

## Polyclonality of *BRAF* Mutations in Acquired Melanocytic Nevi

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**Melanocytic nevi are thought to be senescent clones of melanocytes that have acquired an oncogenic *BRAF* mutation. *BRAF* mutation is considered to be a crucial step in the initiation of melanocyte transformation. However, using immunomagnetic separation or laser-capture microdissection, we examined *BRAF* mutations in sets of approximately 50 single cells isolated from acquired melanocytic nevi from 13 patients and found a substantial number of nevus cells that contained wild-type *BRAF* mixed with nevus cells that contained *BRAF*<sup>V600E</sup>. Furthermore, we simultaneously amplified *BRAF* exon 15 and a neighboring single nucleotide polymorphism (SNP), rs7801086, from nevus cell samples obtained from four patients who were heterozygous for this SNP. Subcloning and sequencing of the polymerase chain reaction products showed that both SNP alleles harbored the *BRAF*<sup>V600E</sup> mutation, indicating that the same *BRAF*<sup>V600E</sup> mutation originated from different cells. The polyclonality of *BRAF* mutations in acquired melanocytic nevi suggests that mutation of *BRAF* may not be an initial event in melanocyte transformation.**

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*v-raf* murine sarcoma viral oncogene homolog B1 (*BRAF*) is a member of the *raf* gene family that encodes a cytoplasmic serine/threonine kinase that signals in the mitogen-activated protein kinase pathway and mediates cell proliferation and differentiation. Mutations in the *BRAF* gene, most commonly including *BRAF*<sup>V600E</sup>, which encodes an amino acid substitution that leads to RAS-independent autoactivation of the BRAF kinase with subsequent stimulation of the downstream kinases and transcription factors, have been frequently found in melanoma lesions and melanocytic nevi (1,2). This finding has been interpreted to suggest that mutation of *BRAF* is a crucial step in the initiation of melanocyte transformation (2). Furthermore, two recent studies have shown that sustained *BRAF*<sup>V600E</sup> expression in normal melanocytes causes either cell cycle arrest accompanied by the induction of both p16<sup>INK4a</sup> protein expression and senescence-associated acidic  $\beta$ -galactosidase activity (3) or substantial reduction of cell proliferation accompanied by the induction of nuclear p16<sup>INK4a</sup> expression (4). Both p16<sup>INK4a</sup> protein expression and acidic  $\beta$ -galactosidase activity were also demonstrated in melanocytic nevi in situ (3,4). These

results suggest that melanocytic nevi are benign tumors that originate from the clonal expansion of a single melanocyte that acquires a *BRAF*<sup>V600E</sup> mutation and temporarily proliferates in response to oncogenic *BRAF* signaling, followed by growth arrest due to oncogene-induced senescence (5).

However, in a recent analysis of *BRAF* mutations in a large series of melanocytic nevi (6), we observed clonal heterogeneity in terms of *BRAF* mutations among the cells in a few small congenital nevi (6). Because of these findings and the possibility that they contradict the currently accepted model of melanocytic neoplastic progression (5), we tested the polyclonality of *BRAF* mutations in acquired melanocytic nevi.

The study was approved by the medical ethics committee of the Shinshu University School of Medicine. All patients gave written informed consent. We obtained acquired melanocytic nevi from 14 patients. After bisection, one-half of the nevus tissue was routinely fixed and embedded in paraffin. The other half was used to isolate single nevus cells. For six nevi (numbers 1–6 in Table 1), we removed the epidermis, minced the tissue, and collected nevus cells using a cocktail of human high molecular weight-

melanoma-associated antigen (HMW-MAA)-specific monoclonal antibodies and magnetic beads (Dynabeads CELLection Pan Mouse IgG Kit; Invitrogen DYNAL AS, Oslo, Norway), as described previously (7). Separated nevus cells were smeared on film-coated glass slides (Meiwafosis Co Ltd, Osaka, Japan) and subjected to laser-capture microdissection using a PALM-MB microdissection system (PALM Microlaser technologies, Bernried, Germany) to isolate approximately 60–65 cells.

Immunohistochemical staining with monoclonal antibodies showed high levels of expression of HMW-MAA on nevus cells (Supplementary Figure 1, available online). No staining of melanocytes was detected. Although a few keratinocytes within the hair follicles and the basal layer of the epidermis expressed HMW-MAA, as reported previously (8), virtually no cells were captured when we performed immunomagnetic cell separation using normal skin. To further confirm the specificity of the nevus cell isolation, we stained the immunomagnetically separated cells with a rabbit polyclonal anti-S-100 antibody (Dako, Glostrup, Denmark) (1:300 dilution) followed by DAKO Envision System (Dako, Carpinteria, CA), for three samples (sample numbers 3–5), and observed positive staining for almost all of the separated cells.

