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BRIEF COMMUNICATION

Clonal *BRAF* Mutations in Melanocytic Nevi and Initiating Role of *BRAF* in Melanocytic Neoplasia

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***BRAF*^{V600E} mutations are frequent in melanomas originating from intermittently sun-exposed skin and also in common acquired melanocytic nevi, suggesting that *BRAF* mutation is an early event in melanocytic neoplasia. All neoplastic melanocytes within such a nevus would be expected to carry the *BRAF* mutation, and thus we evaluated the frequency of cells with *BRAF*^{V600E} mutations within acquired nevi by droplet digital polymerase chain reaction. In *BRAF*-mutant nevi the number of *BRAF* mutant alleles equaled the number of wild-type (WT) alleles in the neoplastic cell population, consistent with a fully clonal heterozygous *BRAF* mutation. The allelic ratio of *BRAF*^{V600E} to *BRAF*^{WT} in the eight VE1-positive nevi, adjusted for degree of stromal contamination, ranged from 0.84 to 1.12 with an average ratio of 1.01. This was confirmed by immunohistochemistry with an antibody specific for *BRAF*^{V600E}, which uniformly labeled the neoplastic cells without any evidence of heterogeneity. We found *BRAF*^{V600E} mutations in the melanocytic nevi to be fully clonal, strongly suggesting that *BRAF*-activating mutations typically are early initiating events in melanocytic neoplasia.**

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Activating *BRAF* mutations in melanocytic neoplasia were originally discovered in melanoma. Subsequent studies identified similar mutations in the majority of melanocytic nevi (82%), suggesting that *BRAF* mutations occur early during melanocytic transformation, possibly as an initiating event (1). The fact that the majority of nevi arise early in life, primarily during the first two decades, and also in multiplicity, with an average 40-year-old having 40 nevi, also suggests that the number of genetic alterations required for their formation must be small (2). This notion is further supported by animal models in which mutant *BRAF* expression in melanocytes leads to benign melanocytic tumors and only in the context of additional genetic alterations progression to melanoma is observed (3,4). Based on these considerations, it appears likely that *BRAF* mutations represent an initiating event, which, in susceptible individuals, is sufficient to generate nevi.

However, Lin et al. genotyped single cells from melanocytic nevi in which a *BRAF*^{V600E} mutation was identified in the bulk population of cells and found that a statistically significant number of neoplastic cells did not carry the *BRAF* mutation (5). On the basis of this result, the authors concluded that activating *BRAF* mutations are not initiating events in neovogenesis. They propose that nevi may arise from cells both with and without *BRAF*^{V600E} that are independently initiated or that *BRAF*^{V600E} arises in a subpopulation of nevus cells after initiation. Lin et al. (5) also found *BRAF*^{V600E} alleles in cis with both alleles of a nearby heterozygous single-nucleotide polymorphism in all four cases examined by Sanger sequencing after long-range polymerase chain reaction (PCR), concluding that multiple distinct *BRAF*^{V600E} mutations commonly occur in nevi.

Resolving these contradicting scenarios is important to understanding the tumor-initiating role of *BRAF*. If *BRAF*^{V600E} were

an initiating event in nevi, it would be expected to be present in all nevus cells, and as a consequence, in all melanoma cells that emanated from one of the nevus cells by means of the acquisition of additional genetic alterations. This has important considerations for understanding the development of resistance to targeted therapies directed at mutant *BRAF*.

We used droplet digital PCR (ddPCR) (6) and a *BRAF*^{V600E}-specific antibody, VE1 (7), to further evaluate the distribution of *BRAF*^{V600E} mutations within the neoplastic melanocytes of nevi (Supplementary Methods, available online). We selected formalin-fixed, paraffin-embedded tissues from 10 acquired melanocytic nevi (compound and intradermal) from the archives of the Dermatopathology Section of the University of California, San Francisco, under approval of the institutional review board. All nevi underwent immunohistochemistry with the VE1 mouse monoclonal antibody (7). Eight of 10 cases demonstrated uniform immunoreactivity for VE1 of the entire neoplastic population of melanocytes, sparing stromal cells (Figure 1), whereas two nevi were entirely negative (Supplementary Figure 1, available online).

For all nevi, DNA was extracted from tissue sections that were manually microdissected to remove the epidermis and hair follicles. *BRAF*^{V600E} and *BRAF*^{WT} alleles were quantitated for each sample by ddPCR. In ddPCR, the DNA sample is equally partitioned into thousands of aqueous droplets suspended in a water in oil emulsion. The emulsion is subjected to thermal cycling, in which the DNA within each droplet undergoes amplification, in the presence of two TaqMan probes for the WT and mutant allele, respectively. At the end, droplets in which sequence complementary to the TaqMan probe was amplified can be detected by specific fluorescence. The number of droplets containing a specific allele is used to quantitate the concentration of that allele within the sample. This method allows the enumeration of alleles in a large number of starting cells, and is not subject to allele dropout (8), which can skew results when DNA is isolated and amplified from a small number of starting cells.

