Polyclonality of *BRAF* mutations in Acquired Melanocytic Nevi

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Abstract

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 laser-capture microdissection, we examined *BRAF* mutations in sets of approximately 50 single cells isolated from acquired melanocytic nevi from 13 patients and found nevus cells that contained wild-type *BRAF* mixed with nevus cells that contained $BRAF^{V600E}$. 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 $BRAF^{V600E}$ mutation, indicating that the same $BRAF^{V600E}$ 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.

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* ^{V600E}, 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*^{V600E} expression in normal melanocytes causes either cell cycle arrest accompanied by the induction of both p16^{INK4a} protein expression and senescence-associated acidic β-galactosidase activity (3) or substantial reduction of cell proliferation accompanied by the induction of nuclear p16^{INK4a} expression (4). Both p16^{INK4a} protein expression and acidic β-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*^{V600E} 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. In order 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 (OCT) compound, cut 6-μm-thick, stained with toluidine blue and subjected to laser-capture microdissection. 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℃ (6). We amplified exon 15 of the gene using hemi-nested 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 (Figures 1,A and 1,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 $BRAF^{V600E}$ (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 $BRAF^{V600K}$, $BRAF^{V600A}$, $BRAF^{V600E/G}$, and $BRAF^{T599I}$, 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 $BRAF^{V600E/G}$ 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 ($BRAF^{T599I}$ and $BRAF^{V600K}$) were shown to have much lower kinase activity compared with $BRAF^{V600E}$ (9). Thus, cell clones harboring these variant mutations may have weaker growth advantage than those with the $BRAF^{V600E}$ 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 $BRAF^{V600E}$ mutation in a few single cells was considered to be due to the failure of PCR to amplify the wild-type allele, ie, allele drop-out, which is a common problem in single-cell PCR (10). To assess the actual frequency of allele drop-out, we performed a control experiment using the melanoma cell line 928mel, which is known to harbor the heterozygous $BRAF^{V600E}$ mutation (11). Using the methodology used to obtain single nevus cells, single melanoma cells were isolated by laser microdissection either from immunomagnetically-captured cell smears or 6-μm cryosections of cells suspended in agarose and embedded in OCT compound. We found wild-type *BRAF* sequences and homozygous $BRAF^{V600E}$ mutations, both of which were thought to be due to allele drop-out, in 3 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 drop-out 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 ^{V600E} 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 drop-out in control melanoma cells as the test proportion, ie, 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 0.05, 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 (GCCGAGA 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 HMA-MMA-specific monoclonal antibodies (for sample numbers 3 and 6) or by laser-capture microdissection of multiple nevus cell nests from 6- μ m 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) mutation and the T allele of SNP rs7801086 and 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 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 (HUMARA) gene has been used previously to show that acquired melanocytic nevi are of clonal origin (12). However, a recent study questioned the validity of HUMARA 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).

Funding: Grant-in-Aid for Cancer Research from the Ministry of Health, Labor, and Welfare of Japan [15–10 to T. S.] and by PHS grant awarded by the National Cancer Institute [R01 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.

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Figure Legends

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 (HMW-MAA)-specific monoclonal antibodies bound to immunomagnetic beads (pink dots). The cells (encircled) were procured by laser-capture microdissection (top). Bar = 20 µm. Polymerase chain reaction amplification and subsequent sequencing of single nevus cells showed wild-type *BRAF* and $BRAF^{V600E}$ 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** µm. Sequencing revealed two of the contiguous single-nevus cells to have the $BRAF^{V600E}$ mutation and one to have a compound heterozygous $BRAF^{V600E}$ (T1799A)/ $BRAF^{V600G}$ (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 microdissection of frozen tissue sections (numbers 11 and 14). A 2859-bp fragment containing both *BRAF* exon15 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.

Supplementary Figure 1. High molecular weight-melanoma-associated antigen (HMW-MAA) expression in acquired melanocytic nevus (AMN). **A)** Immunohistochemical staining of an acquired melanocytic nevus lesion. Staining with a combination of the three anti-HMW-MAA-specific monoclonal antibodies (763.74, VF1-TP41.2 and VT80.12, each at the concentration of 0.5 mg/ml) followed by the CSA II System (CSA II , Biotin-Free Catalyzed Amplification System; Dako Cytomation, Carpinteria, CA) showed extensive expression of HMW-MAA on the membranes of nevus cells. Expression was not detected on normal melanocytes in the basal cell layer of the epidermis. **B-C)** Enlarged images of the boxed areas in (**A)**. (in **A, bar =** 1 mm; in **B and C**, bar = 200 µm.)

