

Analysis of *BRAF* and *N-RAS* Mutations in Metastatic Melanoma Tissues¹

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ABSTRACT

We examined mutations in *BRAF* exons 11 and 15 and *N-RAS* exons 2 and 3, in 77 metastatic melanoma cases and 11 melanoma cell lines. Significant differences in the mutation rates observed at different metastatic sites could not be detected. The most frequent mutation, the V599E amino acid substitution in *BRAF* exon 15, was observed in 31 of 77 (40%) tissues and 5 of 11 (45%) cell lines. Tandem base-pair substitutions encoding V599R and V599K amino acid changes were observed in two cases. Novel findings with respect to melanoma include a cell line possessing a 2 base-pair substitution in *BRAF* exon 11 and a case harboring mutations in both *BRAF* exon 11 and *N-RAS* exon 3. Our data show that *BRAF* mutation is common in melanoma metastases, regardless of their site, that mutations include both exons 11 and 15, and suggest that anti-RAS/RAF strategies may be effective in metastatic melanoma patients.

INTRODUCTION

Novel treatment modalities are clearly needed to improve the dismal outcome of metastatic melanoma patients (1–3). In the treatment of solid tumors, strategies to target specific oncogenes are becoming increasingly important. Efficacy has been demonstrated for trastuzumab (Herceptin) in the treatment of metastatic breast cancer patients with HER-2 overexpression (4), and ST1571 (Gleevec), a tyrosine kinase inhibitor that targets the product of the *BCR-ABL* translocation found in chronic myeloid leukemia, as well as the constitutively active KIT receptor found in gastrointestinal stromal tumors (5). Activation of the RAS/RAF/MAPK³ pathway is a critical component of tumor cell proliferation and survival in general (6, 7) and specifically in melanoma (8–10). Until recently, mutational studies of the RAS/RAF/MAPK pathway in melanoma tissues were limited primarily to *RAS* genes, detecting mutation rates of *N-RAS* in 5–33% of cases (11–13). *BRAF*, a member of the *RAF* family of serine-threonine kinases, activates the MAPK cascade when it is bound to activated RAS proteins (6). Recent studies reported frequent *BRAF* mutation in melanoma cell lines and primary cultures (14, 15). Limited attention has been given to metastatic melanoma clinical specimens (16), which would be more representative of the specific patient population to be treated by anti-RAS/RAF targeted therapy. In this study, we focused on examining the frequency of *BRAF* and *N-RAS* mutations in a large cohort of metastatic melanoma tissues to determine whether patients with this stage of disease could be considered for clinical trials using anti-RAS/RAF-based strategies.

MATERIALS AND METHODS

Tumor Specimens and Cell Lines. Seventy-seven frozen metastatic melanoma samples and corresponding normal tissues were retrieved from the Memorial Sloan-Kettering Cancer Center Tumor Bank. Pathology information, excluding patient-identifying information, was reviewed to determine the anatomical site of metastatic tumor. Fifty of 77 lesions (65%) were from lymph nodes or soft tissue sites; 27 of 77 (35%) were from visceral sites. The Institutional Review Boards of both Memorial Sloan-Kettering Cancer Center and New York University approved the conduct of this study. Human metastatic melanoma cell lines (SK-MEL 19, 29, 85, 94, 100, 103, 147, 173, 187, 192, and 197) were maintained in culture using DMEM with 10% FCS and 1% penicillin/streptomycin (BioWhittaker).

Mutation Detection. DNA was extracted from tumor tissues and cell lines using the QIAgen Tissue Kit (Qiagen, Valencia, CA). *BRAF* exons 11 and 15, and *N-RAS* exons 2 and 3, were amplified by PCR using QIAgen HotStar Taq. The primer sequences for *BRAF* exons 11 and 15 have been published (14); the primer sequences and annealing temperatures used for analysis of *N-RAS* were:

Exon 2: Ta = 63°C

F: 5'-CTGGTTTCCAACAGGTTCTTGC;

R: 5'-CTACCACTGGGCCTCACCT

Exon 3: Ta = 53°C

F: 5'-CATACTGGATACAGCTGGAC;

R: 5'-TGACTTGCTATTATTGATGG, or, Ta = 58°C

F: 5'-GGTGAAACCTGTTTGTGGGA;

R: 5'-ATACACAGAGGAAGCCTTCG

PCR products were subjected to direct sequencing using an ABI PRISM 310 Genetic Analyzer. The resulting traces were analyzed using SeqScape software (Applied Biosystems, Foster City, CA) to identify mutations. Mutations were confirmed by repeat PCR and sequencing using a different primer. For one case containing a 2-bp substitution in *BRAF* exon 15, PCR products from four separate reactions were cloned into Topo TA (Invitrogen, Carlsbad, CA), and 17 colonies were sequenced.