The eight nevi that were positive by VE1 immunohistochemistry showed *BRAF*^{V600E} to *BRAF*^{WT} allelic ratios between 0.61 and 0.79 (Figure 2A). For the two cases negative by VE1 immunohistochemistry, the *BRAF*^{V600E} to *BRAF*^{WT} allelic ratios were less than 0.001, similar to normal control DNA (Figure 2, B and C; Supplementary Figure 2, available online).

To correct for dilution of the mutant allele frequency by normal DNA contributed

by stromal cells, we estimated stromal contamination of the microdissected tissues by two-color fluorescence microscopy with antibodies against Sox-10 or a cocktail of antibodies against HMB-45, MART-1, and tyrosinase (pan-melanoma), both of which highlight melanocytes, and 4',6-diamidino-2-phenylindole counterstain to identify nuclei. At least 300 cells from representative regions corresponding to the microdissected areas were evaluated for Sox-10 or pan-melanoma

positivity (Figure 2D). Stromal contamination was estimated as the proportion of cells that reacted with neither Sox-10 nor pan-melanoma antibodies and the measured number of WT *BRAF* alleles was adjusted accordingly. The corrected allelic ratio of *BRAF*^{V600E} to *BRAF*^{WT} in the eight VE1 positive nevi ranged from 0.84 to 1.12 with an average ratio of 1.01 (Figure 2E). A ratio of 1.0 would be the expected finding, if all neoplastic melanocytes carried one mutant and one WT *BRAF* allele.

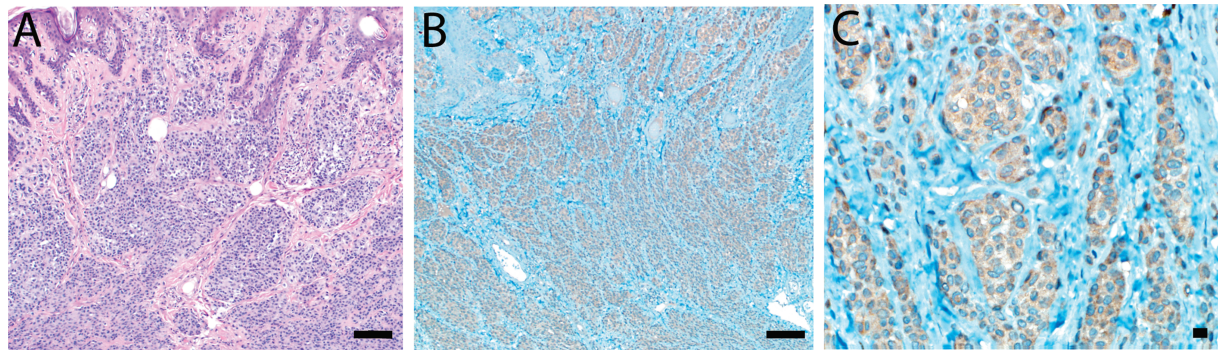


Figure 1. VE1 immunohistochemistry of a nevus with *BRAF*^{V600E} mutation. **A)** Low-power (×10) view of nevus stained with hematoxylin and eosin. Scale bar = 100 μm. **B)** Low-power (×10) view of same nevus by immunohistochemistry with the VE1 antibody, demonstrating uniform positivity throughout the population of nevus cells. Scale bar = 100 μm. **C)** High-power (×40) view of the nevus. Nevus cells demonstrate positive staining whereas stromal cells between nests of melanocytes are negative for VE1 immunoreactivity. Scale bar = 10 μm.

