Selective Loss of Wild-Type p16\(^{INK4a}\) Expression in Human Nevi

Journal of Investigative Dermatology (2011) **131**, 2329-2332; doi:10.1038/jid.2011.197; published online 7 July 2011

TO THE EDITOR
The p16\(^{INK4a}\) cyclin-dependent kinase inhibitor acts as a negative regulator of cyclin D-dependent kinases and is a critical gatekeeper at the G1-S checkpoint (Serrano et al., 1996). Accordingly, p16\(^{INK4a}\) is frequently inactivated in human tumors, and deletions involving this locus occur frequently in melanomas. Inherited mutations in the p16\(^{INK4a}\) gene are also associated with melanoma susceptibility in 40% of multiple-case melanoma families (Goldstein et al., 2006). It is generally acknowledged that the progressive and gradual

L. Scurr et al.
Loss of p16\(^{INK4a}\) in Human Nevi


Selective Loss of Wild-Type p16\(^{INK4a}\) Expression in Human Nevi

Journal of Investigative Dermatology (2011) **131**, 2329-2332; doi:10.1038/jid.2011.197; published online 7 July 2011

TO THE EDITOR
The p16\(^{INK4a}\) cyclin-dependent kinase inhibitor acts as a negative regulator of cyclin D-dependent kinases and is a critical gatekeeper at the G1-S check-
loss of $p16^{INK4a}$ expression correlates with the advancing stages of melanocytic disease progression. Thus, benign nevi show minimal loss of the $p16^{INK4a}$ gene, whereas allelic loss of this locus is common in dysplastic nevi, and in primary and metastatic melanomas (Talve et al., 1997; Sini et al., 2008).

Similarly, there is a progressive decrease in the expression level of $p16^{INK4a}$ protein from melanoma in situ to invasive and metastatic melanomas (Keller-Melchior et al., 1998; Sini et al., 2008).

The repression of $p16^{INK4a}$ expression is likely to commence earlier than disease manifestation, occurring during melanocytic proliferation, in benign and atypical nevi. Initial reports described qualitatively uniform immunohistochemical labelling for $p16^{INK4a}$ in nevi (Keller-Melchior et al., 1998; Sini et al., 2008). Reduced $p16^{INK4a}$ expression in nevi indicates that $p16^{INK4a}$ may contribute to the clonal expansion of nevus cells. This is supported by the clinical observation that patients with heterozygous $p16^{INK4a}$ mutations often develop larger, more numerous, and dysplastic nevi (Bishop et al., 2000). To precisely examine the expression of $p16^{INK4a}$ in nevi, we initially determined the percentage of nevus cells expressing detectable $p16^{INK4a}$ in 20 excised human nevi, comprising 15 compound nevi and 5 dysplastic nevi.

As shown in Figure 1, all nevi displayed a heterogenous pattern of $p16^{INK4a}$ immunopositivity, and although the sample size was small we achieved borderline statistical significance on comparing $p16^{INK4a}$ expression in compound versus dysplastic nevi (Mann-Whitney test; $P = 0.053$; Figure 1b).

Considering that normal melanocytes at the dermal-epidermal junction have undetectable levels of $p16^{INK4a}$ (data not shown; Michaloglou et al., 2005; Gray-Schopfer et al., 2006). Reduced $p16^{INK4a}$ expression in nevi indicates that $p16^{INK4a}$ may contribute to the clonal expansion of nevus cells. This is supported by the clinical observation that patients with full-length recombinant $p16^{INK4a}$ protein, is frequently used for immunohistochemical detection of $p16^{INK4a}$ in human cancers (Redman et al., 2008). Using melanocytic nevi from melanoma-prone individuals from a single family (ID 31220) carrying a germline $p16^{INK4a}$ mutation encoding the R24P variant (Holland et al., 1995), we were able to examine whether wild-type $p16^{INK4a}$ expression was selectively lost in nevus cells. In all, 11 nevi (8 compound nevi and 3 dysplastic nevi) were derived from five R24P carriers and 19 nevi (15 compound nevi and 4 dysplastic nevi) were excised from age-matched melanoma-affected controls, with no known family history of melanoma. The nevi ranged in size from 2 to 22 mm for the controls and from 3 to 20 mm for the R24P carriers. The indication for excision was clinical change within a clinically dysplastic (atypical) nevus to exclude melanoma (with low clinical suspicion of malignancy) in the majority of cases.

We used dual immunofluorescence with JC8 and C20 on paraffin-embedded sections of nevi; the C20 antibody detects both wild-type and mutant $p16^{INK4a}$ (Figure 2). R24P-positive nevus cells expressing any combination

