

Detection of Mutations in the Mitogen-Activated Protein Kinase Pathway in Human Melanoma

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ABSTRACT

Purpose: Recent studies suggest that activating point mutations in *B-RAF* may commonly occur in melanoma. We devised a method to detect point mutations in heterogeneous tissues containing both wild-type and mutant *B-RAF* and *N-RAS* genes by using site-directed mutagenesis to introduce new restriction sites in the cDNA sequence when the specific point mutations are present. We used this technique to determine the incidence of mitogen-activated protein kinase (MAPK) mutations in human melanoma.

Experimental Design: We screened 85 melanoma samples for the most common *B-RAF* and *N-RAS* mutations found in melanoma using a site-directed mutagenesis-based detection technique. Western blotting was used to evaluate downstream up-regulation of the mitogen-activated protein kinase pathway in these tissues.

Results: Thirty-three samples (7 of 25 primaries, 15 of 25 regional metastases, 5 of 25 nodal metastases, and 6 of 10 distant metastases) harbored the V599E *B-RAF* mutation (39%), 12 contained a Q61R *N-RAS* mutation and 5 a Q61K *N-RAS* mutation. Western blotting with antiphosphorylated extracellular signal-regulated kinase 1/2 antibodies demonstrated up-regulation of the MAPK pathway in samples containing activating *B-RAF* or *N-RAS* mutations compared with wild-type samples. This method of detection was sensitive and specific with no false positives.

Conclusions: Activating mutations of the MAPK pathway were present in ~60% of samples tested and caused activation of this cellular pathway that appears to be important in the pathogenesis of melanoma.

INTRODUCTION

Recent evidence suggests that specific defects in mitogen-activated protein kinases (MAPKs) may be responsible for the pathogenesis of the majority of human melanomas. Davies *et al.* (1) recently reported that ~60% of melanomas have a mutation in *B-RAF* that leads to constitutive activation of MAPK pathways. Mutations in *N-RAS*, an upstream component of this same pathway, occur in ~25% of melanomas, and these *B-RAF* and *N-RAS* mutations appear to be mutually exclusive (1–4). Therefore, alterations in MAPK signaling may play a role in the pathogenesis of the majority of melanomas, implying that the development of drugs that antagonize these changes could lead to improved therapies. However, Davies *et al.* (1) and other recent studies describe only a few clinical melanoma specimens screened for MAPK pathway mutations, and the incidence of these mutations in human melanoma is therefore not yet known. Furthermore, a clinically useful screening test for the presence of MAPK pathway mutations will be necessary to monitor and stratify patients undergoing MAPK pathway-targeted therapies. We have developed a sensitive and specific screening test for the presence of the three most common point mutations of MAPK pathway components found in human melanoma, specifically the V599E mutation of *B-RAF* and the Q61K and Q61R mutations of *N-RAS*. We used this test to determine the incidence of these mutations in melanoma specimens. These data validate this screening method and document the incidence of *B-RAF* and *N-RAS* mutations in human melanoma.

MATERIALS AND METHODS

Detecting Point Mutations in Heterogeneous Tissues. We devised a strategy to detect point mutations in heterogeneous tissues containing both wild-type (WT) and mutant *B-RAF* and *N-RAS* genes. WT mRNA was expected to be present in every specimen because clinical melanoma specimens are likely to contain some number of normal cells (lymphocytes, fibroblasts, and so forth) in the tissue samples, and the melanoma cells themselves are likely to be heterozygous in the *N-RAS* and *B-RAF* alleles. Thus, we devised a method capable of detecting a small number of copies of mutant *B-RAF* and *N-RAS* alleles in a WT background. This method uses reverse transcriptase-PCR amplification followed by restriction enzyme digestion to detect point mutations. For this strategy to be successful, a specific restriction site must be present only when the mutation is present, allowing the restriction enzyme to cut the cDNA into identifiable fragments. When the WT allele is present, the restriction site is not present. Restriction sites of this type do not occur in the Q61R *N-RAS*, Q61K *N-RAS*, or the V599E *B-RAF* mutations. However, using site-directed mutagenesis using specifically designed PCR primers, we introduced changes in the sequences near the point mutations to produce new restriction sites.

