

***BRAF* and *RAS* Mutations in Human Lung Cancer and Melanoma¹**

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ABSTRACT

***BRAF* encodes a RAS-regulated kinase that mediates cell growth and malignant transformation kinase pathway activation. Recently, we have identified activating *BRAF* mutations in 66% of melanomas and a smaller percentage of many other human cancers. To determine whether *BRAF* mutations account for the MAP kinase pathway activation common in non-small cell lung carcinomas (NSCLCs) and to extend the initial findings in melanoma, we screened DNA from 179 NSCLCs and 35 melanomas for *BRAF* mutations (exons 11 and 15). We identified *BRAF* mutations in 5 NSCLCs (3%; one V599 and four non-V599) and 22 melanomas (63%; 21 V599 and 1 non-V599). Three *BRAF* mutations identified in this study are novel, altering residues important in AKT-mediated *BRAF* phosphorylation and suggesting that disruption of AKT-induced *BRAF* inhibition can play a role in malignant transformation. To our knowledge, this is the first report of mutations documenting this interaction in human cancers. Although >90% of *BRAF* mutations in melanoma involve codon 599 (57 of 60), 8 of 9 *BRAF* mutations reported to date in NSCLC are non-V599 (89%; $P < 10^{-7}$), strongly suggesting that *BRAF* mutations in NSCLC are qualitatively different from those in melanoma; thus, there may be therapeutic differences between lung cancer and melanoma in response to *RAF* inhibitors. Although uncommon, *BRAF* mutations in human lung cancers may identify a subset of tumors sensitive to targeted therapy.**

INTRODUCTION

Identification of gene mutations in human cancers can lead to the development of effective targeted therapies (1). We reported recently that activating *BRAF* mutations are present in 66% of melanomas and a small percentage of NSCLC³ cell lines (2). In melanoma, most *BRAF* mutations involved codon 599 in the activation segment of the kinase domain (exon 15; Fig. 1); however, four of four *BRAF* mutations found in NSCLCs were non-V599 (exons 11 and 15). Interestingly, none of the 3 of 43 cancer cell lines with both *BRAF* and *RAS* mutations had a codon 599 *BRAF* mutation [one each in NSCLC (*NRAS* and Q61K), colorectal (*KRAS* and G13D), and ovarian cancer lines (*KRAS* and G13D; Ref. 2)]. This (2) and a subsequent report (3) provide evidence that *BRAF* kinase mutations are present in 10% of colon cancers overall and in 31% of mismatch repair-deficient tumors ($P < 10^{-6}$; Ref. 3). In the latter series of colon cancers, *KRAS* and

BRAF mutations were mutually exclusive ($P < 10^{-6}$); our current and previous series have inadequate power to evaluate this interaction.

ERK1 and ERK2, downstream effectors of the RAS-RAF-MEK-ERK-MAP kinase pathway, are constitutively active in many NSCLCs and melanomas (4, 5). MAP kinase pathway activation in these tumors has not been associated with mutations in *ERK1/2* or *MEK* but rather is attributable to constitutive phosphorylation of these proteins. Thus, activating mutations in *BRAF* could explain activation of MEK and ERK1/2 in both melanoma and NSCLC. To follow up on the observation that NSCLCs (particularly adenocarcinomas) may have an inverted ratio of V599/non-V599 *BRAF* mutations as compared with melanoma (2) and to investigate the role of *BRAF* mutations in the unexplained activation of the MEK-ERK-MAP kinase pathway in lung cancer, we evaluated the frequency of *BRAF* kinase domain mutations in primary NSCLCs and early passage melanoma cell lines. On the basis of the initial observation that one NSCLC cell line with a *BRAF* mutation had an *NRAS* mutation (2), we also screened the NSCLCs and melanomas for codon 61 *NRAS* and codon 12/13 *KRAS* mutations, which may be found in these tumors.