RESULTS

Overall, mutations in *BRAF* exons 11 and 15, and *N-RAS* exons 2 and 3, were detected in 36 of 77 (47%) tissues and 8 of 11 (73%) cell lines (Table 1). Among metastatic sites, mutations were seen in 9 of 16 (56%) visceral sites, 14 of 28 (50%) lymph node metastases, 5 of 11 (45%) lung metastases, and 8 of 22 (36%) soft tissue metastases. There was no statistically significant difference between these frequencies (χ^2 , $P = 0.65$). Importantly, no germ-line mutations were identified in normal tissue samples obtained from the patients whose tumors sustained *BRAF* or *N-RAS* mutations ($n = 36$; Table 1).

The most frequent mutation was the T1796A substitution, resulting in the V599E amino acid change in *BRAF* exon 15. This mutation was detected in 31 of 77 (40%) tissues and 5 of 11 (45%) cell lines. Tandem bp substitutions, GT1795-96AG and GT1795-96AA, which encode V599R and V599K amino acid changes, respectively, were detected in two other melanoma tissues (Fig. 1A). Cloning and sequencing of PCR products from the GT/AG tandem mutation confirmed that this case did not possess a compound heterozygous mutation. Both of these tandem mutations occurred in lung metastases. In the cell lines, all five mutations were the T1796A substitution (Table 1). Among specimens that contained an exon 15 mutation, no *BRAF* exon 11 or *N-RAS* mutations were observed.

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³ The abbreviation used is: MAPK, mitogen-activated protein kinase.

Table 1 Characteristics of BRAF and N-RAS mutations in melanoma tissues and cell lines

A. Metastatic site	n	Total mutant (%)	Mutation data		
			n	BRAF	N-RAS
Lymph node	28	14 (50)	14	V599E	WT
Soft tissue	22	8 (36)	7	V599E	WT
Lung	11	5 (45)	1	WT	Q61R
			3	V599E	WT
			1	V599R	WT
			1	V599K	WT
Visceral (except lung)	16	9 (56)	7	V599E	WT
			1 ^a	G468R	Y64N
			1	WT	Q61L
Total metastases	77	36 (47)			
Paired normal tissues ^b	36	0 (0)			

B. Cell line	BRAF	N-RAS
19	V599E	WT
29	V599E	WT
85	G468S	WT
94	V599E	WT
100	V599E	WT
103	WT	Q61R
147	WT	Q61R
173	WT	WT
187	WT	WT
192	V599E	WT
197	WT	WT

^a Both mutations in the same case.

^b Normal tissue from patients whose tumors contained BRAF or N-RAS mutations.

Mutations in BRAF exon 11 were observed, albeit rarely. One tissue sample harbored a G1402A point mutation (Fig. 1B), which encodes a G468R substitution. This mutation was accompanied by a mutation in N-RAS exon 3, T190A, which encodes a Y64N substitution. Among the cell lines, SK-MEL 85 contained a tandem bp substitution in BRAF exon 11, GG1402/1403TC, encoding a G468S amino acid change (Fig. 1C). Interestingly, at this location, a single substitution of G1402T would have resulted in a premature termination, resulting in no BRAF protein expressed. Two of 77 samples had nucleotide changes in exon 11 (G1329A) that did not result in amino acid changes. These noncoding DNA sequence changes were not observed in DNA from normal tissues in these patients.

For N-RAS, 3 of 77 (4%) tissues and 2 of 11 (18%) cell lines

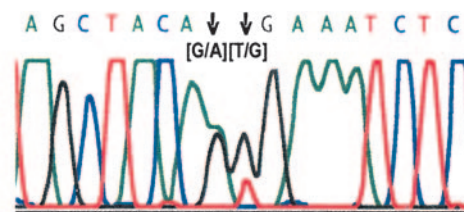
harbored mutations (Table 1). Besides the aforementioned T190A transversion that occurred in the metastatic lesion possessing the BRAF exon 11 mutation, two mutations, A182G and A182T, producing Q61R and Q61L substitutions were also observed. In both of these cases, BRAF exon 11 was wild type. The two N-RAS mutations found in the melanoma cell lines were Q61R. No mutations were observed in N-RAS exon 2 in either the cell lines or tissue samples.