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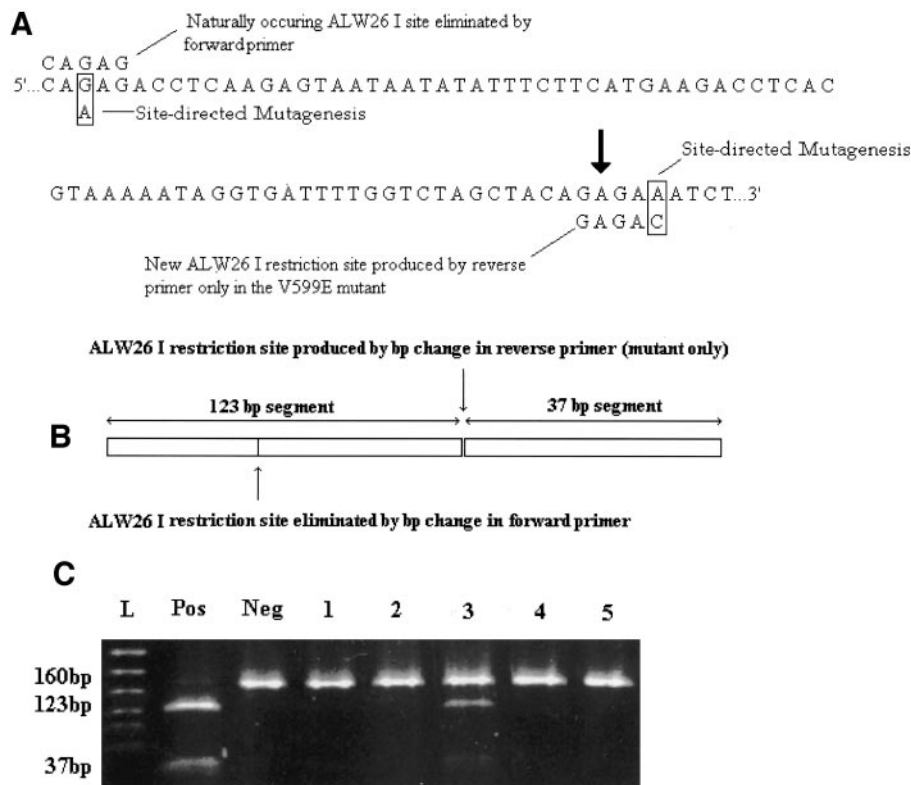
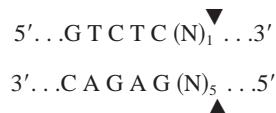


Fig. 1 A, creation of an *Alw26I* restriction site near codon 1796 of *B-RAF* having the V599E mutation. An arrow indicates the thymine to adenine V599E point mutation. Changes introduced by the primers are shown in boxes. An adenine is substituted for guanine near the 3' end of the forward primer to eliminate an *Alw26I* site that spans exons 574 and 575 in both the mutant and wild-type sequences. A substitution of a cytosine for an adenine in the reverse primer produces a new *Alw26I* restriction site in the mutant sequence but not in the wild-type sequence. B, the resultant digestion products. C, representative agarose gel electrophoresis result. L = ladder; Pos = positive control (HT-144 cell line); Neg = negative control (MCF-7 cell line); Lanes 1–5 are tumor samples. Tumor sample 3 shows cutting with *Alw26I*, demonstrating a V599E mutation of *B-RAF* in this tumor sample. A 160-bp band is still seen after digestion of the PCR product from tumor sample 3 and most likely represents wild-type *B-RAF* sequence from contaminating normal cells in that sample.

Detecting *B-RAF* V599E Mutation. The V599E mutation in *B-RAF* is produced by the substitution of an adenine for a thymine in codon 1796 (A1796T) that results in the substitution of a glutamic acid for a valine at position 599 of the protein. Several restriction sites exist near codon 1796 in *B-RAF*. *Alw26I* is a restriction enzyme that cuts the following sequence (arrows indicate the site cut, n = any nucleotide):



An *Alw26I* restriction site spanning codons 574/575 naturally occurs in *B-RAF*. We eliminated this restriction site by site-

directed mutagenesis using a forward primer (Fig. 1A and Table 1). We introduced a new *Alw26I* restriction in the V599E mutant *B-RAF* sequence by site-directed mutagenesis using a reverse primer (Fig. 1A and Table 1). These primers produced a 150-bp product that is cut by *Alw26I* into 123- and 37-bp fragments if the V599E mutation is present but remains a 150-bp product if the WT sequence is present (Fig. 1B). The digested PCR product is run on a 15% polyacrylamide gel and visualized using ethidium bromide staining (Fig. 1C).

