Development of Malignant Melanoma[1]

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Abstract

Overexpression of the p53 gene product has been observed in a high percentage of malignant melanomas. To evaluate the role of this protein in the development of melanoma, we examined p53 expression in benign, premalignant, and malignant melanocytic lesions. Using the antibodies DO-7 and 1801, which recognize both wild-type and most mutant forms of the p53 protein, we analyzed by immunohistochemical staining 26 benign nevi, 34 dysplastic nevi from patients at low risk for the development of melanoma, 22 dysplastic nevi from patients at high risk for the development of melanoma, 61 primary melanomas (including 15 that arose from dysplastic nevi), and 10 metastatic melanomas. Expression of the p53 protein was not observed in any of the benign or dysplastic nevi. Of the primary melanomas only 3 (5%) demonstrated nuclear staining, whereas 70% of the metastatic melanomas showed a positive reaction for p53. These data suggest that overexpression of the p53 gene product is a late event in the progression of melanoma and consequently indicate that expression of this protein cannot be used as a marker to identify patients at high risk for the subsequent development of melanoma.

Introduction

The *p53* gene, located on human chromosome 17, encodes a M_r 53,000 nuclear phosphoprotein which in its native form can suppress cellular proliferation (1). This property of *p53* was first convincingly demonstrated in DNA transfection experiments in which wild-type *p53* was found to block the transformation of rodent cells by oncogenes such as *ras* and *E1A* (2). In marked contrast to the wild-type form of p53, many mutant forms of this protein possess oncogenic potential (3). Collectively, these observations have been explained by a dominant negative hypothesis which proposes that the mutant p53 protein interferes with functioning of the concomitantly expressed wild-type protein (4). The loss of wild-type *p53* gene expression has been implicated in the development of a wide variety of human cancers. This correlation is perhaps best illustrated in patients with the familial Li-Fraumeni syndrome (5). Such individuals manifest a marked susceptibility to many malignancies including sarcomas, cancer of the brain and breast, leukemia, and possibly melanoma. Members of several affected families have been screened and found to carry germ line mutations within conserved regions of the *p53* gene. Alterations within the *p53* gene have also been detected in many varieties of sporadic human cancer and, at present, constitute the most frequently identified genetic abnormality in human cancer (6, 7).

The wild-type form of the p53 protein has a relatively short half-life (approximately 5–30 mins) and is normally present in minute quantities in nonneoplastic cells. In contrast, many *p53* gene point mutations lead to the production of proteins which are significantly more stable than their wild-type counterparts, thus resulting in intracellular overaccumulation (8). For these reasons, wild-type *p53* generally cannot be detected immunohistochemically in normal adult human cells but may be observed readily in those malignant cells that express the mutant form of this protein. Recently, two reports have presented immunohistochemical data which suggest that malignant melanomas overexpress the p53 protein (9, 10).

The development of a melanoma is thought to arise via a multistep process characterized by distinct histopathological stages (11). The first stage, the common acquired nevus, consists of a focal proliferation of morphologically normal cutaneous melanocytes. Dysplastic nevi, which constitute the second stage, exhibit cytological and architectural abnormalities and serve as a marker for increased melanoma risk especially when associated with a family history of melanoma (12). Neoplastic transformation of melanocytes usually results in an initial phase of radial growth, which may be followed by later vertical growth and metastasis. We undertook the present study to determine the timing of p53 overexpression during the development of melanomas and to establish whether patients with dysplastic nevi also showed evidence of p53 abnormalities.

Materials and Methods

Tissue Specimens. In preliminary studies, immunostaining for p53 was carried out on frozen metastatic melanoma specimens. All subsequent immunohistochemical staining of nevi and melanomas was performed on formalin-fixed, paraffin-imbedded tissue sections obtained from the surgical pathology files of Women's College Hospital and Flemingdon Laboratories (Toronto, Ontario, Canada). The histological criteria used to delineate the benign (26 samples) from dysplastic (56 samples) nevi were those of Clark *et al.* (13). The dysplastic nevi were further categorized into low (34 specimens) and high (26 specimens) risk groups according to the classification system of Kraemer *et al.* (Ref. 12; see Table 1 for details). Among the melanomas, 61 were primary tumors (of which 15 exhibited histological evidence of having arisen from a dysplastic nevus) and 10 were obtained from metastatic tumor deposits. In all cases, the histological diagnosis was confirmed on the hematoxylin and eosin sections immediately adjacent to those selected for immunohistochemical analysis. Detailed clinical history and follow-up was available for all cases of dysplastic nevi.