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 *BRAF*	No. of cells with heterozygous V600E mutation	No. of cells with other *BRAF* 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)	---

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

†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 drop-out.

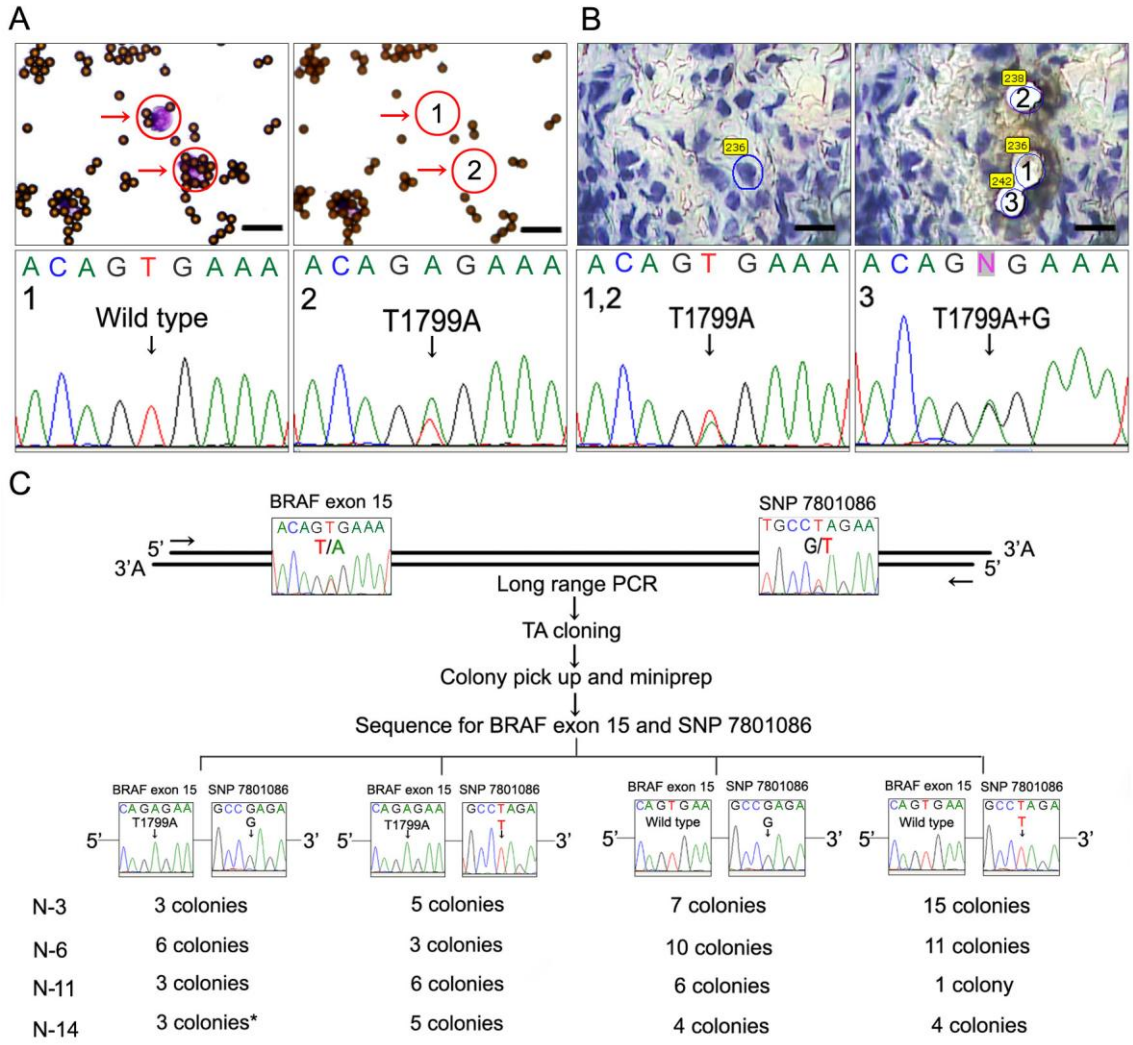
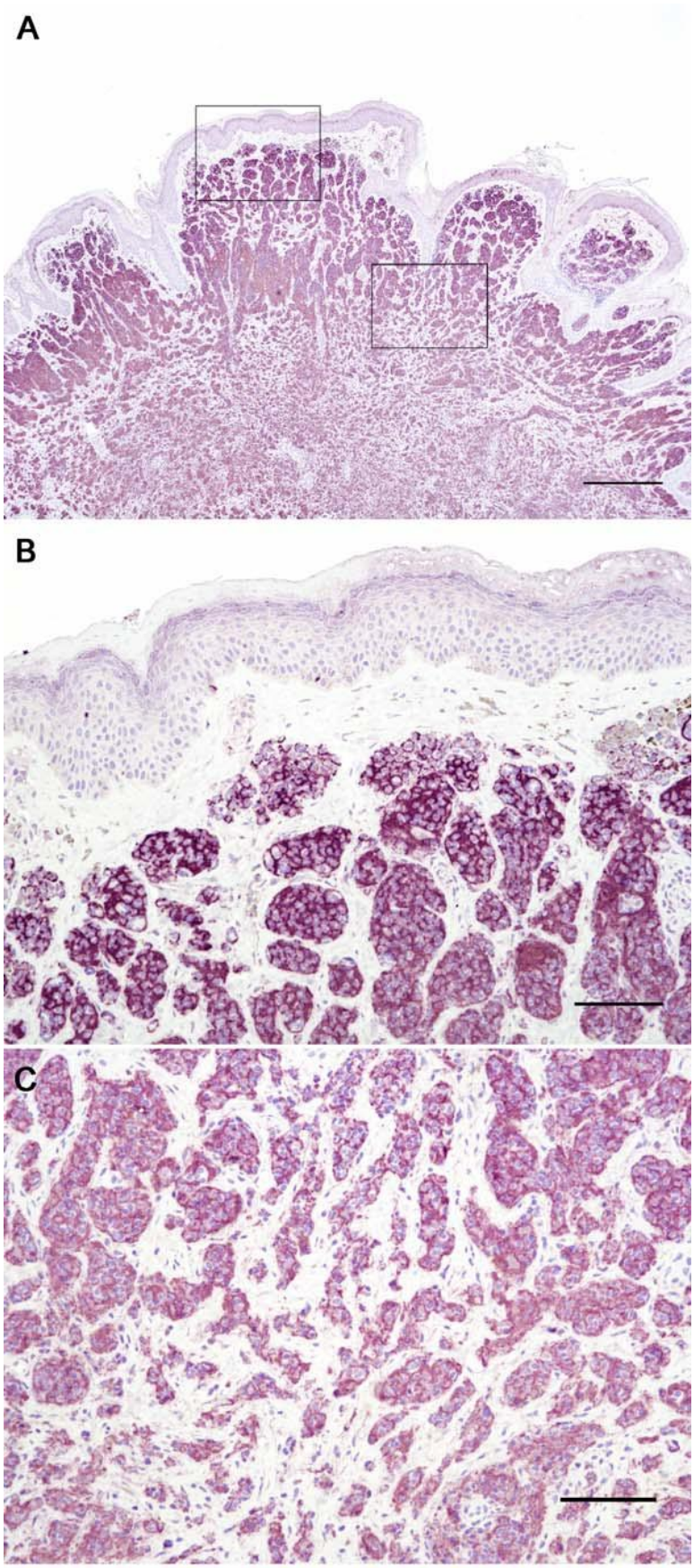

A
B
1
Wild type
2
T1799A
1,2
T1799A
3
T1799A+G
C
BRAF exon 15
SNP 7801086
5'
3'A
T/A
G/T
Long range PCR
TA cloning
Colony pick up and miniprep
Sequence for BRAF exon 15 and SNP 7801086
Wild type
N-3 3 colonies 5 colonies 7 colonies 15 colonies
N-6 6 colonies 3 colonies 10 colonies 11 colonies
N-11 3 colonies 6 colonies 6 colonies 1 colony
N-14 3 colonies* 5 colonies 4 colonies 4 colonies

Figure 1.



Supplementary Figure 1.