Detecting *N-RAS* Q61R and Q61K Mutations. Because the two most common point mutations that result in constitutive activation of *N-RAS* in melanoma (Q61R and Q61K) do not form unique restriction sites, we used site-directed mutagenesis to produce restriction sites that could be

Table 1 Primer sequence used in this study (primers written 5' to 3')

Target	Primer	Sequence	Anneal
β-Actin	Forward	ATGAGATTGGCATGGCTTTA	52°C
	Reverse	GGTGTGCACTTTTATTCAAC	
Tyrosinase	Forward	TTGGCAGATTGTCTGTAGCC	56°C
	Reverse	AGGCATGTGTCATGCTGCTT	
MART-1	Forward	ATGCCAAGAGAAGATGCTCAC	55°C
	Reverse	AGCATGTCTCAGGTGTCTCG	
V599E <i>B-RAF</i>	Forward	ATGGATTACTTACACGCCAAGTCAATCATCCACAAAGACCTCA	68°C
	Reverse	CAACTGTTCAAACCTGATGGGACCCACTCCATCGAGATGTC	
Q61R <i>N-RAS</i>	Forward	ATGACTGAGTACAAACTGGTGGTGGTTGGAGCAGG TGGTGT	68°C
	Reverse	GCCTGTCTCATGTATTGCTGTCTCATGGCACTGCCTCTTC	
Q61K <i>N-RAS</i>	Forward	ATAGATGGTGAAACCTGTTTGGTGGACATACTGGATACAGTTCTGA	60°C
	Reverse	CGCTTAATCTGCTCCCTGTAGAGGTTAATATC	

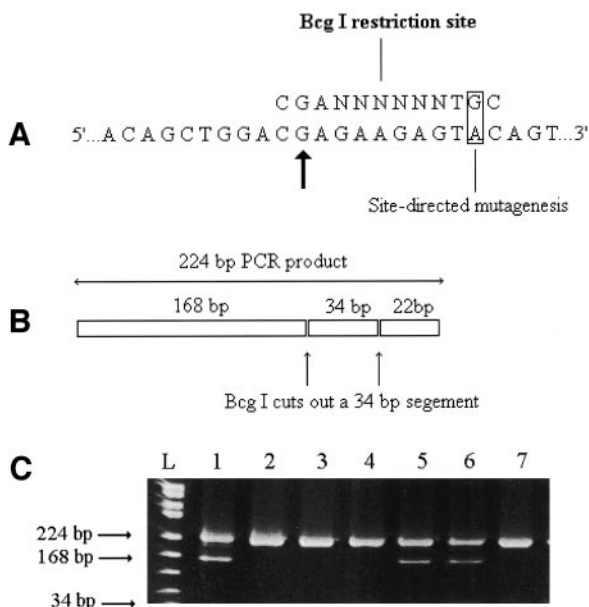
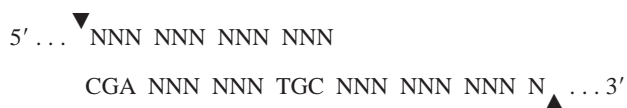


Fig. 2 A, creation of a *BcgI* restriction site near codon 61 of *N-RAS* with a guanine (arrow) replacing an adenine to form the Q61R mutation. The 3' end of the reverse primer introduces a change of a guanine to an adenine (box) in codon 64 to produce a *BcgI* restriction site in the Q61R mutant cDNA that is not present in the wild-type or Q61K mutant. The forward primer binds to the first 40 nucleotides of the cDNA to produce a 224-bp PCR product that is cut by *BcgI* into a 168-, a 34-, and a 22-bp product if the sequence contains the Q61R mutation but remains as a 224-bp product if the sequence contains the Q61K mutation (see Fig. 4) or is wild type. B, the resultant digestion products. C, representative agarose gel electrophoresis result. L = ladder; Lane 1 = positive control (SK-Mel 2 cell line); Lane 2 = negative control (MCF-7 cell line); Lanes 3–7 are tumor samples. Tumor samples 5 and 6 show cutting with *BcgI*, demonstrating a Q61R mutation of *N-RAS* in these two tumor samples (34-bp band is very faint at bottom of gel and 22-bp band is not shown). A 224-bp band is still seen after digestion of the PCR product from tumor samples 5 and 6 that most likely represents wild-type *N-RAS* sequence from contaminating normal cells in that sample.

used to identify these *N-RAS* mutations. Using site-directed mutagenesis in codon 64 of *N-RAS*, we produced a *BcgI* restriction site that is present when the Q61R mutation is present but not when the sequence is WT or when the Q61K mutation is present (Fig. 2A and Table 1). *BcgI* cuts the following sequence (arrows indicate the areas cut, n = any nucleotide):



Digestion with *BcgI* produces a 34-bp fragment, as well as the 5' and 3' ends of the cDNA molecule. Using primers to insert a nucleotide change, we produced a 224-bp PCR product cut by *BcgI* into 168-, 34-, and 22-bp fragments when the Q61R mutation is present (Fig. 2, B and C).

