*Clinical Chemistry* 52:9
1675–1678 (2006)

Molecular Diagnostics and Genetics

# Rapid Screening of 4000 Individuals for Germ-line Variations in the *BRAF* Gene

MICHAEL R. JAMES,* TROY DUMENI, MITCHELL S. STARK, DAVID L. DUFFY, GRANT W. MONTGOMERY, NICHOLAS G. MARTIN, and NICHOLAS K. HAYWARD

***Background:*** **The *BRAF* gene is frequently somatically altered in malignant melanoma. A majority of variations are at the valine 600 residue leading to a V600E substitution that constitutively activates the kinase. We screened 4000 patient and control DNAs for germ-line variations at the valine 600 residue.**

***Methods:*** **We developed a novel assay by adapting single-base variation assays and software for MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry to screen for all 5 reported variants at codon 600 of the *BRAF* gene. We screened a case-control collection comprising samples from 1082 melanoma patients and 154 of their unaffected relatives from 1278 families and from 2744 individuals from 659 unselected twin families with no history of melanoma. A panel of 66 melanoma cell lines was used for variation-positive controls.**

***Results:*** **All melanoma cell lines that we had found previously to carry a codon 600 variation were verified in this study. Three of the 4 possible variants (V600E n = 47, V600K n = 2, V600R n = 1) were detected, but no case of V600D was available. No germ-line variants were found in the samples from the 3980 melanoma patients or from the control individuals.**

***Conclusions:*** **This new assay is a high-throughput, automated alternative to standard sequencing and can be used as a rapid initial screen for somatic variants associated with melanoma. Germ-line variants at valine 600 are unlikely to exist and do not contribute to the reported role of the *BRAF* gene in melanoma predisposition.**



*BRAF*[1] gene somatic variations have been found in a high proportion (40%–88%) of malignant melanoma tissue *(1–9)* and also are common in benign nevi *(6, 10)*, a finding that may suggest that *BRAF* variations play a role in the initiation of melanocytic neoplasia. Most of these somatic variations occur in the kinase domain of B-Raf encoded by exons 11 and 15 of the gene *(6, 10)* and are thought to act by directly increasing the constitutive kinase activity toward its downstream targets MAPKK (mitogen-activated protein kinase kinase) and MAPK (mitogen-activated protein kinase), although other indirect mechanisms exist *(11, 12)*. The valine at codon 600 is involved in the majority of activating variations, most of which are glutamic acid substitutions (V600E). (Note: Because of updated sequence information, National Center for Biotechnology Information has recently renumbered Braf codons such that V599E is now V600E; all codon numbering in this paper conforms to this new numbering.)

Genetic studies have implicated *BRAF* in predisposition to melanoma *(13, 14)*, although no causative variations have been found. By comparison, rare germ-line variants of *CDKN2A* account for ~25% of familial melanoma cases *(15)* but only a tiny proportion of melanoma in the general population *(16)*. Given that B-Raf has relatively high basal kinase activity and that most described variations are activating variations, it is plausible that germ-line variants causing modest activation could exist and could contribute to predisposition to melanoma. Only 1 common germ-line coding variant has been observed, however, and it is a silent change (G643G) in exon 16, which is unlikely to have functional consequences. Nevertheless, a recent study reported 3 different germ-line variants in melanoma cases from Italy, including 1 case of V600E *(17)* that prompted us to analyze our large case-control collection.

Queensland Institute of Medical Research, Brisbane, Australia.
* Address correspondence to this author at: Queensland Institute of Medical Research, 300 Herston Rd, Herston, QLD 4029, Australia. Fax 61-7-33620101; e-mail michaelJ@qimr.edu.au.

Received March 14, 2006; accepted June 30, 2006.
Previously published online at DOI: 10.1373/clinchem.2006.070169

[1] Human genes: *BRAF*, v-raf murine sarcoma viral oncogene homolog B1; *CDKN2A*, cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4).

We studied an Australian case-control sample group made up of samples from 1082 melanoma cases from 1278 families participating in the Queensland Familial Melanoma Project, *(18–20)* and 2744 individuals without melanoma from 659 twin families (476 DZ, 159 MZ) enrolled in the Brisbane Twin Nevus Study *(21, 22)*. Approval to undertake this study was granted by the Human Research Ethics Committee of the Queensland Institute of Medical Research. All participants gave their signed informed consent.

