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Advances in Brief

BRAF Oncogenic Mutations Correlate with Progression rather than Initiation of Human Melanoma[1]

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Abstract

***BRAF* oncogenic mutations have been identified in significant numbers of melanocytic lesions. To correlate *BRAF* mutation and melanoma progression, we screened *BRAF* mutations in 65 melanocytic lesions, including nevi, radial growth phase (RGP), vertical growth phase (VGP) melanomas, and melanoma metastases, as well as 25 melanoma cell lines. PCR and direct sequencing were used to analyze DNA samples extracted from laser capture microdissected tissues. A similar high frequency (62–72%) of *BRAF* oncogenic mutations was identified in melanocytic nevi, VGP, metastatic melanomas, and melanoma cell lines [H. Davies *et al.*, Nature (Lond.), *417:* 949–954, 2002; P. M. Pollock *et al.*, Nat. Genet., *33:* 19–20, 2002; and M. S. Brose *et al.*, Cancer Res., *62:* 6997–7000, 2002]. In striking contrast, we found *BRAF* lesions in only 10% of the earliest stage or RGP melanomas. These findings imply that *BRAF* mutations cannot be involved in the initiation of the great majority of melanomas but instead reflect a progression event with important prognostic implications in the transition from the great majority of RGP melanomas to VGP and/or metastatic melanoma.**

Introduction

BRAF encodes a serine/threonine kinase that acts in the MAPK[3] pathway to transduce regulatory signals from RAS through MEK (MAPK kinase) to MAPK. Recently, mutations in *BRAF* have been reported at high frequency ranging from 59 to 80% in melanoma samples, including tumor cell lines, short-term cultures, and tumor tissues (1). A T1796A transversion in exon 15, resulting in a V599E missense mutation, accounts for >90% of mutations detected in melanoma samples (1). The V599E mutation has been shown to increase BRAF kinase activity and to cause it to acquire transforming activity in the NIH3T3 transfection assay (1). Pollock *et al.* (2) recently reported that the incidence of *BRAF*-activating mutations in nevi, including congenital, acquired, compound, and dysplastic, ranged from 70 to 88%, consistent with the hypothesis that this mutation represents the initiation step in melanocytic neoplasia. However, the involvement of *BRAF* mutations in the earliest stage of melanoma designated RGP was not reported. Such tumors are generally confined to the epidermis or with microinvasion into the dermis and represent frank but low-grade malignancy. The present studies were undertaken in an effort to elucidate whether mutational activation of *BRAF* correlates with initiation or progression of human melanoma.

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[3] The abbreviations used are: MAPK, mitogen-activated protein kinase; RGP, radial growth phase; VGP, vertical growth phase; LCM, laser capture microdissection.

Materials and Methods

Tumor Samples and Melanoma Cell Lines. Formalin-fixed and paraffin-embedded tissues, including 24 melanocytic nevi, 20 RGPs, 8 VGPs, and 13 melanoma metastases, were obtained from the Pathology Department of Mount Sinai School of Medicine. The diagnosis of a melanocytic nevus was based on a well-established constellation of criteria. The epidermis and dermis contained a symmetrical ordered, sharply demarcated melanocytic proliferation. The melanocytes were present as small clusters in the epidermis or dermis, exhibited minimal cytological atypia, and did not show significant mitotic figures. The melanocytes also showed changes in response to the microenvironment in that their nuclei became smaller and more neuroid with descent deeper in the dermis (maturation), and there was more pigment superficially. The diagnosis of RGP and VGP melanoma was made according to well-established criteria. RGP melanomas consisted of cases in which the melanoma cells were confined either to the epidermis only (malignant melanoma *in situ* RGP) or confined to the superficial papillary dermis (malignant melanoma microinvasive RGP). By definition, if the cells entered the dermis, the nests in the dermis could not be larger than the largest intraepidermal cluster and could not exhibit mitoses. The VGP melanoma was defined by the presence of an invasive dermal component in which the cells in the dermis were larger than the largest intraepidermal cluster of melanocytes or showed significant mitotic activity. Other features that were helpful in establishing the VGP diagnosis included that the cells in the dermis were more morphologically atypical, irregular shaped, or showed foci of necrosis, *i.e.,* cytologic evidence that a new, potentially tumorigenic clone of melanocytes was forming (5).