To detect the Q61K mutation, we used a *SfiI* restriction site produced in the Q61K cDNA sequence using primers that change nucleotides in codons 59 and 60 of *N-RAS* (Fig. 3A). *SfiI* cuts the following sequence (arrows indicating the area cut):



Primers that insert the two nucleotide changes shown in Fig. 3A produce a 250-bp PCR product that is cut by *SfiI* into 210- and 40-bp fragments if the Q61K mutation is present in *N-RAS* (Fig. 3, B and C).

Sensitivity and Specificity. To test the sensitivity and specificity of this technique for identifying the V599E mutation, we constructed plasmids that contained either the WT *B-RAF* sequence or the V599E mutant sequence. We designed primers that amplify a 300-bp segment of *B-RAF* cDNA centered on codon 1796. MCF-7 cells (known *B-RAF* WT; Ref. 1) were used to produce the cDNA for the WT plasmid, and HT-144 melanoma cells (known *B-RAF* V599E mutant; Ref. 1) were used to produce the cDNA for the mutant plasmid. These cDNAs were inserted into the plasmid using a protocol supplied by the manufacturer (Invitrogen). Plasmids were cloned and amplified in competent *Escherichia coli* and were sequenced using the sequencing primers supplied with the plasmid to verify the sequence. To determine the sensitivity and specificity of the V599E detection protocol, we serially diluted B-RAF sequence containing plasmids from 10^9 to 10 copies/reaction and used these dilutions in the V599E detection protocol. We confirmed the sensitivity and specificity of detecting *N-RAS* mutations with this system using the same method as that used for the *B-RAF* mutation, constructing plasmids that contained either a WT *N-RAS* cDNA or one of the Q61 mutations. These *N-RAS*-containing plasmids were serially diluted from a concentration of 10^9 down to 10 copies/reaction. Plasmids containing WT and mutant sequences were also used in all experiments as positive and negative controls.

Western Blotting. Cultured cells or homogenized frozen tissue specimens were lysed on ice for 45 min with radioimmunoprecipitation assay buffer [10 mM sodium phosphate (pH 7.2), 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 2 mM EDTA] supplemented with fresh 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 50 $\mu\text{g}/\text{ml}$ leupeptin and centrifuged at $14,000 \times g$ at 4°C for 10 min. Proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. The blots were incubated in blocking solution consisting of 5% milk in TBS-T (0.1% Tween 20) for 1 h at 25°C , then immunoblotted with polyclonal anti-extracellular signal-regulated kinase (ERK) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-phospho-MAP/ERK kinase antibody (Sigma, St. Louis, MO). Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Experiments were done under conditions of linearity with respect to protein concentration.

Clinical Melanoma Specimens. Eighty-five melanoma samples were used in this study and included primary tumors (25), regional dermal metastases (25), nodal metastases (25), and distant metastases (10). The Tissue Retrieval Service of the Cancer Institute of New Jersey banked these specimens, under a blanket Institutional Review Board-approved protocol, in a fashion that preserves patient confidentiality and conforms to Health Insurance Portability and Accountability Act regulations. Each