We used a modified genotyping assay via a primer extension reaction and MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry (MassARRAY, Sequenom Inc) to perform a sequence survey of codon 600. A 2.5-$\mu$L PCR was performed oil-free in standard 384-well plates (TwinTec, Eppendorf Inc.) with 10 ng genomic DNA and the PCR-L (left) and PCR-R (right) primers as shown in Fig. 1A. Primers were at 100 nmol/L and deoxynucleoside triphosphate (dNTP) at 100 $\mu$mol/L, plus 0.05 units HotStar Taq polymerase (Qiagen) in 1X standard buffer provided with the enzyme supplemented to 3 mmol/L $MgCl_2$. Thermal cycling in an ABI-9700 instrument was 15 min at 95 °C, followed by 45 cycles of 20 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C. To the completed PCR reaction, we added 0.06 units shrimp alkaline phosphatase (in 1$\mu$L) and then subjected the mixture to incubation for 30 min at 37 °C, followed by inactivation for 5 min at 85 °C. The reaction was evaporated to dryness (20 min at 80 °C in a fan-driven oven) and resuspended in 2.5 $\mu$L containing 300 nmol/L each of the forward and reverse extension primers as shown in Fig. 1A, 10 $\mu$mol/L each of ddATP, dGTP, dATP, and dCTP, and 0.064 units/$\mu$L ThermoSequenase (Amersham) in 0.5 × Qiagen PCR buffer (final in reaction 1.7x PCR buffer). The reactions were heated in an ABI-9700 thermocycler for 30 s at 95 °C, then cycled 75 times for 15 s at 52 °C, 5 s at 80 °C, and after every 5th cycle for 10 s at 95 °C. Samples were diluted with 12 $\mu$L water, 3 $\mu$L desalting resin (Bio-Rad AG50W-X8 fine) in water, mixed by rotation for 15-min on a carousel, and then spotted on a SpectroChip (Sequenom) and analyzed in an AutoFlex mass spectrometer per standard procedures *(23, 24)*.

The standard assay for single-base variations [also termed single-nucleotide polymorphisms (SNPs)] on the

A


B

| codon 600 | | Forward Primer Products | | | Reverse Primer Products | | | F+R |
|---|---|---|---|---|---|---|---|---|
| code | seq | bases added | primer mass | assay code | bases added | primer mass | assay code | "genotype" |
| | | none | 7084.6 | (P) | none | 6695.4 | (P) | |
| wt=V | GTG | GTGA | 8344.4 | T | CA | 7281.8 | C | TT+CC |
| E | GAG | GA | 7711.0 | G | CTCTGTA | 8812.8 | T | GT+TC |
| K | AAG | A | 7381.8 | A | CTTTGTA | 8827.8 | G | AT+GC |
| R | AGG | A | 7381.8 | A | CCTTGTA | 8812.8 | T | AT+TC |
| D | GAT | GA | 7711.0 | G | A | 6992.6 | A | GT+AC |