MATERIALS AND METHODS

DNA Isolation. DNA was obtained from 35 early passage melanoma cell lines, 2 snap-frozen metastatic melanomas, and 139 snap-frozen lung tumors (>40% tumor) using the Wizard Genomic Purification Kit (Promega). DNA from 165 paraffin-embedded lung tumor specimens was prepared from 10 × 30- μ m sections after macrodissection, resulting in selection of $\geq 90\%$ tumor cells. DNA was isolated after xylene extraction and proteinase K digestion as described previously (6).

Mutation Screening and Confirmation. Genomic DNA was screened for mutations using conformation-sensitive capillary electrophoresis as described previously (2). The following intron-based PCR primers were designed to amplify the exons of interest and the associated splice junctions:

KRAS exon2:F-GTGTGACATGTTCTAATATAGTCA.
KRAS exon2:R-GAATGGTCCTGCACCAAGTAA.
NRAS exon3:F-GGTGAAACCTTTGTGGGA.
NRAS exon3:R-AACCTAAAACCAACTTCCCA.
BRAF exon11:F-TCCCTCTCAGGCATAAGGTAA.
BRAF exon11:R-CGAACAGTGAATATTTCTTTTGTAT.
BRAF exon15:F-TCATAATGCTTGCTCTGATAGGA.
BRAF exon15:R-GGCCAAAAATTTAATCAGTGGGA.

The template consisted of 120 ng of genomic DNA. PCR reactions were performed using standard PCR conditions [95°C × 15 min; 95°C × 45 s, 56°C (RAS) or 58°C (BRAF) × 45 s, 72°C × 45 s, for 40 cycles; then 72°C × 10 min, 50- μ l reactions] with fluorescence-labeled primers. We combined 3 μ l of tumor PCR product and 3 μ l of wild-type PCR product in a total volume of 30 μ l of 1 × PCR buffer to ensure heteroduplex formation in the event of allele loss in a tumor. Heteroduplex formation was performed as follows: 96°C for 10 min, 72 cycles of 96°C for 20 s decreasing temperature by 0.5°C/cycle, followed by incubation at 60°C for 30 min, and then cooling to room temperature. The samples were analyzed on an ABI PRISM 3100 automated capillary sequencer under semidenaturing conditions using polymer provided by ABI

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³ The abbreviations used are: NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma.

BRAF

Kinase Domain

G-Loop Activation Segment

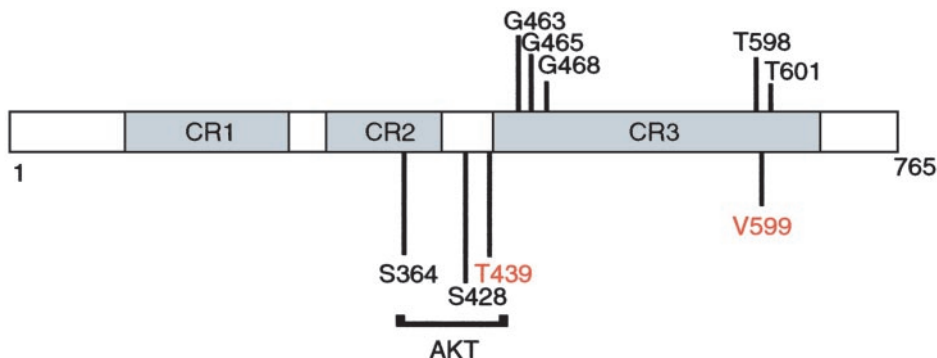


Fig. 1. Schematic drawing of *BRAF* showing the location of the G-loop (exon 11) and activation segment (exon 15) of the kinase domain. Additionally shown are the invariant glycine residues that make up the backbone of the glycine loop (G463, G465, and G468) and phosphorylation sites in the activation segment (T598 and T601). AKT-phosphorylated residues are as shown (S364, S428, and T439). Codon 599 and the Thr439 AKT phosphorylation site are highlighted in red.

and optimized run conditions (2). Data were captured using GeneScan to identify samples with a shift in peak migration relative to a wild-type control, indicating the presence of a putative sequence variation. PCR products selected by the presence of a heteroduplex shift were gel purified and sequenced on the ABI 3100 to confirm the presence of a mutation.