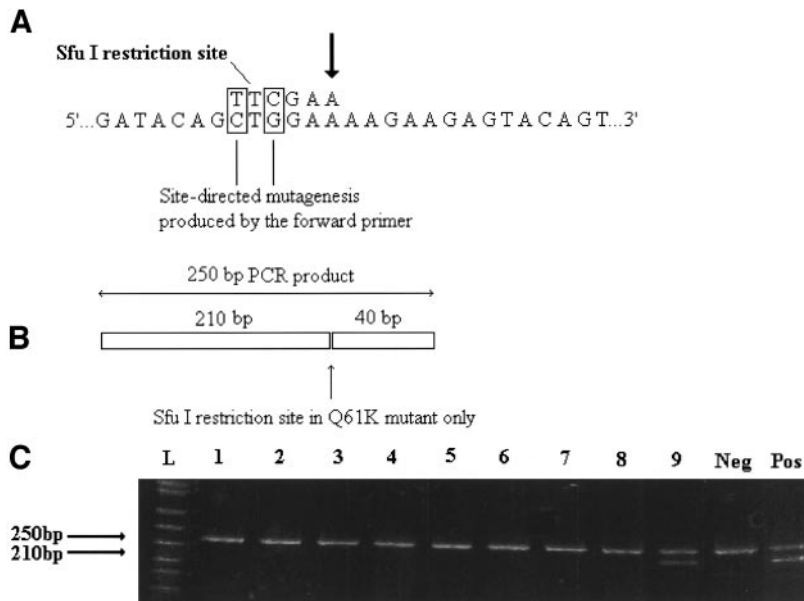


Fig. 3 A, creation of a *SfuI* restriction site near codon 61 of *N-RAS*. An adenine (arrow) replaces a cytosine to form the Q61K mutation. The 5' end of the forward primer introduces a change of a thymine to a cytosine and a cytosine for a guanine (boxes) in codons 59 and 60 to produce a *SfuI* restriction site in the Q61K mutant that is not present in the wild-type or Q61R mutant. The reverse primer binds downstream to produce a 250-bp PCR product that is cut by *SfuI* into a 210- and a 40-bp product if the sequence contains the Q61K mutation but remains as a 250-bp product if the sequence contains the Q61R mutation or is wild type. B, the resultant digestion products. C, representative agarose gel electrophoresis result. L = ladder; Neg = negative control (MCF-7 cell line); Pos = positive control; Lanes 1–9 are tumor samples. Tumor sample 9 shows cutting with *SfuI*, demonstrating a Q61K mutation of *N-RAS* in this tumor sample (40-bp band is not shown). A 250-bp band is still seen after digestion of the PCR product from tumor sample 9 that most likely represents wild-type *N-RAS* sequence from contaminating normal cells in that sample.

specimen was obtained in the operating room and snap frozen in liquid nitrogen within 5 min of resection. The specimens were stored at -80°C until processed. Total RNA was extracted from the specimens using a standard spin-column-based system (RNAeasy; Qiagen), and first strand synthesis was performed using a standard kit (Superscript First Strand Synthesis kit; Invitrogen). All melanoma specimens used in this study were positive by PCR for β -actin (5), tyrosinase (6, 7), and MART-1 (7, 8), demonstrating RNA integrity and that each specimen contained melanoma cells.

Primers and PCR Conditions. The primers and PCR conditions used for the detection of β -actin, tyrosinase, and MART-1, as well as the primers used in the site-directed mutagenesis detection protocol for the V599E *B-RAF* and the Q61 *N-RAS* mutations are shown in Table 1. PCR reactions (25 μl reactions) were run using a standard Taq polymerase (MasterTaq; Eppendorf), with an initial denaturing step at 94°C for 3 min followed by 40 cycles consisting of denaturing at 94°C for 30 s, annealing at the specific temperature for each set of primers for 30 s, and extension at 72°C for 1 min. A final extension at 72°C for 8 min was followed by storage at 4°C . Restrict digestions were carried out on 10 μl of the resultant PCR product at 36°C for 2 h with the appropriate enzymes and buffers as per manufacture's instructions. Digests were run on 15% polyacrylamide gels and visualized by ethidium bromide staining and UV transillumination. Controls included the use of known mutant and WT specimens in each run. Blast searches confirmed that the primer sequences used in these reactions do not anneal to other known genes.

Statistical Analysis. Members of the Division of Biometrics at The Cancer Institute of New Jersey carried out the statistical analysis. Comparison of the incidence of the V599E point mutation in melanoma samples from different stages of progression was performed using contingency tables and the χ^2 test with P s < 0.05 considered significant.

RESULTS

Determining the Sensitivity and Specificity of Site-Directed Mutagenesis. To determine sensitivity and specificity, we tested the site-directed mutagenesis procedure on plasmids containing either mutant or WT *B-RAF* cDNA, serially diluted from 10^9 to 10 copies/reaction as described in "Materials and Methods." Fig. 4 demonstrates that we were able to detect as few as 100 copies of mutant *B-RAF* sequence. Moreover, up to 10^{11} copies/reaction WT sequence produced no detectable mutant-specific bands when digested with *Alw26I* (data not shown). Next, we tested plasmids that contained either a WT *N-RAS* cDNA or one of the Q61 mutations and were able to detect as few as 100 copies of either the Q61R or the Q61K mutation. Furthermore, no detectable mutant-specific band was produced when as many as 10^{11} copies/reaction WT sequence were used in PCR reactions and digestion with *SfuI* or *BcgI* (data not shown).