Immunohistochemistry. The streptavidin biotin technique and 2 different anti-p53 antibodies were used to detect p53 protein. The monoclonal antibodies DO-7 (Dako Corp., Santa Barbara, CA) and 1801 (Becton-Dickinson, Mountain View, CA) were raised against recombinant wild-type p53 protein and react with both the wild-type and most mutant forms of p53 protein (14). Briefly, tissue sections (4 μm thick) were cut from the appropriate specimens and mounted on glass slides. Endogenous peroxidase activity was blocked with 0.03% H_2O_2 in methanol. The primary antibody was applied for 4 h and the sections were subsequently incubated with biotinylated swine multilinked anti-goat, mouse, rabbit immunoglobulin followed by streptavidin-biotin-peroxidase complex. Peroxidase activity was visualized as a brown reaction product using 3% H_2O_2 and 3,3'-diaminobenzidine tetrahydrochloride. In all cases scored as positive using the peroxidase containing complex, immediately adjacent sections were stained with streptavidin-alkaline phosphatase and a CAS red chromogen. Various breast carcinomas (see "Discussion") which expressed p53 were used as positive controls.

Results

It has been previously reported that formalin fixation may result in a lower frequency of binding by anti-p53 antibodies compared to that

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Table 1 *Expression of p53 protein in dysplastic nevi and melanomas*

Lesion	Total no. examined	*p53* positive	
		DO-7	1801
Benign nevi	26	0	0
Dysplastic nevi (DN[a])			
Low risk			
A	28	0	0
B	6	0	0
C	13	0	0
High risk			
D_1	9	0	0
D_2	4	0	0
Malignant melanoma			
Primary			
Arising in a DN	15	0	0
Not associated with DN	46	3 (7%)	3 (7%)
Metastatic			
Skin	4	2 (50%)	2 (50%)
Lymph node	6	5 (83%)	5 (83%)

[a] The Kraemer (12) classification of dysplastic nevis (DN) syndrome kindreds is described here by type, name, and characteristics: A, sporadic DN, one family member with DN; B, familial DN, two or more family members with DN; C, sporadic DN and melanoma, only one family member with DN and melanoma; D_1, familial DN and melanoma, two or more family members with DN, one with melanoma; D_2, familial DN and melanoma, two or more family members with DN and melanoma. The risk of developing melanoma increases progressively from type A to type D_2. The melanoma risk for D_2 kindreds has been estimated to be 148 times the normal caucasian population (12).

observed in frozen specimens (15). We thus initially compared the binding of both anti-p53 antibodies to a series of metastatic melanomas prepared using both forms of preservation. Among the 7 metastatic melanomas analyzed, no difference in the frequency of p53 staining was observed between the frozen and formalin-fixed specimens. Moreover, both the pattern of p53 immunostaining and the number of positively stained cells within each specimen were very similar in both the frozen and formalin-fixed tissues. We consequently used formalin-fixed, paraffin-embedded samples for all subsequent analyses.

The results of p53 immunostaining in the formalin-fixed melanocytic lesions are summarized in Table 1. No significant difference in immunostaining was observed between the two different anti-p53 antibodies. Among the benign nevi we found no evidence for overexpression of p53 protein. Similarly, none of the dysplastic nevi from either the low or high risk groups expressed detectable levels of this protein. Among the 15 primary melanomas arising in dysplastic nevi none displayed p53 staining. Of the remaining 46 primary tumors, only three deeply invasive specimens showed a positive reaction for p53 in the nuclei (Fig. 1). In contrast with the primary melanomas, the majority (70%) of the metastatic melanomas displayed positive staining for p53 (Fig. 2).

In all of the positive cases immunoreactivity was restricted to the nuclei of the neoplastic cells. Normal cells within the stroma surrounding the tumor and the adjacent normal tissue were negative for p53 immunostaining. Occasionally, tumor cells with positively stained cytoplasm were observed, but these were not scored as positive unless concomitant staining of the nucleus was detected. Within the positive tumor specimens the number of neoplastic cells stained with either antibody ranged 5–20%. In three cases, primary melanomas which were initially scored as positive using deaminobenzidine tetrahydrochloride were ultimately read as negative upon restaining with the red chromogen. Those areas of the tumor which had been falsely scored as positive corresponded to melanin granules which were of the shape and size of nuclei (Fig. 3).

Discussion

Previous immunohistochemical reports have indicated that the p53 gene product is overexpressed in a high percentage of malignant melanomas (9, 10). These observations raised the possibility that the loss of normal p53 functioning played a pivotal role in the early development of this malignancy. In the present study, we found that while most metastatic melanomas exhibit abnormally high levels of p53 protein, only a small minority of the primary melanomas and none of the preneoplastic dysplastic nevi specimens displayed detectable levels of this protein. Since overexpression of p53 was first detected only after neoplastic transformation and was most commonly observed in metastatic lesions we conclude that overexpression of the p53 gene product is unlikely to initiate melanoma development. Rather, abnormal functioning of this protein is likely to promote tumor progression only after the neoplastic process has already been initiated.