For the remaining eight nevi (numbers 7–14 in Table 1), one-half of the nevus tissue was snap frozen in optimal cutting temperature compound, cut 6  $\mu$ m thick, stained with toluidine blue, and subjected to laser-capture microdissection. Approximately 60–65

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## CONTEXT AND CAVEATS

### Prior knowledge

Oncogenic *BRAF* mutation has been considered to be an early step in the formation of melanocytic nevi, but recently, clonal heterogeneity was found in a few nevi.

### Study design

Approximately 50 single cells were separated from each of 13 melanocytic nevi either by using immunomagnetic beads or by laser-capture microdissection and were subjected to single-cell polymerase chain reaction and sequencing to determine *BRAF* mutations. In another experiment, *BRAF* and a neighboring single-nucleotide polymorphism were simultaneously amplified from nevi of four patients who were heterozygous for the polymorphism.

### Contribution

Although *BRAF* mutations were always found among nevus cells, cells that contained only wild-type *BRAF* predominated in nine of 13 nevi. When *BRAF* was sequenced from both alleles of four patients heterozygous for an adjacent polymorphism, both alleles harbored *BRAF* mutations.

### Implications

These results suggest that *BRAF* may be mutated as a late event in melanocytic tumorigenesis.

### Limitations

A small number of nevi of only two histological types were examined.

*From the Editors*

isolated single nevus cells were collected into lysis buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mg/mL proteinase K (Roche Diagnostics, Basel, Switzerland), and 3% Tween 20 and incubated for 16 hours at 50°C (6). We amplified exon 15 of the gene using heminested polymerase chain reaction (PCR) (Supplementary Table 1, available online). PCR products were purified and sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). To prevent potential contamination, aerosol resistant tips with filters were used and gloves and a mask were worn at all times. Samples without target DNA and/or DNA from single epidermal keratinocytes containing wild-type *BRAF* (similarly isolated by laser-capture microdissection) were always

included as negative controls to monitor PCR contamination.

We successfully isolated single nevus cells from immunomagnetically captured cell smears and from frozen tissue sections (Figure 1, A and B). The success rate of PCR amplification from single cells ranged from 74% to 85%, and we obtained *BRAF* sequences from approximately 50 single nevus cells for each patient sample. Sequence analyses revealed that all the 13 samples of the acquired melanocytic nevi contained nevus cells that had wild-type *BRAF* and cells that were heterozygous for *BRAF*<sup>V600E</sup> (Table 1). Nevus cells containing wild-type *BRAF* predominated in nine of 13 nevi. Consistent with the results of our previous study (6), nevus cells harboring *BRAF* mutations were very rarely detected in the two acral nevi that we examined (sample numbers 12 and 13) compared with nevi from other sites, which suggested a possible role for UV exposure in the acquisition of *BRAF* mutations in acquired melanocytic nevi. Cells with rare *BRAF* mutations, such as *BRAF*<sup>V600K</sup>, *BRAF*<sup>V600A</sup>, *BRAF*<sup>V600E/G</sup>, and *BRAF*<sup>T599I</sup>, all of which have been previously described in melanoma lesions (<http://www.sanger.ac.uk/genetics/CGP/cosmic>), were found in three nevi (numbers 4, 7, and 9). Except for one compound heterozygous mutation of *BRAF*<sup>V600E/G</sup> that was identified in sample number 9 (Figure 1, B), all of the other variant mutations were heterozygous. The protein products of two of these variant mutations (*BRAF*<sup>T599I</sup> and *BRAF*<sup>V600K</sup>) were shown to have much lower kinase activity compared with *BRAF*<sup>V600E</sup> (9). Thus, cell clones harboring these variant mutations may have weaker growth advantage than those with the *BRAF*<sup>V600E</sup> mutation, and may have been overlooked in the previous conventional sequence analyses, because the heterozygous mutation could be reliably discernible only when the samples contained more than 20% of clonal mutant cells (6).

Detection of the homozygous *BRAF*<sup>V600E</sup> mutation in a few single cells was considered to be due to the failure of PCR to amplify the wild-type allele, that is, allele dropout, which is a common problem in single-cell PCR (10). To assess the actual frequency of allele dropout, we performed a control experiment using the melanoma cell line 928mel, which is known to harbor the heterozygous *BRAF*<sup>V600E</sup> mutation (11).