Incidence of *B-RAF* and *N-RAS* Mutations in Melanoma. We used the site-directed mutagenesis technique to detect V599E *B-RAF* mutations and the Q61K and Q61R *N-RAS* mutations in 85 clinical melanoma samples. We found that 33 of the 85 samples (7 of 25 primary tumors, 15 of 25 regional dermal metastases, 5 of 25 nodal metastases, and 6 of 10 distant metastases) had the V599E mutation (39%). None of the sam-

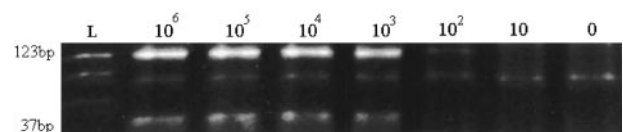


Fig. 4 Agarose gel electrophoresis of serially diluted plasmids containing the V599E mutant sequence. Dilutions are from 10^6 to 10 copies/reaction. L = Ladder; 0 = no plasmid in the reaction. A 123-bp band is visible at a concentration as low as 10^2 plasmids/reaction.

ples with a V599E mutation contained a Q61K or Q61R *N-RAS* mutation. Of the 85 samples tested, we found that 17 (20%) contained a *N-RAS* mutation (5 primary tumors, 7 regional dermal metastases, 3 nodal metastases, and 2 distant metastasis), 12 contained a Q61R mutation, and 5 contained a Q61K mutation. Therefore, either a *N-RAS* or *B-RAF* mutation was seen in 50 of 85 samples tested (59%). Thirty-five of the 85 melanoma samples contained neither of the Q61 *N-RAS* mutations nor the V599E *B-RAF* mutation in this study (41%). There was a significant difference in the incidence of the V599E *B-RAF* mutation between melanoma samples representing different stages of progression with regional dermal metastatic lesion and distant metastases having a higher incidence of the V599E mutation than primary tumors or nodal metastatic lesions ($P < 0.01$).

Downstream Effects of Activating MAPK Mutations in Cell Lines. We used anti-phospho-MAP/ERK kinase and ERK antibodies (9–12) to detect MAPK activation in cell lines known to be WT for *N-RAS* and *B-RAF* [human umbilical vascular endothelial cells and SK-Mel 31 melanoma cells (1)], mutant in *N-RAS* [SK-MEL 2 melanoma cell line (1)], or mutant for the V599E *B-RAF* mutation [HT-144, Mel 501, and A2058 melanoma cell lines (1)]. The level of phosphorylation of MAP/ERK kinase and ERK in the WT cell lines was used as the

baseline level. Mutant cell lines had different levels of increase of phosphorylated MAP/ERK kinase and ERK over the WT cell lines (Fig. 5A).

Downstream Effects of Activating MAPK Mutations in Clinical Specimens. The same anti-phospho-ERK antibodies were used to detect MAPK activation in clinical melanoma specimens (Fig. 5B). We found increases in the phosphorylated forms of ERK in melanoma samples containing the V599E *B-RAF* or one of the two Q61 *N-RAS* mutations as compared with specimens containing WT alleles.

DISCUSSION

We confirm the findings of Davies *et al.* (1) that activating point mutations in components of the MAPK pathway are common in human melanoma. We also demonstrate that these activating point mutations lead to increased levels of the phosphorylated form of ERK 1 and 2 in clinical melanoma samples (Fig. 5). ERK activation is thus likely to play an important role in the pathogenesis of melanoma. Activated ERK can phosphorylate a number of cytoplasmic targets and can enter the nucleus and activate many different transcription factors (9, 10, 13, 14). The MAPK signaling cascade controls many different cellular functions, including growth, apoptosis, and differentiation (10,

