SUPPLEMENTARY INFORMATION

NRAS and *BRAF* Mutational Analysis

Separate PCR amplifications were performed using primers encompassing *NRAS* codons 12, 13, and 18 (forward: 5'-GACTGAGTACAAACTGGTGG-3', reverse: 5'-GGGCCTCACCTCTATGGTG-3') or *NRAS* codon 61 (forward: 5-GGTGAAACCTGTTTGTTGGA-3', reverse: 5'-ATACACAGAGGAAGCCTTC-3'). Each fragment was amplified in a 25 μl reaction containing 1-3 μl tumor DNA lysate, 400 nM each primer, 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1.5mM $MgCl_{2)}$, 125 uM each of the four deoxyribonucleotide triphosphates (dNTPs), and 0.5 units AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, California). The following PCR cycle parameters used were: 1 cycle of 94^{o}C for 3 min., then 35-40 cycles of 94^{o}C for 30 sec., 60^{o}C for 1 min., 72^{o}C for 1 min., then 1 cycle of 72^{o}C for 10 min. The quantity of each PCR product obtained was evaluated on a 10% acrylamide gel prior to performing SSCP.

For *BRAF* at exon 15; forward: 5'-TCATAATGCTTGCTCTGATAGGA-3' and reverse: 5'-GGCCAAAAATTTAATCAGTGGA-3' primers were used.*(1)* For *BRAF* at exon 11, forward: 5'-CTGTTTGGCTTGACTTGAC-3' and reverse: 5'-GACTTGTCACAATGTCACC-3' primers were used. For some laser capture microdissected samples, inner primer sets were used for both *BRAF* exon 15 and exon 11. The *BRAF* exon 11 and 15 regions were amplified by PCR in 25-50 μl reactions containing 1- 5 μl of tumor DNA lysate, 400 nmol each of *BRAF* exon 15 or 11 forward and reverse primers, 0.5-1 unit of AmpliTaq Gold DNA polymerase (ABI), with 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl), 1.5 mM $MgCl_2$, and 125 μM each dNTP. PCR was performed with 1 cycle of 94^{o}C for 3 min.; followed by 35-40 cycles of 94^{o}C for 30 sec. and 60^{o}C or 57^{o}C (exon 11 or 15) for 1 min. and 72^{o}C for 1 min.; and a final extension of 72^{o}C

for 10 min. for 1 cycle. The quantity of each PCR product obtained was analyzed on a 10% acrylamide gel prior to performing SSCP.

SSCP was performed in a 20μl reaction containing 300nM of each of the two primers, 1X PCR buffer (50mM KCl, 10mM Tris-HCl, pH 8.3), 1.5mM $MgCl_2$, 150μM each dNTP, except 22.5μM dCTP and 0.2 μl of α-^{32}P-dCTP (MP Biomedical, Irvine, California), and 0.5 unit AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, California). First round PCR product was diluted approximately 1 to 75 in dH_2O (to equalize concentrations) and 2 μl used in the SSCP PCR reaction with the same cycling parameters as for the initial PCR (33 cycles). Amplified PCR product (2μl) was diluted in 70μl of 0.1% SDS/10mM EDTA, then mixed with 92% formamide/40mM EDTA stop dye at a 2:1 ratio, denatured and analyzed by polyacrylamide gel electrophoresis. Each *NRAS* SSCP product was run on two different 6% (5.8/0.2 acrylamide/BIS ratio) polyacrylamide gels, one with 10% glycerin and each *BRAF* product was run on two different 4.8% (4.6/0.17 acrylamide/BIS ratio) polyacrylamide gels, one with 10% glycerin. The glycerin gels were run at room temperature with cooling fans directed on them and the other gel was run at 4^{o}C. Both were run in 1XTBE at 40 watts until the bromophenol blue dye fronts were a few inches from the bottom of the gel. The gels were then transferred to chromatography paper, dried and exposed to film (Hyperfilm MP) at –80^{o}C for 2 days.

Positive and negative SSCP controls, as well as an undenatured DNA control, and a no-DNA control were loaded on all SSCP gels. The following served as positive controls: the Colo205 cell line (ATCC, Manassas, Virginia) containing a G<u>T</u>G→G<u>A</u>G point mutation codon600 for *BRAF* exon 15, the H1395 cell line (ATCC, Manassas, Virginia) containing a G<u>G</u>A→G<u>C</u>A point mutation in codon 469 for *BRAF* exon 11, the HL60 cell line (ATCC,

Manassas, Virginia) containing a CAA→CTA mutation in codon 61 for *NRAS* exon 3, and a non-study melanoma sample containing a GGT→TGT mutation in codon 12 for *NRAS* exon 2. Negative controls were cell lines or non-study tissues not exhibiting these mutations.

All gene fragments showing band shifts on SSCP were considered potentially positive for mutation and were sequenced. In addition, as a quality control measure, a random 10% of SSCP-negative samples were sequenced and all were negative for any sequence variation.

PCR products were incubated at 37^{o}C with ExoSAP-IT (USB, Cleveland, Ohio) (2ul/ 5ul PCR product) for 15 minutes prior to sequencing to remove remaining dNTPs and primers and then heated to 80^{o}C for 15 minutes to inactivate the enzyme. Sequencing was then performed using either the method previously described *(2)* or the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB) according to the standard protocol. Samples were run on an 8 or 9% polyacrylamide standard sequencing gel. All mutations were independently confirmed by sequencing of a separately-amplified aliquot of DNA to rule out artifactual mutations.

Silent Mutations

The following silent mutations were found: One melanoma had *NRAS* A18A (GCA to GCC), L19L (CTG to TTG), T20T (ACA to ACG), V29V (GTA to GTG). Two melanomas had *BRAF* R444R (CGG to CGA). Another melanoma had *BRAF* R603R (CGA to AGA).

References

1. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417:949-54.
2. Thomas NE, Alexander A, Edmiston SN, et al. Tandem BRAF mutations in primary invasive melanomas. J Invest Dermatol 2004;122:1245-50.