Using the methodology used to obtain single nevus cells, single melanoma cells were isolated by laser-capture microdissection either from immunomagnetically captured cell smears or 6- $\mu$ m cryosections of cells suspended in agarose and embedded in optimal cutting temperature compound. We found wild-type *BRAF* sequences and homozygous *BRAF*<sup>V600E</sup> mutations, both of which were thought to be due to allele dropout, in three of 50 (6%) single melanoma cells isolated by immunomagnetic beads and in 12 of 50 (24%) of those obtained from 6- $\mu$ m frozen sections. The much higher frequency of allele dropout in the latter is explained by the nuclear damage during tissue sectioning. This was reflected in the results that showed a lower frequency of homozygous *BRAF*<sup>V600E</sup> mutations in samples obtained by immunomagnetic isolation (numbers 1–6) than frozen tissue sections (numbers 8–13). Then, we conducted a two-sided binomial test for each nevus sample using the 95% confidence limits of allele dropout in control melanoma cells as the test proportion, that is, 0.11 for immunomagnetic cell separation and 0.27 for frozen tissue sections. We used SPSS 15.0 software package (SPSS Inc, Tokyo, Japan) for this analysis. At the statistical significance level of .05, the possibility that all of the wild-type sequences were due to drop out of the mutant alleles was denied in all but one nevus (number 11), indicating that most acquired melanocytic nevi were composed of both *BRAF*-mutated cells and *BRAF*-wild-type cells.

We then further investigated whether *BRAF* mutant cells in acquired melanocytic nevi are monoclonal or polyclonal. For this purpose, we examined four acquired melanocytic nevi (numbers 3, 6, 11, and 14) that were excised from patients who were heterozygous for the single nucleotide polymorphism (SNP) rs7801086 (GCCG AGA vs GCCTAGA) (Figure 1, C). This SNP maps to chromosome 7, about 2 kb telomeric from exon 15 of the *BRAF* gene. We obtained pure nevus cell populations (but not single nevus cells) from bisected nevus tissues either by using immunomagnetic beads with HMW-MAA-specific monoclonal antibodies (for sample numbers 3 and 6) or by laser-capture microdissection of multiple nevus cell nests from 6- $\mu$ m frozen tissue sections (for sample numbers 11 and 14). After simultaneous amplification of

**Table 1.** Polyclonality of *BRAF* mutations in acquired melanocytic nevi as revealed by single-cell polymerase chain reaction (PCR) and sequencing of DNA from approximately 50 cells per nevus\*

Sample No.	Age, y	Sex	Site	Histology (Type)	Method	No. of cells with wild-type <i>BRAF</i>	No. of cells with heterozygous V600E mutation	No. of cells with other <i>BRAF</i> mutations†	No. of cells with homozygous V600E mutation	PCR failure	Total No. of cells (valid PCR)	P‡
N-1	34	F	Nose	Intradermal nevus (Miescher)	IMS	15	34	0	1	9	59 (50)	<.001
N-2	28	F	Abdomen	Intradermal nevus (Unna)	IMS	11	38	0	1	9	59 (50)	.018
N-3	31	F	Nose	Intradermal nevus (Miescher)	IMS	26	22	0	2	12	62 (50)	<.001
N-4	44	M	Face	Intradermal nevus (Miescher)	IMS	33	11	2 (V600K) 3 (T599I)	1	14	64 (50)	<.001
N-5	52	F	Neck	Intradermal nevus (Unna)	IMS	28	21	0	1	13	63 (50)	<.001
N-6	35	F	Face	Intradermal nevus (Miescher)	IMS	30	20	0	2	10	62 (52)	<.001
N-7	13	F	Abdomen	Intradermal nevus (Unna)	FTS	29	16	1 (V600A)	2	15	63 (48)	<.001
N-8	65	F	Nose	Compound nevus (Miescher)	FTS	31	14	0	5	9	59 (50)	<.001
N-9	81	M	Face	Compound nevus (Miescher)	FTS	21	23	1 (V600E/G)	4	16	65 (49)	.012
N-10	28	F	Arm	Intradermal nevus (Unna)	FTS	31	16	0	3	15	65 (50)	<.001
N-11	27	F	Face	Intradermal nevus (Miescher)	FTS	13	30	0	7	13	63 (50)	.510
N-12	25	F	Sole	Junctional nevus (Acral)	FTS	42	3	0	1	16	62 (46)	<.001
N-13	37	M	Sole	Compound nevus (Acral)	FTS	45	5	0	0	13	63 (50)	<.001
N-14	27	F	Abdomen	Intradermal nevus (Unna)	FTS	—	—	—	—	—	—	—
928mel	—	—	—	Melanoma cell line	IMS	1	47	0	2	13	63 (50)	—
928mel	—	—	—	Melanoma cell line	FTS	7	38	0	5	12	62 (50)	—

\* FTS = Frozen tissue section. Six- $\mu$ m cryosections were prepared from the nevus tissues or melanoma cell suspension embedded in agarose; IMS = Immunomagnetic cell separation using antibodies against human high molecular weight-melanoma-associated antigen.