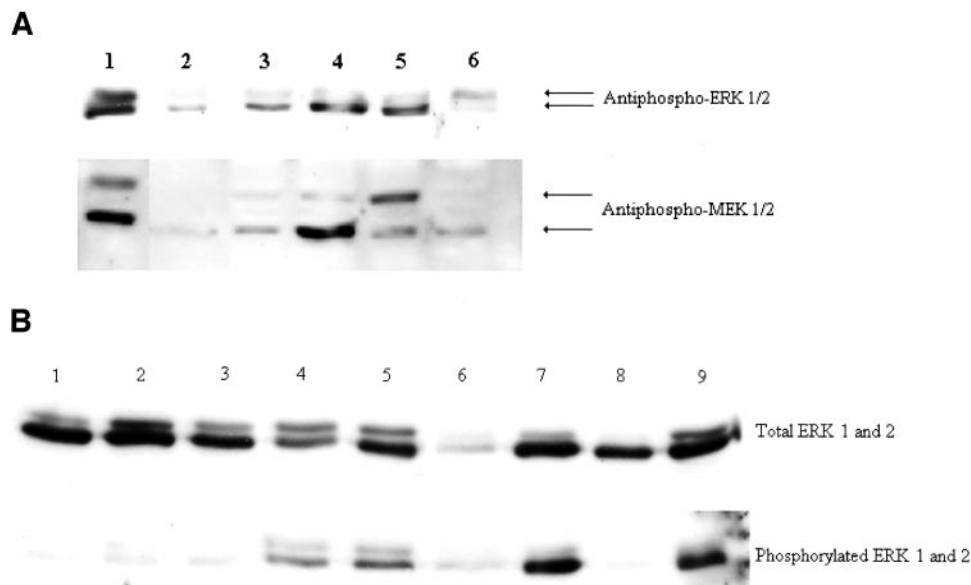


Fig. 5 A, Western blot using protein extracted from cell lines with either wild-type *N-RAS* and *B-RAF* or a mutation in one or the other of these genes. Antibodies against extracellular signal-regulated kinase (ERK) 1/2 (top panel) or mitogen-activated protein/ERK (MEK) 1/2 (bottom panel) were used. SK-Mel 31 melanoma cells (Lane 2) and human umbilical vascular endothelial cells (Lane 6) are known to be wild type for both *N-RAS* and *B-RAF* and are used as the baseline level of phosphorylated MEK and ERK. A2058 melanoma cells (Lane 3), HT-144 melanoma cells (Lane 4), and MEL 501 melanoma cells (Lane 5) have known V599E *B-RAF* mutations and show varying degrees of increased levels of phosphorylated MEK and ERK over baseline. SK-Mel 2 cells (Lane 1) have a known *N-RAS* mutation (Q61R) and shows a similar increase in the phosphorylated forms of MEK and ERK as the cell lines with V599E *B-RAF* mutations. Arrows indicate the two bands representing ERK1 and 2 (top panel) and the two bands that represent MEK1 and 2 (bottom panel). Loading controls were run demonstrating equal protein loading in each lane (data not shown). B, Western blot using protein extracted from seven human melanoma specimens. Tumors 1–3 were found to be wild type in both B-Raf and N-Ras and show little increase of the phosphorylated forms of ERK 1 and 2. Tumors 4 and 5 were found to harbor the V599E B-Raf mutation and each shows increased phosphorylation of ERK 1 and 2. Tumor 6 harbors a Q61K N-Ras mutation and tumor 7 harbors a Q61R N-Ras mutation and both demonstrated increase of the phosphorylated form of ERK 1 and 2. Lane 8 is a negative control (MCF-7 cell line) and Lane 9 a positive control (HT-144 cell line, known V599E mutant). Total ERK 1 and 2 staining demonstrates that increases in total ERK 1 and 2 do not account for the increase seen in the phosphorylated forms.

13, 15). Because the constitutive activation of the MAPK pathway can affect many different cellular processes, it likely plays a pivotal role in the oncogenic activity of melanoma.

We found that 39% of our clinical melanoma specimens harbored a V599E *B-RAF* mutation and 20% harbored one of the two known Q61 *N-RAS* mutations, demonstrating that ~60% of our melanoma specimens contain one of these mutations. The incidence of *N-RAS* mutations in this study was similar to past studies (2). The incidence of the V599E *B-RAF* mutation that we found in this study is lower than reported by Davies *et al.* (1) who found 5 of 9 melanoma specimens and 11 of 15 short-term melanoma cultures contained a point mutation in *B-RAF* with ~80% occurring as the V599E mutation (1). Our study examines a significantly larger number of melanoma specimens, and we have included specimens from different levels of progression. We found that primary tumors (7 of 25, 28%) and nodal metastasis (5 of 25, 20%) had a lower incidence of the V599E mutation than did regional dermal metastases (15 of 25, 60%) or distant metastases (6 of 10, 60%; $P < 0.01$). The incidence of the V599E mutation that we found in the regional dermal metastases and the distant metastatic lesions is similar to the incidence of this mutation found by Davies *et al.* (1) in their specimens. Furthermore, this incidence of this mutation in distant metastases is similar to that recently found by another group (16). Dong *et al.* (16) examined distant metastatic lesions of melanoma and found that 8 of 13 specimens harbored a *B-RAF* mutation (62%).