In addition, 25 human melanoma cell lines obtained from American Type Culture Collection, Dr. Meenhard Heryln (Wistar Institute, Philadelphia, PA), or established by us were included in the study.

LCM and DNA Extraction. Formalin-fixed and paraffin-embedded tissue sections were histologically evaluated for the presence of melanocytic lesions, including melanomcytic nevus, RGP, VGP, and metastatic melanomas. Pure populations of cells were carefully microdissected using a PixCell II Laser Capture Microdissection System (Arcturus, Mountain View, CA) according to the manufacturer's instructions. A total of >200 cells was collected in each case from serial tissue sections. Collected cells were transferred to an Eppendorf tube and resuspended in 20–50 μl of lysis buffer containing 10 mM Tris, 1 mM EDTA, 0.5% Tween 20 (pH 8.3), and 5 μl of proteinase K (20 mg/ml). Samples were incubated 1–2 days at 55°C followed by boiling for 10 min to inactivate proteinase K. Microdissection and DNA extraction were repeated as required. The Puregene Cell and Tissue Kit was used to extract DNA from cultured cells according to the manufacturer's protocol (Gentra, Minneapolis, MN).

PCR and Direct Sequencing. *BRAF* exon 15, *NRAS* exon 2, and exon 3 were PCR amplified using forward and reverse primer sequences as described previously (1). PCR amplification was carried out with genomic DNA in a volume of 50 μl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphate, 50 pmol of forward and reverse primers, 20 μg/ml BSA, 5 units of TaqDNA polymerase (Invitrogen, Carlsbad, CA), and 3–10 μl of crude DNA extract of microdissected cells or 100 μg of purified DNA from cultured cells. PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The purified PCR products were directly sequenced on both strands using a Prism Model 3700 Capillary Array Sequencer and Big Dye Terminator Chemistry (Applied Biosystems, Foster City, CA). Sequence analysis was based on *BRAF* and *NRAS* cDNA sequences (GenBank access nos. NM_004333 for *BRAF*, and NM_002524 for *NRAS*).

Results and Discussion

We screened *BRAF* exon 15 sequences in LCM RGP melanomas (Fig. 1*A*) and compared results with a similar analysis of VGP melanomas, which commonly arise within the RGP and exhibit a high potential for metastatic spread, as well as metastatic melanomas and melanoma cell lines. The frequencies of *BRAF* mutations in nevi, VGP, and metastatic melanomas were high, ranging from 62 to 71% (Table 1). However, RGP melanomas exhibited *BRAF* mutations in only 2 of 20 (10%) analyzed. *NRAS*-activating lesions are also found in melanomas (1, 2, 5, 6), but do not generally overlap with *BRAF* mutations in the same lesion (1, 2, 5). Thus, we also sequenced *NRAS* exon 2 and exon 3 surrounding codons 12, 13, and 61, the hot spots for oncogenic-activating mutations (1, 2, 5, 6), in all *BRAF* wild-type samples. *NRAS* mutations were not detected in RGP samples but were found in other lesions and increased the combined *BRAF* and *NRAS* mutation frequencies to 75% in VGP, 69% in metastasis, and 84% in melanoma cell lines (Table 1). The great disparity in the frequency of *BRAF* mutations in nevi and RGP melanomas was highly statistically significant (Fisher's exact test, $P < 0.00006$). The difference between the frequency of *BRAF* mutations in VGP (63%) or metastatic melanoma (62%) and RGP melanoma was also statistically significant whether compared separately ($P < 0.01$ or $P < 0.005$) or pooled ($P < 0.001$).