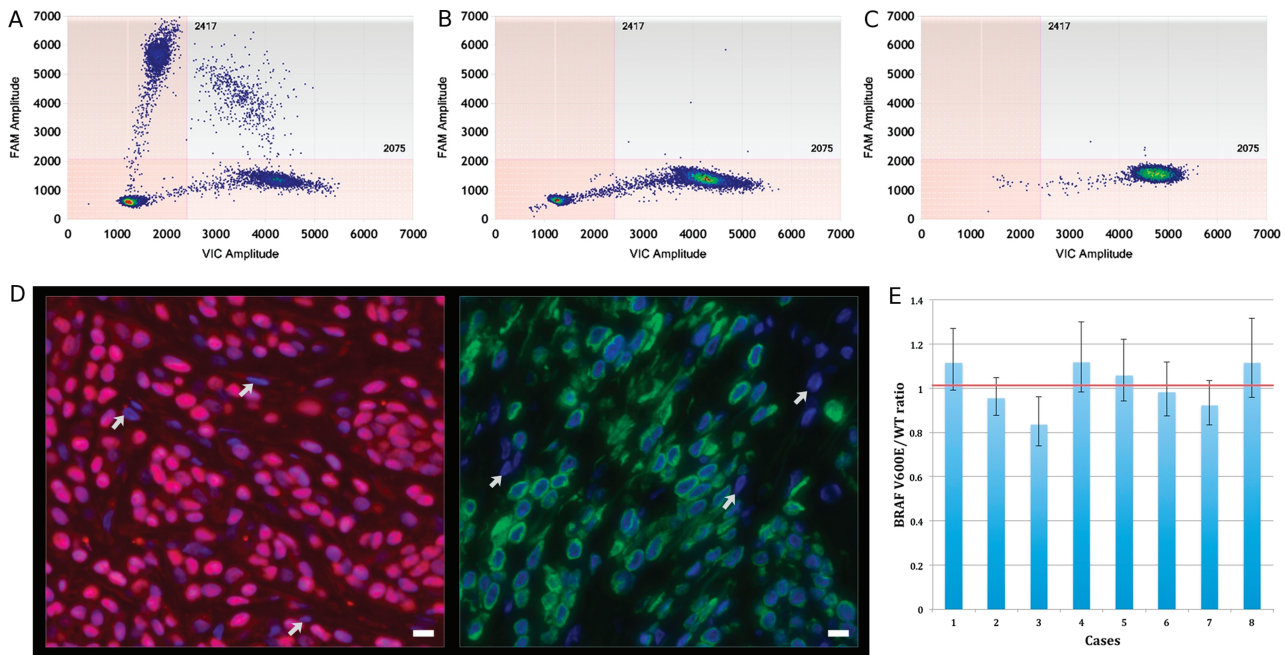


Figure 2. Assessing *BRAF* allelic ratios by digital droplet polymerase chain reaction (ddPCR). **A)** ddPCR results for a nevus with *BRAF* mutation. **B)** ddPCR results for a nevus without *BRAF* mutation. **C)** ddPCR results for a normal control DNA sample. The y-axis shows the fluorescence intensities of the 6-carboxyfluorescein (FAM)-labeled probe for *BRAF*^{V600E}, and the x-axis shows those of the VIC-labeled probe detecting wild-type (WT) *BRAF*. Each dot represents one droplet. The droplets in the upper left quadrant contain *BRAF*^{V600E} alleles only, those in the upper right quadrant contain both *BRAF*^{V600E} and *BRAF*^{WT} alleles, and the droplets in the lower right

quadrant contain *BRAF*^{WT} alleles only. Droplets in the lower left quadrant contain neither *BRAF*^{V600E} nor *BRAF*^{WT} alleles. The calculated concentrations for *BRAF*^{V600E} and *BRAF*^{WT} were 272 and 348 copies/μL, respectively for (A), 0.472 and 1720 copies/μL for (B), and 0.369 and 6920 copies/μL for (C). **D)** Immunofluorescence for Sox10 (red) and pan-melanoma (green) with 4',6-diamidino-2-phenylindole (DAPI) counterstain (blue). Stromal cells label only with DAPI and not with Sox10 or pan-melanoma antibodies (arrows). Scale bars = 10 μm. **E)** Allelic ratios, adjusted for the degree of stromal contamination, with 95% confidence intervals of *BRAF*^{V600E} and *BRAF*^{WT}.

Our results are at variance with the genotyping performed by Lin et al. (5) where PCR was performed on DNA extracted from single cells. As not all template molecules in the starting material can be successfully amplified, this approach can miscalculate allelic ratios, when, for example, the mutant allele “drops out” (8). This problem is accentuated in the presence of normal cells, where the likelihood of drop-out of the mutant is increased over the WT allele. The methods used for nevus cell isolation in Lin et al. (5) are not 100% specific, and thus stromal contamination could be a potential source of error. In contrast, our methods sample a large number of nevus cells, and we subsequently correct for stromal contamination.

In our study, the data from both methods—the expression of *BRAF*^{V600E} protein as determined by immunohistochemistry and the allelic ratios determined by ddPCR—strongly suggest that the *BRAF*^{V600E} mutation is present in the majority, if not all

melanocytes in *BRAF*^{V600E} nevi. Although our sample size was small, the consistency of the results obtained supports the model that *BRAF* mutation is an early initiating event that drives clonal expansion.

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