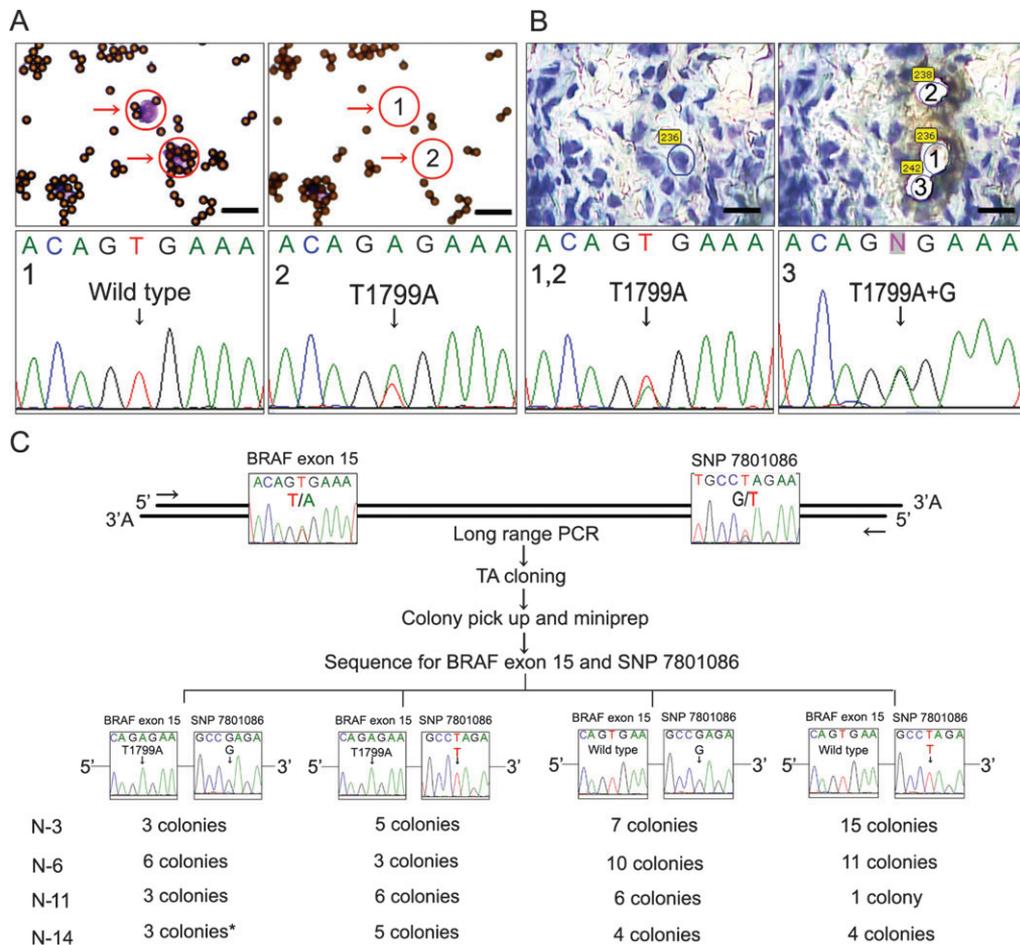
† V600K, GT1798-99→AA; T599I, C1796→T; V600A, T1799→C; V600E/G, T1799→A + T1799→G.

‡ Two-sided binomial tests were conducted using the 95% confidence limit of each method (0.11 for IMS and 0.27 for FTS) with the null hypothesis that all the wild types were due to allele dropout.

DNA fragments containing both *BRAF* exon 15 and SNP rs7801086 using the long-range Expand High Fidelity<sup>PLUS</sup> PCR System (Roche Applied Science, Mannheim, Germany), we subcloned the PCR prod-

ucts of separate alleles in bacteria. We then sequenced *BRAF* exon 15 and SNP rs7801086 from 16 to 30 individual bacterial colonies (for PCR primers, see Supplementary Table 1, available online).