DNA was prepared from 292 NSCLCs, 12 neuroendocrine lung cancers, and 35 melanomas. Amplicons of all four exons (*BRAF* exons 11 and 15, *KRAS* exon 2, and *NRAS* exon 3) were obtained from 158 NSCLCs, 10 neuroendocrine lung cancers, and 33 melanomas (Table 1). Data on both *BRAF* exons were available for 179 NSCLCs, 11 neuroendocrine lung cancers, and 35 melanomas (Table 1). Mutation frequencies did not vary significantly when tumors lacking data on one *BRAF* exon were included in the analysis (“All results” versus “Complete Sets;” Fig. 2). Tumor DNA from failed reactions was re-purified, and a minimum of three attempts was made to amplify the exon. Success rates using DNA from frozen tissue and cell lines were almost 100%, but amplification of DNA from archival material was inconsistent. Of the 165 paraffin-embedded lung tumors, 30 failed to yield any amplifiable DNA, and 54 yielded DNA that could not be amplified using at least one primer pair. In only three paraffin-embedded lung cancers, neither *BRAF* exon, but both *RAS* exons, amplified, suggesting that frequent large genomic deletions involving *BRAF* are an unlikely explanation for these results. PCR failures were not consistently attributed to a specific primer pair, suggesting they are a result of random cross-linking during fixation that interferes with primer annealing.

RESULTS AND DISCUSSION

BRAF mutations were identified in 5 NSCLCs (3%), *KRAS* mutations in 14 of 147 adenocarcinomas (10%), and *NRAS* mutations in none (0%; Fig. 2). No NSCLC had both a *BRAF* and a *RAS* mutation, but the number of mutations identified in either gene is too small to evaluate a genetic interaction. *BRAF* mutations were identified in 22 of 35 (63%) melanomas, 1 in exon 11, and the remaining 21 in exon 15 (Fig. 2 and Table 2). Twenty of 21 exon 15 mutations involved

codon 599. One *NRAS* mutation of uncertain significance (S65C) was identified in a melanoma that also had a *BRAF* mutation (V599E); no *KRAS* mutations were identified in the melanomas.

In our previous study, the initial analysis of six NSCLC cell lines yielded two *BRAF* mutations (neither V599), one in exon 15 and one in the exon 11 G-loop (2). That prompted analysis of 125 additional NSCLC cell lines and 14 primary lung tumors. Two more *BRAF* mutations were identified in the second analysis, for a total of 4 of 131 mutations in NSCLC cell lines but none in the primary tumors. Interestingly, zero of four *BRAF* mutations identified in NSCLC cell lines were V599 mutations (2). These data were in contrast to that from melanomas, where 100% of the melanoma cell lines and 91% of primary melanoma mutations involved V599, raising the possibility that *BRAF* mutations in NSCLC are qualitatively different from those in melanoma. Additionally of interest, all four *BRAF* mutations identified in NSCLC cell lines were in cell lines derived from adenocarcinomas, with none identified in those from SCCs. Given these findings, we evaluated data from the present study for codon mutation frequency between NSCLC and melanoma and for differences between adenocarcinomas and SCCs of the lung.

In this series, we identified five NSCLCs with mutations in *BRAF*, only one of which involves V599. Taken together with data from the previous study (2), we have identified nine *BRAF* mutations in NSCLCs, eight of which are non-V599 ($P < 10^{-7}$). These data provide a high level of statistical support to the hypothesis that *BRAF*-related tumorigenesis in NSCLC is qualitatively different from that in melanomas with codon 599 mutations, raising the possibility that therapeutic response to RAF inhibitors may be different in these two tumor types, despite the presence of activating *BRAF* mutations in both.