As is the case with melanoma, overexpression of the p53 gene product has also been detected at a late stage in the development of

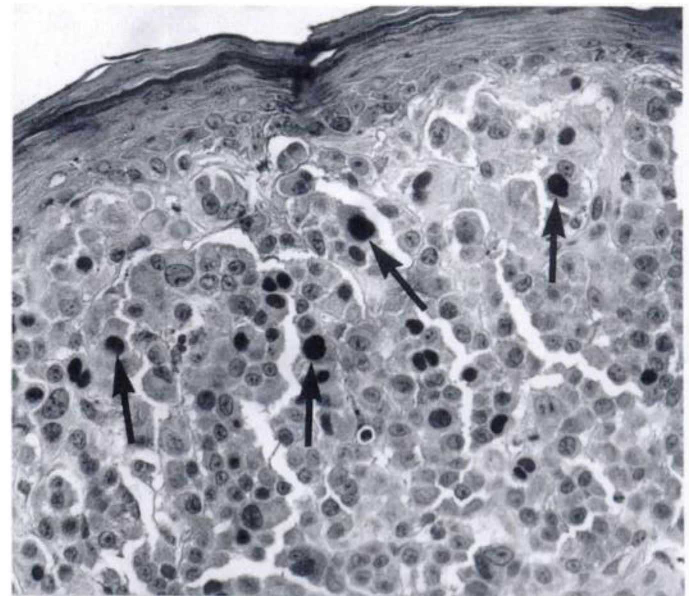

Fig. 1. Immunohistochemical detection of p53 protein in a formalin-fixed, paraffin-embedded section of a primary malignant melanoma. Immunoreactivity is localized in the nucleus of the tumor cells (*arrows*; ×200).

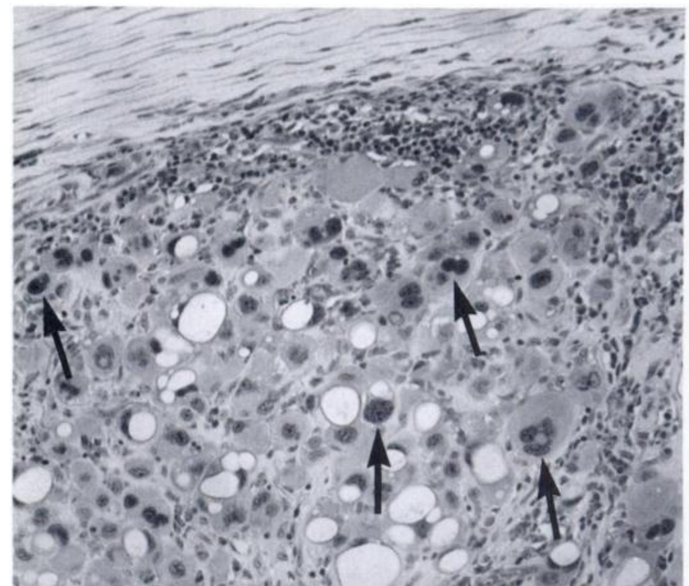

Fig. 2. Metastatic melanoma in a regional lymph node. In this focal area of the tumor more than 50% of the melanoma cells show positive nuclear staining for p53 protein (*arrows*; ×200).

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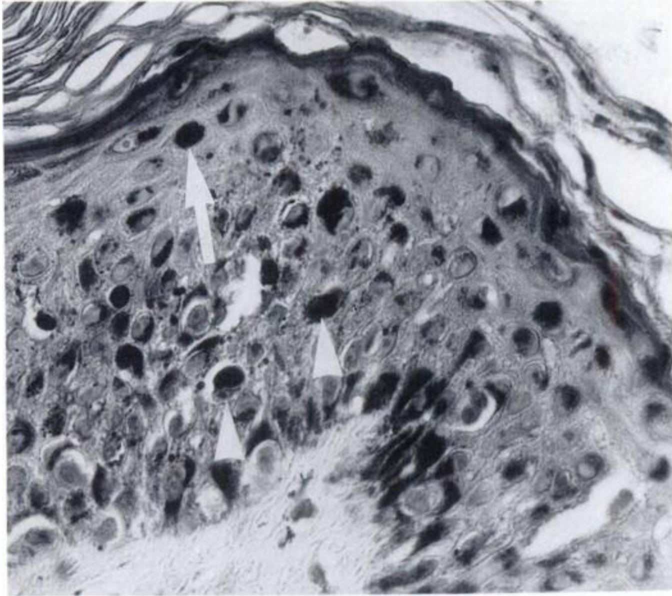