Among the clones containing PCR products from each of the four acquired melanocytic nevi, we found both colonies that harbored the *BRAF*<sup>V600E</sup> (T1799→A) mutation and the T allele of SNP rs7801086 and



**Figure 1.** Polyclonality of *v-raf* murine sarcoma viral oncogene homolog B1 (*BRAF*) mutations in acquired melanocytic nevi. **A**) Selection of single nevus cells after immunomagnetic separation. Single nevus cells (purple dots with arrows) were captured by high molecular weight-melanoma-associated antigen-specific monoclonal antibodies bound to immunomagnetic beads (pink dots). The cells (encircled) were procured by laser-capture microdissection (top; bar = 20  $\mu$ m). Polymerase chain reaction (PCR) amplification and subsequent sequencing of single nevus cells showed wild-type *BRAF* and *BRAF*<sup>V600E</sup> mutations (bottom). **B**) Laser-capture microdissection of frozen tissue section of acquired melanocytic nevi followed by direct sequencing of *BRAF* exon 15 (top; bar = 20  $\mu$ m). Sequencing revealed two of the contiguous single nevus cells to have the *BRAF*<sup>V600E</sup> mutation and one to have a compound heterozygous *BRAF*<sup>V600E</sup> (T1799A) and *BRAF*<sup>V600G</sup> (T1799G) mutation, showing a heterogeneous pattern of *BRAF* mutations in proximal cells on a single-cell level (bottom). **C**)

Subcloning and subsequent sequencing of *BRAF* exon 15 and the single nucleotide polymorphism (SNP) rs7801086. This SNP maps approximately 2 kb telomeric from *BRAF* exon 15. Four nevi (numbers 3, 6, 11, and 14) were excised from patients who were heterozygous for this SNP. DNA was extracted from hundreds of nevus cells isolated either by using immunomagnetic beads (numbers 3 and 6) or laser-capture microdissection of frozen tissue sections (numbers 11 and 14). A 2859-bp fragment containing both *BRAF* exon 15 and the SNP rs7801086 was amplified by long-range PCR. Subcloning was carried out using this fragment as an insert. Sixteen to 30 colonies were randomly picked from each patient sample and analyzed for the sequence of both *BRAF* exon 15 and rs7801086. In all four patient samples, colonies with *BRAF*<sup>V600E</sup> as well as wild-type *BRAF* were accompanied by different SNP alleles, some harboring the G allele and others harboring the T allele. In sample number 14, one colony (\*) showed a tandem *BRAF*<sup>V600E/K601E</sup> (T1799→A and A1802→G) mutation.

colonies that harbored the *BRAF*<sup>V600E</sup> (T1799→A) mutation and the G allele of SNP rs7801086. Colonies that contained wild-type *BRAF* also harbored both G and T alleles. We used a high fidelity *Taq* polymerase in the PCR and did not find any base substitutions other than those encoding T1799A in exon 15 of the *BRAF* gene, except in one bacterial subclone from sample number 14 that carried a *BRAF*<sup>V600E/K601E</sup> (T1799→A and A1802→G) tandem mutation. These results indicate that even the same type of *BRAF*<sup>V600E</sup> mutation could

originate from different cells in the same nevus and that multiple *BRAF* mutations were possible among the cells within a given nevus. Collectively, our data strongly suggest marked polyclonality of *BRAF* mutations in acquired melanocytic nevi.

This result suggests that acquired melanocytic nevi may be polyclonal lesions, of multicellular origin, that result from random proliferation of cells containing wild-type *BRAF* as well as cells containing mutant *BRAF*. The polymorphic X-linked human androgen receptor gene has been

used previously to show that acquired melanocytic nevi are of clonal origin (12). However, a recent study questioned the validity of the human androgen receptor gene as a marker of tumor clonality because the clonal patch is relatively large in humans, often greater than 4 mm in diameter in the aorta, and even larger in the colon and breast, and because polyclonality can only be demonstrated at the borders of X-inactivation patches (13).

The reason why melanocytes in acquired melanocytic nevi are so susceptible to

mutation is unknown. One possibility is that genetically aberrant clones of melanocytes might already exist in the lesional skin of acquired melanocytic nevi, which would expand and acquire multiple mutations from stimuli such as UV radiation. Alternatively, the *BRAF* mutation might be a second hit after the clonal proliferation of nevus cells, which is initiated by either an as yet unknown mutation or other mechanisms. Cell proliferation itself may render melanocytes prone to mutation by the leakage of genotoxic species, such as reactive oxygen species (14).

It should be noted that most of the melanocytic nevi we examined were Unna's nevi and Miescher's nevi. We did not examine Clark's nevi, which are most commonly seen in adult Caucasians and are sometimes seen in association with melanoma (15). Nevertheless, polyclonality of *BRAF* mutations in the lesions of acquired melanocytic nevi suggests an alternative to the view that *BRAF* mutation is an initial event in melanocytic neoplasia (5).

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