Of the 104 adenocarcinomas evaluable for both *BRAF* exons, two

Table 1 Tumors prepared and analyzed for *BRAF* and *RAS* mutations

Tissue type	Total DNA prepared	<i>BRAF</i> exons 11 and 15	Complete sets <i>BRAF</i> exons 11 and 15, <i>KRAS</i> , <i>NRAS</i>
Non-small cell lung cancers (n = 292)			
Adenocarcinoma	171	104	89
SCC	117	72	67
Poorly differentiated adeno/squamous carcinoma	4	3	2
Total	292	179	158
Neuroendocrine lung tumors	12	11	10
Melanoma primary cell lines	35	35	33

	All Samples Tested			Complete Sets
	N	Mut	%	%
Braf Exon 11				
Lung Adenocarcinoma	112	2	2%	1%
Lung Squamous Cell Carcinoma	81	1	1%	1%
Lung Poorly Differentiated CA	2	0	0%	0%
Lung Neuroendocrine Carcinoma	12	0	0%	0%
Melanoma Cell Lines	35	1	3%	3%
Braf Exon 15				
Lung Adenocarcinoma	145	0	0%	0%
Lung Squamous Cell Carcinoma	94	2	2%	1%
Lung Poorly Differentiated CA	3	0	0%	0%
Lung Neuroendocrine Carcinoma	11	0	0%	0%
Melanoma Cell Lines	35	21	60%	58%
Kras Exon 2				
Lung Adenocarcinoma	147	14	10%	12%
Lung Squamous Cell Carcinoma	98	0	0%	0%
Lung Poorly Differentiated CA	3	1	33%	50%
Lung Neuroendocrine Carcinoma	12	0	0%	0%
Melanoma Cell Lines	35	0	0%	0%
Nras Exon 3				
Lung Adenocarcinoma	125	1	1%	0%
Lung Squamous Cell Carcinoma	87	0	0%	0%
Lung Poorly Differentiated CA	4	0	0%	0%
Lung Neuroendocrine Carcinoma	11	2	18%	20%
Melanoma Cell Lines	35	1	0.3%	0%

Fig. 2. Frequency of BRAF mutations. High-lighted mutation frequencies are discussed in the Results and Discussion section.

had novel mutations in exon 11. V458L is five codons upstream of the first glycine in the G-loop (Fig. 1). It is possible that this leucine residue changes the conformation of the adjacent G-loop (2), resulting in activation; however, this remains speculative. The second mutation, K438T, alters the basic residue next to a threonine phosphorylated by AKT (Fig. 1). BRAF has three AKT phosphorylation sites: (a) Thr439 is one of two unique to BRAF; (b) the other being Ser428; and (c) Ser364 is conserved in RAF1 (Ref. 7; Fig. 1). *In vitro* alanine substitution at Thr439 leads to BRAF activation through loss of

AKT-induced inhibition (7), with progressively increased BRAF activity as the additional two sites are mutated. The inhibitory effect of AKT-induced phosphorylation on RAF1 also has been demonstrated (8). Thus, K438T, as well as K438Q found in a melanoma as described below, is likely to inhibit AKT-dependent phosphorylation of the adjacent Thr439, as adjacent basic residues are commonly required in AKT Ser/Thr phosphorylation consensus sequences (9). These data suggest that transformation-related BRAF activation may occur through multiple mechanisms and that mutations of Thr439, as well as the two additional AKT consensus sites (Ser364 and Ser428; Fig. 1; Ref. 10), could play a role in tumorigenesis. Although AKT-BRAF cross-talk has been documented *in vitro*, to our knowledge, this is the first report of mutations involving this interaction in human cancers.

Fourteen of 147 lung adenocarcinomas had *KRAS* mutations (Fig. 2), all but one in codon 12, the exception being A18T, the significance of which is unknown. Constitutional DNA was not available from this patient; thus, a germ-line polymorphism cannot be excluded. The overall *KRAS* mutation frequency in this series of lung adenocarcinomas is 10%, lower than the 17% detected in NSCLCs in the previous series (2) and lower than the frequently cited rate of 30% (11, 12). Multiple studies suggest that the frequency of *KRAS* mutations in nonsmokers (and dogs) is in the range of 10% (11, 13–15). We have no information on the smoking status of the patients whose tumors were evaluated in our studies, but a bias toward young nonsmokers might occur because of various factors related to selectivity of referrals to a tertiary care hospital.