As has been recently reported (1, 2, 5), we observed (Fig. 1*B*) that the great majority of *BRAF* mutations identified were T1796A transversions, which change the wild-type codon GTG (valine) to GAG (glutamate) at position 599 (V559E). In several tested cases, these mutations were not present in normal cells from the same individuals, indicating that they were somatically acquired. One VGP melanoma and one metastasis (Fig. 1*B*) showed the same two-nucleotide substitution, which changes the wild-type codon GTG (valine) to AAG (lysine), at position 599 (V599K). To examine the transforming ability of the V599E and V599K *BRAF* mutants, pCEV29 expression constructs for ^{T1796A}*BRAF*, $^{GT1795–1796AA}$*BRAF*, and $^{wild\text{-}type}$*BRAF* cDNAs were transfected into NIH3T3 cells. Comparable transforming activities were observed for the two mutants (~5000 foci/μg DNA compared with <1.0 focus/μg DNA for wild-type *BRAF*). Mass cultures of marker selected NIH3T3-transformed cells with either ^{T1796A}*BRAF* or $^{GT1795–1796AA}$*BRAF* produced rapidly growing tumors in SCID mice (data not shown). Mutations in *BRAF* V599 codon have been shown to create an acidic or basic amino acid substitution, V599E, D, K, or R (1, 2, 5), and our results show that either type of substitution can comparably activate BRAF. We also identified loss of heterozygosity in 3 metastatic melanomas, including one with a V599K mutation (Fig. 1*B*), and in 16 of 18 (89%) melanoma cell lines carrying *BRAF* mutations (data not shown). It is possible that deletion of the wild-type allele represents an additional genetic selection in aggressive melanoma cells.

Table 1 BRAF *mutation frequencies in different stages of melanoma progression*

Sample	*BRAF* mutation frequency	*NRAS* mutations in *BRAF* wild-type lesions	Combined mutation frequency
Melanocytic nevi (benign)	17/24	0/7	71%
RGP (early melanoma)	2/20	0/18	10%
VGP (invasive melanoma)	5/8	1/3	75%
Metastatic melanoma	8/13	1/5	69%
Melanoma cell lines	18/25	3/7	84%

Although our present results confirm the high frequency of *BRAF* mutations both in nevi and later stage melanomas, our demonstration that early stage (RGP) melanomas exhibit a very low frequency of *BRAF* mutation argues strongly that *BRAF* mutation cannot be involved in the initiation of the great majority of melanomas but instead reflects a progression event. There are profound differences between RGP and VGP melanoma cells at the cellular and functional level. In RGP, neoplastic cells spread in the epidermis or invade dermis only as single cells or small clusters of nonproliferating tumor cells. In VGP, cancer cells expand in the dermis and generate tumor nodules. Unlike VGP cells, RGP melanoma cells are difficult to maintain in long-term tissue culture, have low colony-forming ability, are nontumorigenic in nude mice, and do not have the competence to metastasize (7, 8). When melanoma is diagnosed as RGP, the disease can be cured by proper surgical excision. However, tumors in VGP have acquired metastatic capability and may be resistant to current methods of therapy. Therefore, the distinction between RGP *versus* VGP has