C

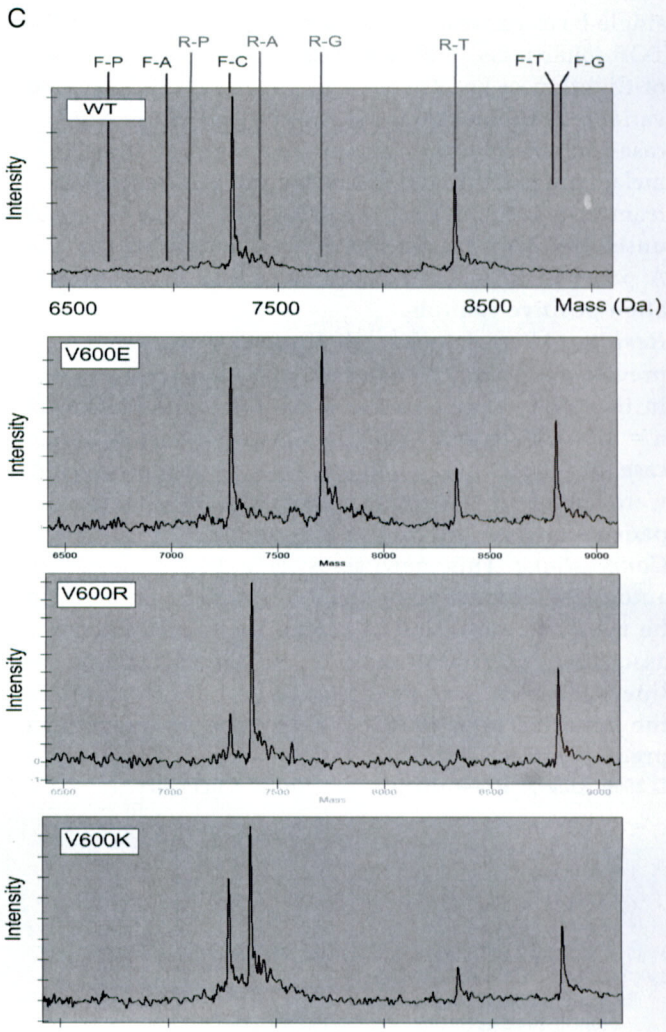


Fig. 1. Variation screening assay.

*(A)*, Sequence of exon 15 of the *BRAF* gene is shown with some flanking intronic sequence. Left (L) and right (R) primers used to amplify the 196 bp genomic fragment are indicated. *Lower case* sequence represents intron and *upper case* represents exon. The forward and reverse primers used in the primer extension assays are indicated flanking codon 600. *(B)*, Products of the forward and reverse primers when extended in a reaction containing ddATP plus dGTP, dATP, dCTP, their corresponding mass and assigned genotype code are shown. *(C)*, Mass spectra of extension products for wild-type (wt) and each type of variant (except for V600D) at codon 600. Abbreviations: R, reverse extension primer; F, forward extension primer; P, unextended primer; G, A, T, C are codes for their respective mass peaks as listed in (*B*). *Boxed letters* above spectra indicate the presence of that peak. Intensity on the Y axis is an arbitrary scale.

Sequenom MassArray system involves the extension of primers by DNA polymerase in the presence of a mixture of ddNTPs and dNTPs such that primers are extended by 1, 2, or 3 nucleotides depending on the single-base variation and the few nucleotides 3′ to it *(23, 25)*. In the current assay, primers immediately adjacent to codon 600 on the forward and reverse strands were extended in a ddATP and dGTP, dCTP, and dTTP mixture that gave extensions of 1 to 7 nucleotides, depending on the sequence (Fig. 1, A and B). Although neither the forward nor the reverse primer extension products alone give unambiguous assignment of all types of variants, the combined results should correctly detect all 5 known codons (Fig. 1B). The software for SNP analysis (Sequenom Inc.) was used to automatically assign to the predicted extension peaks arbitrary "genotype" names (Fig. 1B). Examples of spectra for 4 of the 5 wild-type and variant codons are shown in Fig. 1C; the V600D variant was not found in our samples. Any variant peaks were automatically flagged by the software and their spectra inspected for verification. Although primary results were very clear in this assay, we repeated the PCR and assay on any detected variants or any ambiguous results. All 50 samples previously analyzed by standard sequencing and found to contain variants (see the Supporting Table 2 in Pavey et al. *(9)* and our unpublished data) were confirmed with the new assay; indeed 1 variant, previously V600K, was reassigned as a V600R variant on the basis of the new assay (Fig. 1C) and subsequent review of sequence traces. Most of these samples showed the wild-type codon as well as the variant, but in 4 samples only the variant was visible. An example of weaker signals from the wild-type gene can be observed in the V600R spectrum in Fig. 1C. Although these results suggest possible genomic amplification of the mutant allele, in contrast to reports of amplification of the wild-type copy in melanoma cell lines *(26)*, careful normalization for the peak heights and mass effects in mass spectra plus the use of internal controls would be required to substantiate this explanation.