Figure 1. **$p16^{INK4a}$ expression in human melanocytic tumors.** $p16^{INK4a}$ expression was determined using immunohistochemical analysis of 20 benign nevi. (a) Representative examples of compound nevi stained with $p16^{INK4a}$ antibody (N20; Santa Cruz, Santa Cruz, CA) and positive cells detected using Permanent Red (DAKO, Glostrup, Denmark). Bar = 50 μm. (b) The results for 15 compound and 5 dysplastic nevi are represented graphically. Horizontal bars indicate the median $p16^{INK4a}$ expression values.
of R24P and wild-type p16\textsuperscript{INK4a}, stained positively with C20, while the JC8 antibody stained positively only in cells in which wild-type p16\textsuperscript{INK4a} expression was retained (see Figure 2b). The distribution of p16\textsuperscript{INK4a} was both nuclear and cytoplasmic in all p16\textsuperscript{INK4a}-positive nevus samples. In R24P-positive-only cells (JC8\textsuperscript{−}/C0\textsuperscript{−}/C20\textsuperscript{+}) the distribution of mutant p16\textsuperscript{INK4a} varied and showed nuclear and cytoplasmic, predominantly cytoplasmic, or predominantly nuclear localization (data not shown). As expected, nearly all p16\textsuperscript{INK4a}-positive control nevus cells stained positively with both the JC8 and C20 antibodies. In contrast, a substantial proportion of nevus cells from R24P carriers showed strong positivity with only the C20 antibody (i.e. no JC8 positivity was seen) (Figure 2c) and thus, these nevus cells were negative for wild-type p16\textsuperscript{INK4a} expression. As shown in Figure 2d, there was a highly significant difference between the control nevi and R24P-positive nevi (Mann-Whitney test; \( P < 0.001 \)); in the latter, wild-type p16\textsuperscript{INK4a} expression was selectively lost in a subset (up to 31\%) of nevus cells. These data confirm that the loss of wild-type p16\textsuperscript{INK4a} expression commences early in melanocyte proliferation, with common benign nevi frequently containing cells with no p16\textsuperscript{INK4a}. 

**Figure 2.** Selective loss of wild-type p16\textsuperscript{INK4a} expression in human nevi. The activity of C20 and JC8 p16\textsuperscript{INK4a} antibodies was examined using stable melanoma clones with IPTG-inducible expression of wild-type p16\textsuperscript{INK4a}, R24P, or both (McKenzie et al., 2010) in (a) western analysis or (b) dual fluorescence staining of paraffin-embedded cell pellet sections derived from the indicated IPTG-induced melanoma clones. (c) Representative nevi from control individuals or individuals carrying a germline R24P mutation were stained as indicated. Nuclei were stained with DAPI. Arrows indicate C20-positive/JC8-negative nevus cells. Bar = 20 μm. (d) Percent C20-positive/JC8-negative nevus cells (control nevi, \( n = 19 \); R24P nevi, \( n = 11 \)). Horizontal bars indicate median expression values; the control median was 0. AF-488, Alexa Fluor 488; AF-594; Alexa Fluor 594; DAPI, 4′,6-diamidino-2-phenylindole; IPTG, isopropyl β-D-thiogalactopyranoside.
The mosaic pattern of p16INK4a expression in acquired nevi is reminiscent of the heterogeneity of B-RAF mutations found in human nevi (Lin et al., 2009). It remains to be determined whether the mutant B-RAF is coexpressed with p16INK4a or if activated B-RAF and loss of p16INK4a contribute separately to the clonal expansion of melanocytes. Alternatively, activated B-RAF and p16INK4a deficiency may co-exist in a small percentage of nevus cells to enhance melanocyte proliferation and drive neoplastic transformation. The absence of a mutant-specific B-RAF antibody has so far precluded the execution of such an analysis. It is well established that activated B-RAF can promote nevus formation in murine and fish melanoma models (Patton et al., 2005; Goel et al., 2009), and the arrested state of nevi does not appear to require p16INK4a (Dhomen et al., 2009). Nevertheless, the influence of p16INK4a loss on the development of B-RAFV600E-induced nevi needs to be investigated in human melanocytic tumors. This is particularly relevant as p16INK4a loss regulates the penetrance and latency of B-RAF-induced murine melanomas (Dhomen et al., 2009).

CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
This work was supported by program grant 402761 of the National Health and Medical Research Council of Australia (NHMRC), the Cancer Institute NSW, Cancer Council NSW, and an infrastructure grant to the Westmead Millennium Institute by the Health Department of NSW through the Sydney West Area Health Service. The Westmead Institute for Cancer Research is the recipient of capital grant funding from the Australian Cancer Research Foundation. HR is a recipient of a Cancer Institute NSW Research Fellowship and an NHMRC Senior Research Fellowship. RAS is a Cancer Institute NSW Clinical Research Fellow.

Lyndee L. Scurr1, Heather A. Mckenzie1, Therese M. Becker1, Mal Irvine1, Ken Lai2, Graham J. Mann1,2, Richard A. Scolyer2,3,4 Richard F. Kefford2,3 and Helen Rizos1
1Westmead Institute for Cancer Research and Melanoma Institute of Australia, University of Sydney at Westmead Millennium Institute, Westmead Hospital, Westmead, New South Wales, Australia; 2Melanoma Institute Australia, Sydney, New South Wales, Australia; and 3Department of Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, Sydney, New South Wales, Australia and 4Discipline of Pathology, Sydney Medical School, University of Sydney, Sydney, New South Wales, Australia
E-mail: helen.rizos@sydney.edu.au

REFERENCES
Patton EE, Widlund HR, Koutok JL et al. (2005) BRAF mutations are sufficient to promote nevus formation and cooperate with p53 in the genesis of melanoma. Curr Biol 15: 249–54

TO THE EDITOR
UVB is the main factor in skin cancer (Bath-Hextall et al., 2007). Low-dose UBV (280–320 nm) radiation is sufficient for cutaneous photosynthesis of previtamin D from 7-dehydrocholesterol (Tian and Holick, 1999). Some epidemiologic studies suggest that a poor vitamin D status increases the risk for several cancers and autoimmune diseases (Holick, 2008). Vitamin D activity in peripheral tissues, such as