Polyclonality of \textit{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 \textit{BRAF} mutation. \textit{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 \textit{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 \textit{BRAF} mixed with nevus cells that contained \textit{BRAF}^{V600E}. Furthermore, we simultaneously amplified \textit{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 \textit{BRAF}^{V600E} mutation, indicating that the same \textit{BRAF}^{V600E} mutation originated from different cells. The polyclonality of \textit{BRAF} mutations in acquired melanocytic nevi suggests that mutation of \textit{BRAF} may not be an initial event in melanocyte transformation.

\textit{J Natl Cancer Inst} 2009;101:1423–1427
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 

We found wild-type BRAF sequences and homozygous BRAF 
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-µm 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 
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-µm frozen tissue sections (for sample numbers 11 and 14). After simultaneous amplification of
DNA fragments containing both *BRAF* exon 15 and SNP rs7801086 using the long-range Expand High Fidelity PLUS PCR System (Roche Applied Science, Mannheim, Germany), we subcloned the PCR products 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* V600E (T1799→A) muta-
tion and the T allele of SNP rs7801086 and

![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*](https://academic.oup.com/jnci/article-abstract/101/20/1423/2581330)

<table>
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<tr>
<th>Sample No.</th>
<th>Age, y</th>
<th>Sex</th>
<th>Site</th>
<th>Histology (Type)</th>
<th>Method</th>
<th>No. of cells with wild-type <em>BRAF</em></th>
<th>No. of cells with heterozygous V600E mutation</th>
<th>No. of cells with other <em>BRAF</em> mutations†</th>
<th>No. of cells with homozygous V600E mutation</th>
<th>PCR failure</th>
<th>Total No. of cells (valid PCR)</th>
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</table>

* FTS = Frozen tissue section. Six-µm 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.
colonies that harbored the $BRAF^{V600E}$ (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 $BRAF^{V600E/K601E}$ (T1799→A and A1802→G) tandem mutation. These results indicate that even the same type of $BRAF^{V600E}$ 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 proximal cells on a single-cell level.

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 $BRAF^{V600E}$ 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 $BRAF^{V600E/K601E}$ (T1799→A and A1802→G) mutation.

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 \( \text{BRAF} \) 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 \( \text{BRAF} \) mutations in the lesions of acquired melanocytic nevi suggests an alternative to the view that \( \text{BRAF} \) mutation is an initial event in melanocytic neoplasia (5).

**References**


**Funding**

Grant-in-Aid for Cancer Research from the Ministry of Health, Labor, and Welfare of Japan (15–10 to T.S.) and by Public Health Service grant awarded by the National Cancer Institute (ROI CA105500 to S.F.). J.L. is a PhD student supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**Notes**

The study sponsor(s) had no role in the design of the study; the collection, analysis, or interpretation of the data; the writing of the manuscript; or the decision to submit the manuscript for publication.

Manuscript received November 4, 2008; revised July 21, 2009; accepted August 10, 2009.