Analysis of 4000 individuals has revealed val-600 variants only in the cell lines derived from melanoma tumors, i.e., no germ-line variants were found. A study of 569 Italian cases had identified 1 germ-line case of the V600E variant *(17)*, but these data were subsequently retracted *(27)*. The same study found 2 types of germ-line variants that were previously unreported codon changes (M116R, Q608H). The M116R variant is of uncertain functional significance, but the Q608H variant is in exon 15, near the hotspot for somatic variations that affect the kinase domain of the protein. Neither of these variants nor previously described noncoding variants *(8)* occur at sufficient frequency to account for a genetic predisposition to melanoma in the general population. Given the number of controls screened here, this conclusion can also be extended to thyroid, colorectal, and lung carcinomas in which somatic variations in *BRAF* have also been found at significant frequencies *(1, 2, 28–32)*. Recently germ-line variants in BRAF have been associated with the rare genetic disease cardio-facio-cutaneous syndrome *(33)*. The severity of this syndrome, however, and the fact that the majority of associated BRAF variations differed from somatic variations in tumors, are consistent with our conclusion that germ-line BRAF variations are not found in the general population.

This new assay offers a high-throughput, automated alternative to standard sequencing. It also provides definitive unambiguous results, partly by virtue of the accuracy of mass spectrometry combined with interrogation of both DNA strands. Because >90% of all *BRAF* variations implicated in melanoma are of the type screened by this assay, it is a rapid initial screen of suspect material. Furthermore, this approach can be generalized to rapidly screen for defined variants. Indeed we have extended the method to several other genes and variants (work in progress).

We thank Ann Eldridge, Marlene Grace, Megan Campbell, and Anjali Egan for technical assistance, and we thank the melanoma patients, twins, and their families for their cooperation. This work was supported by grants from the Australian National Health and Medical Research Council (961061, 981339, and 199600), the Queensland Cancer Fund, the Cooperative Research Centre for Discovery of Genes for Common Diseases, and the US National Cancer Institute (CA88363). N.G.M. is a former member of the Scientific Advisory Board of Sequenom Inc. (he is no longer on the SAB) and holds share options in Sequenom Inc.

## References

1. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417:949–54.
2. Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, Gerrero R, et al. BRAF and RAS mutations in human lung cancer and melanoma. Cancer Res 2002;62:6997–7000.
3. Dong J, Phelps RG, Qiao R, Yao S, Benard O, Ronai Z, et al. BRAF oncogenic mutations correlate with progression rather than initiation of human melanoma. Cancer Res 2003;63:3883–5.
4. Gorden A, Osman I, Gai W, He D, Huang W, Davidson A, et al. Analysis of BRAF and N-RAS mutations in metastatic melanoma tissues. Cancer Res 2003;63:3955–7.
5. Kumar R, Angelini S, Czene K, Sauroja I, Hahka-Kemppinen M, Pyrhonen S, et al. BRAF mutations in metastatic melanoma: a possible association with clinical outcome. Clin Cancer Res 2003;9:3362–8.
6. Pollock PM, Harper UL, Hansen KS, Yudt LM, Stark M, Robbins CM, et al. High frequency of BRAF mutations in nevi. Nat Genet 2003;33:19–20.
7. Satyamoorthy K, Li G, Gerrero MR, Brose MS, Volpe P, Weber BL, et al. Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. Cancer Res 2003;63:756–9.
8. Laud K, Kannengiesser C, Avril MF, Chompret A, Stoppa-Lyonnet D, Desjardin L, et al. BRAF as a melanoma susceptibility candidate gene? Cancer Res 2003;63:3061–5.