There are many reasons why we may have found a lower incidence of the V599E mutation in primary melanomas and nodal metastases than originally reported by Davies *et al.* (1). First, we only screened our specimens for the V599E *B-RAF* and Q61R and Q61K *N-RAS* mutations. The site-directed mutagenesis detection method can only detect the specific mutation that is being screened for, and the less common *B-RAF* mutations reported by Davies *et al.* (1) would be missed using this method. Furthermore, Dong *et al.* (16) also reported that only 10% of radial growth phase primary melanoma specimens in their study harbored a *B-RAF* mutations, whereas the incidence of these mutations in vertical growth phase specimens was 63% (16). Because we did not distinguish between vertical and radial growth phase primary melanoma specimens in our study, we likely included a mixture of samples representing both phases of growth. This may also account for the lower than expected incidence of *B-RAF* mutations in our study. Another recent report by Thomas *et al.* (17) documents the occurrence of tandem *B-RAF* mutations in primary human melanomas. They examined 18 primary melanoma specimens and found that 3 (17%) harbored the V599E *B-RAF* mutation and 3 (17%) harbored one of three different tandem mutations in the same codon. We are currently designing site-directed mutagenesis methods to detect the less common single and tandem *B-RAF* mutations and have begun to group our primary melanoma specimens by phase of growth. We will soon reexamine our bank of melanoma specimens to determine the incidence of all of the different *B-RAF* mutations in human melanoma. It is also possible that mutations in components of the MAPK pathway occur at different frequencies at different stages of melanoma progression. We are screening a larger number of melanoma specimens representing different stages of progression from

individual patients in an effort to better understand the relationship between MAPK mutations and progression in melanoma.

Activating mutations in the MAPK pathway may play a prominent role in the development of melanoma. Therefore, targeting this pathway with specific therapies becomes an important therapeutic goal. Clinical trials will require a specific and sensitive method to detect these mutations. However, sequencing is expensive, time consuming, and requires sophisticated equipment and trained staff and personnel. Single-strand conformation polymorphism and denaturing gradient gel electrophoresis are two other methods used to detect gene mutations in tumor specimens and their relative ease and low cost have made them widely used for prescreening specimens before sequencing. However, the sensitivity and specificity of single-strand conformation polymorphism and denaturing gradient gel electrophoresis have been questioned and the need to confirm the presence of the mutation by sequencing limits the usefulness of these methods (18–20). Detecting the mutant protein with specific antibodies using either immunohistochemical or Western blotting techniques would also be useful. Immunohistochemistry would be especially useful because screening of archival, paraffin-embedded specimens would then be simplified. We describe a new method of screening for MAPK mutations based on the creation of new restriction sites in the *B-RAF* and *N-RAS* cDNA by site-directed mutagenesis. This technique is simple, uses inexpensive techniques, and multiple samples can be screened quickly. It is also sensitive; we were able to detect as few as 100 copies of the mutant sequences using this technique, even against a high background of WT sequence. Furthermore, this technique is specific; we obtained no false positive bands from known WT tissues and from WT plasmid concentrations as high as 10^{11} copies/reaction. These qualities make this method suitable for use in clinical specimens in which tissue is limited and contains a mixture of tumor and normal cells. We are currently adapting this technique for use in paraffin-embedded tissues. Mutations in *B-RAF* and *N-RAS* should be detectable in archival tissues using this technique, making screening of large numbers of archival specimens possible. Treatment protocols that target MAPK pathway components have been examined in many different tumor types (9, 21–24). Many of the methods that have been used to target this pathway in other types of cancer will soon be tested in patients with melanoma. It will thus be important to be able to stratify patients by MAPK component mutation status in these protocols and our site-directed mutagenesis technique is one method that is likely to prove useful in these studies.

In summary, we have shown that *B-RAF*- and *N-RAS*-activating point mutations are frequent in human melanoma using a novel, sensitive, and specific method of detection.

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