Table 2. BRAF and RAS mutations identified in lung cancers and melanomas

	BRAF exon 11	BRAF exon 15	KRAS2 exon 2	NRAS exon 3
Lung cancer				
Lung adenocarcinoma	V458L (1) K438T (1)		G12R (1) G12A (3) G12L (1) G12D (1) G12S (2) G12F (1) G12C (4) A18T (1)	S65R (1)
Lung SCC	T439P (1)	L596V (1) V599E (1)		
Lung poorly differentiated carcinoma			G12L (1)	
Lung neuroendocrine carcinoma				S65C (2)
Malignant melanoma	K438Q (1)	V599E (19) V599D (1) K600E (1)		S65C (1)

In the 117 SCC, we identified one novel mutation in exon 11 (T439P) and two mutations in exon 15 (L596V and V599E). As expected based on the existing literature, we did not identify *RAS* mutations in SCCs. The exon 15 *BRAF* mutations are both in the activation segment and cause *BRAF* activation (2). However, the exon 11 mutation is novel. This mutation alters Thr439, an AKT phosphorylation site (described above), further supporting the suggestion that transformation-related *BRAF* activation may occur through multiple mechanisms.

No *BRAF* or *KRAS* mutations were identified in the 12 neuroendocrine lung cancers, but two had codon 65 *NRAS* mutations (20%). Analysis of this mutation in a melanoma (described below) suggests this mutation may not be transforming. There are few data in the literature on the prevalence of *NRAS* mutations in neuroendocrine lung cancers, and our series is small as well, but additional investigation may be warranted.

Data from the 35 early passage primary melanoma cell lines confirm our previous report that the majority of melanomas have *BRAF* mutations (63% in this series), predominantly V599 (20 of 22, 91%; Fig. 2 and Table 2; Ref. 2). One of the two non-V599 mutations is in exon 15 and is in the adjacent residue (K600E). The other non-V599 mutation is in exon 11 (K438Q), similar to the novel K438T mutation identified in a lung adenocarcinoma. This mutation results in substitution of Lys438, likely required for AKT phosphorylation of the adjacent Thr439, thus likely to be an activating mutation (7, 8, 10).

No *KRAS* mutations and one *NRAS* mutation of unknown significance (S65C) were identified in the 35 melanomas. Specifically, no *NRAS* codon 61 mutations, reported in 15% of melanoma cell lines (16), were identified. The codon 65 *NRAS* mutation was further investigated in three additional melanoma cell lines from the same individual, all derived from axillary lymph node metastases. This mutation (S65C) is not present in the primary tumor nor in a metastatic deposit sampled in June 1983 and is present in only one of two cell lines established from lymph node biopsies performed 2 months later. These data provide evidence that this mutation is not a germ-line polymorphism, nor did it contribute to malignant transformation in this tumor; whether it is important in disease progression is unknown.

In summary, we have confirmed the high prevalence of *BRAF* mutations in melanoma, shown that *BRAF* exon 11 and 15 mutations are not a common cause of the MAP kinase pathway activation in NSCLC, and identified four novel *BRAF* mutations. These mutations provide the first *in vivo* evidence that activation of *BRAF* through loss of AKT-induced inhibitory phosphorylation is associated with tumorigenesis, and studies to understand this relationship may uncover additional therapeutic targets. We provide strong statistical evidence that *BRAF* mutations in NSCLCs are qualitatively different that those in melanoma, with possible therapeutic implications when considering *BRAF* inhibitors. These results define the frequency of *BRAF* mutations in NSCLC (2–3%) and in melanomas (>60%). In both cases,

future studies will determine whether these *BRAF* mutations represent promising new therapeutic drug targets for these chemoresistant tumors. Even if the *BRAF* mutations are rare, as in lung cancer, they may identify a tumor sensitive to targeted therapy, resulting in significantly improved outcomes for the patients that harbor them.

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