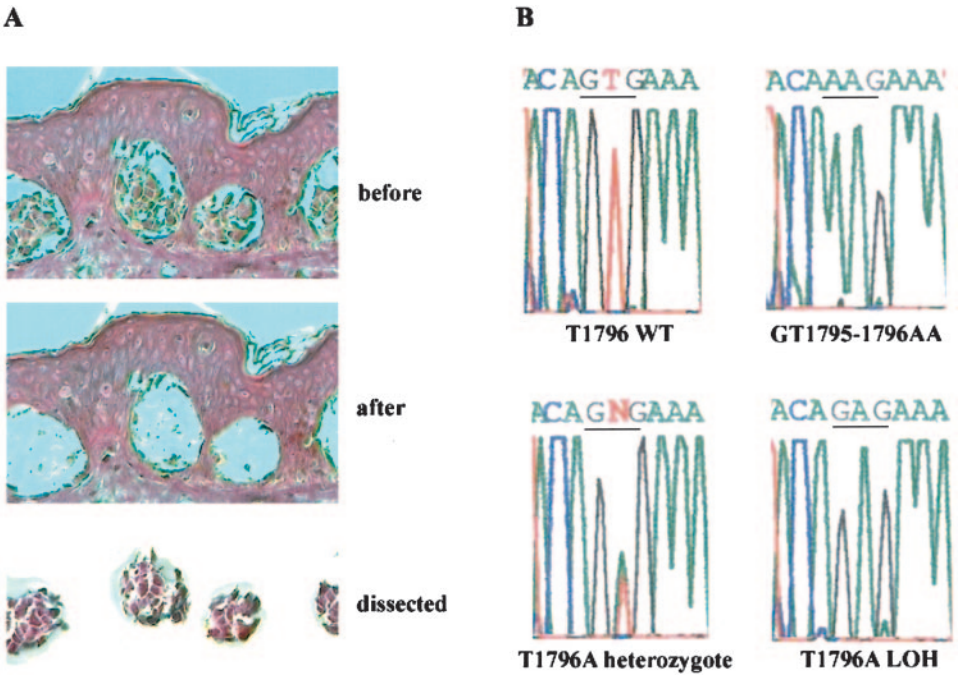

Fig. 1. Microdissection of RGP melanoma and mutations in the *BRAF* gene. *A*, representative LCM of a RGP melanoma. Before, after microdissection, and dissected tumor cells. LCM achieved >80% tumor cell population in each case. *B*, mutations in the *BRAF* codon 599, wild-type T1796, heterozygous T1796A mutation, 2-base GT1795-1796AA substitution, and loss of heterozygosity of T1796A mutation. Formalin-fixed and paraffin-embedded tissue sections were evaluated by histopathology for the presence of melanocytic nevus, RGP, VGP, and metastasis. The sensitivity of the mutation analysis was examined using mixtures of different ratios of *BRAF* wild-type and mutant alleles. It was possible to detect the *BRAF* mutation in a 3:1 mixture of wild-type and heterozygous mutant DNA.

profound prognostic and therapeutic implications. Our present findings strongly imply that *BRAF* mutations most commonly represent genetic progression events in melanomas and thus may serve as biomarkers for disease progression in the important clinical distinction between RGP and VGP melanomas.

It should be noted that comparable *BRAF* mutations frequencies of 86, 88, 70, and 80% were reported in various nevi specimens, including congenital, intradermal, compound, and dysplastic (also termed atypical; Ref. 2), suggesting that *BRAF* mutations do not likely contribute to possible differences in the propensity to progress to melanoma among these nevi groups. There are a very large number of melanocytic nevi in the general population compared with the relatively low incidence of melanoma (7–9). It is known clinically that nevi very often regress over time. In tissue culture, oncogenically activated *RAS* or *RAF* has been shown to induce permanent growth arrest/senescence rather than unrestricted proliferation in normal fibroblasts through a mechanism involving p53, $p21^{Cip1}$, or p16 upregulation (10–13). For example, RAS or RAF oncogenic stress mediated by extracellular signal-regulated kinase leads to increased alternative reading frame (ARF) at the level of transcription and ARF inactivates mouse double minute 2 homolog (MDM2), which targets p53 for proteosomal degradation (14, 15). Thus, such stress upregulates p53 and its transcriptional target, $p21^{Cip1}$, which is growth inhibitory to cells (16, 17). There are also reports that $p21^{Cip1}$ upregulation plays an important role in the terminal differentiation of a number of cell types (18). Whether this explains the absence of progression of the vast majority of *BRAF* mutation containing nevi to melanoma awaits additional study.