Fig. 3. Malignant melanoma (intraepidermal component). Melanin pigment is observed in the form of "balls" which overly the nuclei of keratinocytes (*arrow*) and malignant melanocytes (*arrowheads*). These aggregates of melanin pigment were initially misinterpreted as evidence for positive p53 immunostaining using diaminobenzidine tetrahydrochloride as the chromogen, but were ultimately scored as negative when adjacent sections were stained with a red chromogen (×400).

several other human tumors. For example, the progression from low grade astrocytoma to high grade glioblastoma has been correlated with the clonal expansion of tumor cells bearing mutations within the *p53* gene (16). Similarly, high grade invasive tumors of the thyroid, bladder, and colon exhibit a greater number of *p53* mutations than their low grade counterparts (17–19). In contrast, in some other human malignancies, abnormal functioning of the p53 product appears to occur much earlier in tumor evolution. For example, immunohistochemical evidence of p53 overexpression has been observed in premalignant lesions of the breast, testes, and esophagus (20–22). Thus, while abnormalities in p53 expression are frequently observed in human malignancies, the onset of p53 overexpression appears to vary in different tumor types.

Two prior immunohistochemical studies have provided evidence for elevated levels of p53 in both primary and metastatic melanomas. Using the polyclonal antiserum CM-1, which recognizes both the wild-type and most mutant forms of p53, Bártek *et al.* (9) screened 38 primary and metastatic melanoma samples and found 92% to stain positively with this antibody. In a subsequent study (10), 14 of 20 primary melanomas showed positive immunostaining using a monoclonal antibody (Mab-240) directed against an epitope found only in mutated p53 protein. As in these previous reports, we also found a majority of metastatic melanoma specimens to exhibit readily detectable levels of p53 protein. In contrast, however, we observed only a relatively small percentage of the primary melanomas to contain detectable levels of p53 protein. While we do not fully understand the reasons for the discrepancy between our results and those of Bártek *et al.* (9) and Stretch *et al.* (10), it may be explained in part by differences in the specificity of the anti-p53 antibodies or by misinterpretation of melanin deposits as evidence for p53 immunostaining, as we observed in several cases. It is unlikely that this discrepancy could be attributed to differences in the sensitivity of p53 immunostaining: in a parallel experiment using the same technique we detected p53 overexpression in 21 of 50 (42%) of primary breast carcinoma samples (data not shown). Previous immunohistochemical studies using the 1801 antibody have reported that 20–45.5% of primary breast carcinomas overexpress the p53 protein (23).

To date, only one report has provided direct genetic evidence for *p53* mutations in human melanoma. Volkenandt *et al.* (24) screened 9 different metastatic melanoma cell lines and found only one to contain a point mutation within any of the four highly conserved regions in the *p53* gene commonly mutated in human cancer. In addition, only the melanoma cell line carrying a *p53* mutation displayed immunohistochemical evidence of *p53* overexpression. The apparent discrepancy between the incidence of p53 staining in metastatic specimens found *in vitro* by Volkenandt *et al.* (24) and our *in vivo* data can be reconciled in two ways. First, p53 expression in melanomas is heterogeneous; no more than 20% of the tumor cells in any specimen show positive immunostaining. Since tumor cell lines arise from the clonal expansion of a single neoplastic cell, it may not be surprising that most cell lines derived from such melanomas will fail to overexpress *p53*. Secondly, it is likely that in some cases overexpression of *p53* is the result of epigenetic phenomena and is not a consequence of a mutation in the coding sequence of this gene. Consistent with this possibility, it is noteworthy that elevated levels of p53 protein have been detected immunohistochemically in a patient with familial breast cancer who showed no evidence of a mutation in the p53 coding sequence (25). Abnormal overexpression of this protein could arise as a result of increased mRNA levels. Alternatively, the p53 protein could be stabilized by binding to other gene products such as MDM2 which regulate p53 functioning (26).

The lack of detectable *p53* expression in preneoplastic melanocytic lesions derived from patients at high risk for the development of melanoma indicates that immunohistochemical evidence of p53 cannot be used as a biomarker to predict the subsequent development of melanoma. However, our findings that expression of p53 is associated with deep primary tumors and metastases suggests that overexpression of this protein may be of prognostic significance in this neoplasm. The level of invasion of the primary melanoma has been inversely correlated with survival and it is possible that evidence of p53 expression may identify a subgroup of patients at particular risk for the development of metastatic disease. Additional studies with a larger series of patients will be required to address this question.

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