9. Pavey S, Johansson P, Packer L, Taylor J, Stark M, Pollock PM, et al. Microarray expression profiling in melanoma reveals a BRAF mutation signature. Oncogene 2004;23:4060–7.
10. Yazdi AS, Palmedo G, Flaig MJ, Kutzner H, Sander CA. SP-11 Different frequencies of a BRAF point mutation in melanocytic skin lesions. Pigment Cell Res 2003;16:580.
11. Wan PTC, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 2004;116:855–67.
12. Morrison DK. Enzymes play molecular tag. Nature 2004;428: 813–4.
13. Meyer P, Sergi C, Garbe C. Polymorphisms of the BRAF gene predispose males to malignant melanoma. J Carcinogen 2003; 2:7.
14. James MR, Roth RB, Shi MM, Kammerer S, Nelson MR, Stark MS, et al. BRAF polymorphisms and risk of melanocytic neoplasia. J Invest Dermatol 2005;125:1252–8.
15. Pollock PM, Trent JM. The genetics of cutaneous melanoma. Clin Lab Med 2000;20:667–90.
16. Aitken JF, Welch J, Duffy DL, Milligan A, Green A, Martin N, et al. CDKN2A variants in a population-based sample of Queensland families with melanoma. J Natl Cancer Inst 1999;91:446–52.
17. Casula M, Colombino M, Satta MP, Cossu A, Ascierto PA, Bianchi-Scarra G, et al. BRAF gene is somatically mutated but does not make a major contribution to malignant melanoma susceptibility: the Italian melanoma intergroup study. J Clin Oncol 2004;22: 286–92.
18. Aitken JF, Duffy DL, Green A, Youl P, MacLennan R, Martin NG. Heterogeneity of melanoma risk in families of melanoma patients. Am J Epidemiol 1994;140:961–3.
19. Aitken JF, Green A, MacLennan R, Youl P, Martin NG The Queensland Familial Melanoma Project: study design and characteristics of participants. Melanoma Res 1996;6:155–65.
20. Palmer JS, Duffy DL, Box NF, Aitken JF, O'Gorman LE, Green AC, et al. Melanocortin-1 receptor polymorphisms and risk of melanoma: is the association explained solely by pigmentation phenotype? Am J Hum Genet 2000;66:176–86.
21. Zhu G, Duffy DL, Eldridge A, Grace M, Mayne C, O'Gorman L, et al. A major quantitative-trait locus for mole density is linked to the familial melanoma gene CDKN2A: a maximum-likelihood combined linkage and association analysis in twins and their sibs. Am J Hum Genet 1999;65:483–92.
22. Zhu G, Evans DM, Duffy DL, Montgomery GW, Medland SE, Gillespie NA, et al. A genome scan for eye color in 502 twin families: most variation is due to a QTL on chromosome 15q. Twin Res 2004;7:197–210.
23. Bansal A, van den Boom D, Kammerer S, Honisch C, Adam G, Cantor CR, et al. Association testing by DNA pooling: an effective initial screen. Proc Natl Acad Sci U S A 2002;99:16971–874.
24. James MR, Haywayd NK, Dumenil T, Montgomery GW, Martin NG, Duffy DL EGF polymorphism and risk of melanocytic neoplasia. J Invest Dermatol 2004;123:760–2.
25. Sun X, Ding H, Hung K, Guo B A new MALDI-TOF based mini-sequencing assay for genotyping of SNPs. Nucleic Acids Res 2000;28:e68.
26. Tanami H, Hirasawa A, Yuki Y, Sonoda I, Inoue J, Yasui K, et al. Involvement of overexpressed wild-type BRAF in the growth of malignant melanoma cell lines. Oncogene 2004;23:8796–804.
27. Casula M, Colombino M, Satta MP, Cossu A, Ascierto PA, Bianchi-Scarra G, et al. Errata to :BRAF gene is somatically mutated but does not make a major contribution to malignant melanoma susceptibility: the Italian melanoma intergroup study. J Clin Oncol 2005;23:936.
28. Cohen Y, Xing M, Mambo E, Guo Z, Wu G, Trink B, et al. BRAF mutation in papillary thyroid carcinoma. J Natl Cancer Inst 2003; 95:625–7.
29. Kimura ET, Nikiforova MN, Zhu Z, Knauf JA, Nikiforov YW, Fagin JA. High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res 2003;63:1454–7.
30. Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature 2002;418:934.
31. Yuen ST, Davies H, Chan TL, Ho JW, Bignell GR, Cox C, et al. Similarity of the phenotypic patterns associated with BRAF and KRAS mutations in colorectal neoplasia. Cancer Res 2002;62: 6451–5.
32. Naoki K, Chen TH, Richards WG, Sugarbaker DJ, Meyerson M. Missense mutations of the BRAF gene in human lung adenocarcinoma. Cancer Res 2002;62:7001–3.
33. Rodriguez-Viciana P, Tetsu O, Tidyman We, Estep AL, Conger BA, Santa Cruz M, et al. Germline mutations in genes within the MAPK pathway cause Cardio-facio-cutaneous syndrome. Science 2006; 311:1287–90.