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References

1. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G. J., Bigner, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., and Futreal, P. A. Mutations of the BRAF gene in human cancer. Nature (Lond.), *417:* 949–954, 2002.
2. Pollock, P. M., Harper, U. L., Hansen, K. S., Yudt, L. M., Stark, M., Robbins, C. M., Moses, T. Y., Hostetter, G., Wagner, U., Kakareka, J., Salem, G., Pohida, T., Heenan, P., Duray, P., Kallioniemi, O., Hayward, N. K., Trent, J. M., and Meltzer, P. S. High frequency of BRAF mutations in nevi. Nat. Genet., *33:* 19–20, 2002.
3. Brose, M. S., Volpe, P., Feldman, M., Kumar, M., Rishi, I., Gerrero, R., Einhorn, E., Herlyn, M., Minna, J., Nicholson, A., Roth, J. A., Albelda, S. M., Davies, H., Cox, C., Brignell, G., Stephens, P., Futreal, P. A., Wooster, R., Stratton, M. R., and Weber, B. L. BRAF and RAS mutations in human lung cancer and melanoma. Cancer Res., *62:* 6997–7000, 2002.
4. Guerry, D., IV, Synnestvedt, M., Elder, D. E., and Schultz, D. Lessons from tumor progression: the invasive radial growth phase of melanoma is common, incapable of metastasis, and indolent. J. Investig. Dermatol., *100:* 342S–345S, 1993.
5. Crowson, A. N., Magro, C. M., and Mihm, M. C. The melanocytic proliferations. A comprehensive textbook of pigmented lesions, pp. 281–398, New York: Wiley-Liss, 2001.
6. Jiveskog, S., Ragnarsson-Olding, B., Platz, A., and Ringborg, U. N-Ras mutations are common in melanomas from sun-exposed skin of humans but rare in mucosal membranes or unexposed skin. J. Investig. Dermatol., *111:* 757–761, 1998.
7. Clark, W. H., Jr., and Tucker, M. A. Problems with lesions related to the development of malignant melanoma: common nevi, dysplastic nevi, malignant melanoma *in situ*, and radial growth phase malignant melanoma. Hum. Pathol., *29:* 8–14, 1998.
8. Elder, D. Tumor progression, early diagnosis and prognosis of melanoma. Acta Oncol., *38:* 535–547, 1999.
9. Kanzler, M. H., and Mraz-Gernhard, S. Primary cutaneous malignant melanoma and its precursor lesions: diagnostic and therapeutic overview. J. Am. Acad. Dermatol., *45:* 260–276, 2001.
10. Zhu, J., Woods, D., McMahon, M., and Bishop, J. M. Senescence of human fibroblasts induced by oncogenic Raf. Genes Dev., *12:* 2997–3007, 1998.
11. Lin, A. W., Barradas, M., Stone, J. C., van Aelst, L., Serrano, M., and Lowe, S. W. Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. Genes Dev., *12:* 3008–3019, 1998.
12. Sewing, A., Wiseman, B., Lloyd, A. C., and Land, H. High-intensity Raf signal causes cell cycle arrest mediated by p21Cip1. Mol. Cell. Biol., *17:* 5588–5597, 1997.
13. Woods, D., Parry, D., Cherwinski, H., Bosch, E., Lees, E., and McMahon, M. Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip1. Mol. Cell. Biol., *17:* 5598–5611, 1997.
14. Groth, A., Weber, J. D., Willumsen, B. M., Sherr, C. J., and Roussel, M. F. Oncogenic Ras induces p19ARF and growth arrest in mouse embryo fibroblasts lacking p21Cip1 and p27Kip1 without activating cyclin D-dependent kinases. J. Biol. Chem., *275:* 27473–27480, 2000.
15. Zhang, Y., and Xiong, Y. Control of p53 ubiquitination and nuclear export by MDM2 and ARF. Cell Growth Differ., *12:* 175–186, 2001.
16. Levine, A. J. p53, the cellular gatekeeper for growth and division. Cell, *88:* 323–331, 1997.
17. Vogelstein, B., Lane, D., and Levine, A. J. Surfing the p53 network. Nature (Lond.), *408:* 307–310, 2000.
18. Boulaire, J., Fotedar, A., and Fotedar, R. The functions of the cdk-cyclin kinase inhibitor p21WAF1. Pathol. Biol., *48:* 190–202, 2000.

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