DISCUSSION

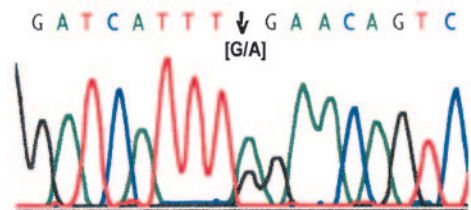
We observed a high rate of alteration to the RAS/RAF/MAPK pathway in metastatic melanoma tissues and cell lines. This was primarily the result of a high mutation rate in the BRAF exon 15 activation domain, accounting for 33 of 36 (92%) mutations detected. Thirty-one of 33 mutant cases encoded the V599E amino acid substitution. These results do not differ substantially from previous studies using cell lines and short-term cultures (14, 15) or a smaller cohort of metastatic cases (16). Two of the 33 mutant cases harbored tandem bp mutations that encoded V599R and V599K amino acid substitutions. Both cases were lung metastases, accounting for 2 of 5 mutant tumors at this site. These tandem mutations have been observed previously, but their site of occurrence was not mentioned (16).

Our analysis did not reveal a significant association between BRAF mutations and specific sites of metastases (lymphatic versus hematogenous). It is possible that BRAF mutations may occur early in melanocyte transformation, contributing to proliferation and survival, but not to metastatic spread. This might explain why the rates of BRAF mutation do not differ significantly between metastases from different sites. This postulate is supported by the report of Pollack et al. (16), who studied BRAF exon 15 mutations in a collection of melanocytic neoplasms, including metastatic melanomas and nevi (benign proliferations of melanocytes). They observed mutations in 37 of 55 (67%) metastases and 63 of 77 (82%) nevi. In addition, mutant N-RAS genes have been reported in nevi, ranging from 14 to 56% of cases (16, 17). Interestingly, the vast majority of nevi are quite stable once they are formed, suggesting that many of these skin lesions may be senescent, as is observed when oncogenic RAS is introduced into otherwise normal cells (18). It should be noted that both the current

A) BRAF 15 Two Base Change



B) BRAF 11 Single Mutation



C) BRAF 11 Two Base Change

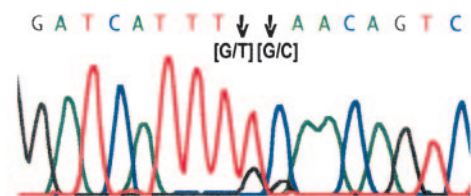


Fig. 1. Sample electropherograms showing mutations in the BRAF gene. A, tumor-derived tandem bp substitution in exon 15, GT1795-96AG, encoding the V599R amino acid change; B, tumor-derived sequence from exon 11 containing the single bp substitution, G1402A, encoding the G468R amino acid change; C, tumor-derived tandem bp substitution, GG1402/1403TC, encoding a G468S amino acid change.

study and study of Pollock *et al.* are limited by relatively small sample sizes for each metastatic site. Larger and more adequately powered studies using prospectively gathered materials to reduce possible selection biases would be required to confidently detect significant differences in the *BRAF* mutation rates at different metastatic sites, should they exist.

Novel observations in the current study were the individual case and single cell line with mutations in the *BRAF* exon 11 kinase domain. Although published reports have documented mutations at amino acid 468, the specific amino acid substitutions in this report (G468R, detected in one visceral metastasis, and G468S, observed in one cell line) have not been reported previously in melanoma. Interestingly, the G468R mutation was accompanied by a mutation in *N-RAS*, Y64N. A similar finding, mutation at *BRAF* amino acid 463 accompanied by a *K-RAS* mutation, was observed by Davies *et al.* (14) in two colorectal and ovarian cell lines. As they speculated, it is likely that the *BRAF* exon 11 mutation does not provide sufficient stimulus to the MAPK pathway and that the addition of RAS activity is required for adequate activation. In contrast, we detected the G468S mutation in a cell line with no accompanying *N-RAS* mutation, suggesting that the G468S mutation is sufficiently active that additional RAS activity is not required, or, that alterations to other components of this pathway exist in this cell line. Among the eight cell lines with mutations to either *BRAF* or *N-RAS*, no line had more than one mutation of any of these genes, suggesting that the activity of these mutant proteins provides sufficient activation to the MAPK pathway.

In conclusion, *BRAF* mutations occur frequently in metastatic melanoma tissues and may include both exons 11 and 15. *N-RAS* mutations were less common. In the large subset of patients whose metastases possess mutated *BRAF* proteins, strategies that either block *BRAF* function or accelerate its degradation (*e.g.*, 17AAG) have significant potential to impact favorably on the otherwise dismal course of their disease.

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