High frequency of *BRAF* mutations in nevi

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To evaluate the timing of mutations in *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) during melanocytic neoplasia, we carried out mutation analysis on microdissected melanoma and nevi samples. We observed mutations resulting in the V599E amino-acid substitution in 41 of 60 (68%) melanoma metastases, 4 of 5 (80%) primary melanomas and, unexpectedly, in 63 of 77 (82%) nevi. These data suggest that mutational activation of the RAS/RAF/MAPK pathway in nevi is a critical step in the initiation of melanocytic neoplasia but alone is insufficient for melanoma tumorigenesis.

Melanoma incidence and mortality rates in fair-skinned populations are increasing worldwide. Epidemiological data suggest that high numbers of benign acquired nevi and dysplastic nevi are associated with higher risk of melanoma[1]. Histopathological analysis also indicates that a proportion of melanomas show evidence of a histologically contiguous nevus. Melanocytic nevi can be present at birth (congenital) or arise during an individual's lifetime (common acquired or dysplastic). Conceptually, the premise that all nevi are premalignant is controversial, owing in part to the clinical and histopathological heterogeneity of these lesions. Histopathologists classify melanocytic nevi on the basis of tissue architectural patterns. Nevi may be defined as junctional (lesional cells at the epidermal–dermal interface), intradermal (cells located exclusively in the papillary or reticular dermis), compound (cells in both epidermis or junction and dermis) or congenital (specialized dermal patterns)[2]. The dysplastic or atypical nevus also bears junctional architecture but, unlike other nevi, shows a degree of nuclear atypia[2].

Recently, mutations in *BRAF* have been reported in 38 of 58 melanoma samples, primarily cell lines and short-term cultures[3]. *BRAF* encodes a serine/threonine kinase that acts in the MAPK pathway to transduce regulatory signals from RAS to MEK1/2. Activation of the MAPK pathway, through both receptor tyrosine-kinases and G-protein–coupled receptors, has a central role in melanocyte proliferation[4,5]. A 1796T→A transversion in exon 15 of *BRAF*, resulting in a V599E amino-acid missense mutation, accounts for 92% of the mutations detected in melanoma samples[3]. The $BRAF^{V599E}$ mutant possesses tenfold greater basal kinase activity and induces focus formation in NIH3T3 cells 138 times more efficiently than does wild-type BRAF[3].

To establish a base frequency of mutations in melanoma biopsies and to determine the timing of *BRAF* mutations in melanoma pathogenesis, we carried out mutation analysis of only exon 15 on individual biopsy samples obtained from both tumors and nevi. We used a melanoma progression tissue microarray[6] constructed with a robotic arrayer and laser-microdissected the relevant tissue biopsy samples. The array contained 325 unique tissue samples from distinct stages of melanoma progression, including congenital, acquired and dysplastic nevi, primary melanomas and melanoma metastases from a variety of body sites. Given the prevalence of the 1796T→A (V599E) mutation, we designed two sets of allele-specific primers (Fmutant and Rmutant) to amplify and sequence exon 15 of *BRAF* (primer sequences and PCR conditions available from authors upon request).

We identified the prevalent 1796T→A missense mutation by both allele-specific PCR (AS–PCR) and sequencing in metastatic melanomas obtained from 37 of 55 (67%) individuals (Table 1). We identified three additional tandem mutations in exon 15 by sequence analysis: a GT→AA resulting in V599K substitution in two metastases; a GT→AG resulting in V599R substitution in one metastasis; and a TG→AT resulting in a V599D substitution in another metastasis. The change to a basic rather than an acidic amino acid in three metastases raises the possibility that the substitution of either a positively or negatively charged amino acid may result in activation of BRAF.

To investigate the timing of BRAF activation, we analyzed a series of primary melanomas, dysplastic nevi and benign nevi in a similar fashion. Although only a small number of primary melanomas and dysplastic nevi yielded analyzable DNA, we found the V599E mutation in 4 of 5 (80%) cases of each lesion. This rate is similar to that seen in metastases (Table 1). A large number of intradermal, congenital and compound nevi were analyzed, including 36 from the tissue microarray and an additional 8 samples microdissected from conventional paraffin blocks. Notably, we observed the V599E activating mutation in 39 of 44 (89%) of these benign nevi (Fig. 1 and Table 1). The *BRAF* exon 15 sequence obtained from all 44 samples was in agreement with the AS–PCR results in all but 4 cases (in which the more sensitive AS–PCR returned a positive finding), indicating that the proportion of mutant cells was high in the majority of samples. We identified one TG→AA tandem substitution, also resulting in a V599E mutation, in a congenital nevus, but no other mutations in exon 15 were identified. For ten samples, microdissection of adjacent nevus cells from the original donor block confirmed the results obtained from the microdissected tissue microarray samples. In two nevi carrying *BRAF*-activating mutations, analysis of adjacent normal skin indicated that the mutations had arisen somatically.

To confirm the high frequency of *BRAF* mutations in nevi, we carried out PCR amplification and subsequent sequencing on a different cohort of nevi in another laboratory. We detected the 1796 T→A mutation in 14 of 17 intradermal nevi and 10 of 16 compound nevi from Australian samples. The mutation was not present in

Table 1 • Frequency of *BRAF* mutation resulting in V599E substitution in melanocytic nevi and melanomas

	Tumor type	*BRAF* (V599E)
Nevi	Congenital	6/7 (86%)
	Intradermal	37/42[a] (88%)
	Compound	16/23[b] (70%)
	Dysplastic	4/5[b] (80%)
	Total	63/77 (82%)
Melanoma	Primary	4/5[c] (80%)
	Dermal/subcutaneous metastases	18/29 (63%)
	Lymph metastases	10/12 (83%)
	Distant organ metastases	9/14 (64%)
	Total	41/60 (68%)

AS–PCR indicated that the mutated allele was present, but it was undetectable by sequencing in [a]two samples, [b]one sample each and [c]three samples.

the germ line in eight cases for which adjacent normal skin was available.

Considering the closely related functions of BRAF and NRAS in the MAPK pathway, we investigated samples for which sufficient DNA was available for activating mutations in exons 1 and 2 of *NRAS* (neuroblastoma RAS viral (v-ras) oncogene homolog), the *RAS* gene most commonly mutated in melanoma. We detected activating mutations in *NRAS* in 7 of 11 (64%) metastases carrying a wild-type BRAF but in none of 13 tumors carrying the V599E mutation. Mutations in *NRAS* were also seen in 5 of 36 (14%) nevi, 2 congenital and 3 intradermal. Notably, we observed activating mutations in *NRAS* not only in 2 of 4 (50%) nevi with wild-type BRAF but also in 3 of 32 (9%) nevi carrying the $BRAF^{V599E}$ mutant. The concomitant activation of both BRAF and NRAS was only observed in samples microdissected from original paraffin blocks, and is probably due to their occurrence in distinct cell populations that were not resolved by our microdissection technique.

In conclusion, our finding of *BRAF* mutations in 63 of 77 (82%) histologically diverse nevi implicates mutation of this gene and activation of the RAS/RAS/MAPK pathway as a crucial step in the initiation of melanocytic neoplasia. These data also demonstrate that BRAF activation alone is insufficient for the development of melanoma, highlighting the requirement for additional molecular defects during the progression to invasive melanoma. Notably, melanocytic nevi can be relatively indolent for decades despite the presence of activating BRAF mutations. It will be important to elucidate the mechanisms responsible for this behavior, in addition to the molecular events necessary to transform a nevus containing a *BRAF* mutation into a melanoma.

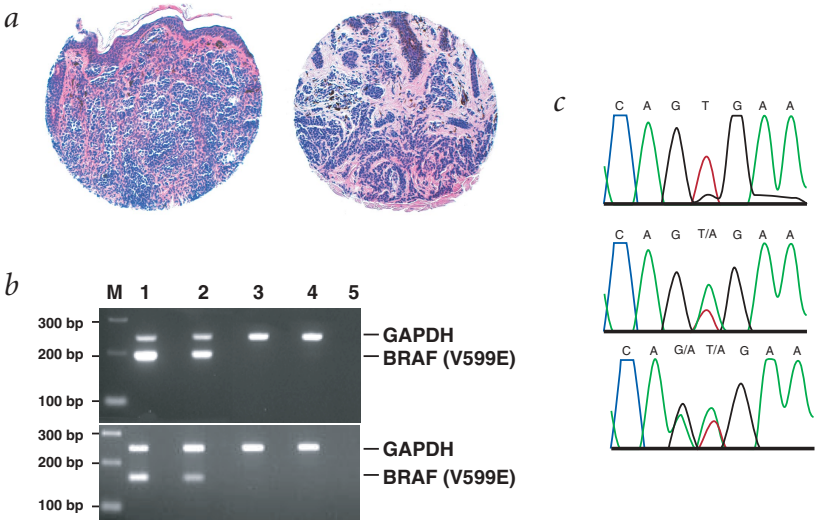

Fig. 1 Identification of *BRAF* mutations in microdissected nevi. ***a***, Representative core biopsies 0.6 μm in diameter on the melanoma progression tissue microarray. Left panel, intradermal nevus; right panel, compound nevus. ***b***, Duplex PCR comprising *GAPD* (upper band) and allele-specific primers to detect the 1796 T→A mutation in exon 15 of *BRAF* (lower band in both panels). Upper panel, *GAPD* duplexed with Fmutant allele-specific primer set. Lower panel, *GAPD* duplexed with the Rmutant allele-specific primer set. M, 100-bp marker (Invitrogen); lanes 1,2, intradermal nevi; lane 3, compound nevus; lane 4, congenital nevus; lane 5, water. ***c***, Sequence chromatographs from *BRAF* exon 15 showing wild-type sequence (top), 1796 T→A transversion resulting in a V599E substitution (from intradermal nevus; middle) and a 1795/96 GT→AA tandem mutation resulting in a V599K substitution (from a melanoma metastasis; bottom).

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Competing interests statement

The authors declare that they have no competing financial interests.

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