Incidence of *BRAF* Oncogene Mutation and Clinical Relevance for Primary Cutaneous Melanomas

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ABSTRACT

Purpose: **The purpose of the study was to clarify the incidence of *B-raf* oncogene (*BRAF*) mutations in primary cutaneous melanomas, their relation to tumor progression, and effect on disease outcome. Somatic mutations of BRAF kinase, a component of the *Ras*-mitogen-activated protein/extracellular signal-regulated kinase kinase-mitogen-activated protein kinase pathway, are frequently reported (>65%) in nevi and malignant melanomas.**

Experimental Design: W**e assessed *BRAF* mutation frequency in exons 11 and 15 in primary ($n = 59$) and metastatic ($n = 68$) melanomas. Direct sequencing of PCR products was performed on DNA isolated and purified from microdissected tumors.**

Results: **Eighteen mutations (31%) at exon 15 were detected in primary melanoma with a significantly ($P = 0.001$) higher frequency in patients < 60 years old. Incidence of *BRAF* mutation did not correlate with Breslow thickness. Presence of *BRAF* mutation of primary tumors did not effect overall disease-free survival. *BRAF* mutation frequency in metastatic lesions was 57% and significantly ($P = 0.0024$) higher than primary melanomas.**

Conclusions: **The study suggests that *BRAF* mutation may be acquired during development of metastasis but is not a significant factor for primary tumor development and disease outcome.**

INTRODUCTION

Sequential genetic aberrations have been correlated with the development and progression of a variety of tumors but are not well understood in cutaneous melanoma. There are two major hypotheses for the transformation of normal melanocytes to primary melanomas: (*a*) one is that melanomas originate from nevi; and (*b*) the other is that melanomas develop *de novo, i.e.*, transformation of normal melanocytes (1, 2). To date, no significant genetic changes have been identified to support either one of the hypotheses. Recently, *B-raf* oncogene (*BRAF*) mutation was found in >65% of melanomas (3, 4), 82% of 77 nevi of diverse histopathologies (5), and >65% of primary melanomas (3). This has led to the controversy of whether *BRAF* mutations may be acquired during primary tumor progression and metastasis development. Because *BRAF* mutation frequency in melanoma has been reported to be essentially the same among nevi, primary tumors and metastases (3, 5), it would be important to investigate *BRAF* mutations in relation to tumor progression and their effects on disease outcome.

The mitogen-activated protein kinase (Ras-mitogen-activated protein/extracellular signal-regulated kinase kinase-mitogen-activated protein kinase) pathway is a membrane-to-nucleus signaling system that controls cell proliferation, differentiation, and apoptosis in mammalian cells (6). The pathway activation has been associated with melanocyte and melanoma proliferation (7, 8). In this pathway, phosphorylation of *Raf* is followed by sequential activation of mitogen-activated protein/extracellular signal-regulated kinase kinase extracellular signal-regulated kinase and mitogen-activated protein kinase. In vertebrates, there are three known Raf proteins: (*a*) *A-Raf*; (*b*) *BRAF*; and (*c*) *C-Raf*. *BRAF* encodes a serine/threonine kinase, which is a key factor in the mitogen-activated protein kinase pathway for transduction of signals from the oncogene *Ras* (7, 8). *BRAF* mutations have been reported in melanoma and various carcinomas (3, 4, 9, 10). The mutation frequency was highest in melanomas and predominantly in exon 15 V600E (3). *BRAF* mutation occurs at two different sites in the kinase domain. A 1799T→A transversion in exon 15 of *BRAF* results in a V600E amino acid missense mutation in 92% of melanomas with *BRAF* mutation (3). Because this mutation significantly increases kinase activity (3, 8, 11), it can lead to continuous transcription-mediated proliferation, which supports neoplastic growth. The frequency of *BRAF* mutations significantly exceeds the frequency of known mutations of other major genes in cutaneous malignant melanomas, such as N-*Ras*, $p16^{INK4a}$, and *p53*.

We investigated the timing of *BRAF* mutations in melanoma progression by correlating the frequency of mutations in primary melanomas and metastases with known prognostic factors. Our studies demonstrate that the frequency of *BRAF* mutations is significantly lower in primary melanomas than in metastases, and it is significantly more common in younger patients. We also found *BRAF* mutation occurrence to be independent of Breslow thickness of the primary melanomas, may be acquired during metastasis, and does not correlate to disease outcome when the mutation is found in the primary tumor lesion.

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MATERIALS AND METHODS

Specimens. Fifty-nine primary melanoma tumor specimens were assessed from 41 males and 18 females who underwent surgical treatment of clinically localized cutaneous melanoma. Human subjects Institutional Review Board approval was obtained for the use of human subjects in this study from Saint John's Health Center and John Wayne Cancer Institute joint committee. The age of these patients ranged from 19 to 92 years and has a median of 67 years. Fifty-two of the 59 primary tumors assessed were excised from the extremities, head and neck, or trunk; 7 primaries were from acral sites. Breslow thickness was obtained for all but one primary tumor. Regional lymph node metastasis was diagnosed in 18 patients; 2 of these patients also had distant metastasis. Fifty-six patients were observed for 1–87 months, with a median of 32 months; follow-up information was not available in 3 patients. Sixty-eight metastatic melanomas were assessed from 39 males and 29 females; 13 were from patients whose primary tumors were also assessed in this study. The 68 metastatic lesions were from regional and distant anatomical sites. Of the 13 primary metastasis pairs of tumors assessed, 12 of the metastatic tumors were regional lymph node metastasis, and 1 specimen was a lung metastasis.

For genomic analysis of the *BRAF* mutation, 5-μm-thick sections were prepared from paraffin-embedded tissue blocks. Tumor cells were microdissected using the PixCell II Laser Capture Microdissection System (Arcturus Engineering, Mountain View, CA; Ref. 12). Captured cells were digested with proteinase K (Qiagen, Valencia, CA) in buffer at 50°C overnight, followed by heat inactivation at 95°C for 10 min. PCR was performed with *Taq* high-fidelity polymerase (Roche Diagnostics, Mannheim, Germany) in a Perkin-Elmer 9600 thermal cycler (Perkin-Elmer, Norwalk, CT) as described previously (13). A number of *BRAF* missense mutations in codons 585–600 of exon 15 and codons 463–468 of exon 11 have been reported, and V600E in particular is more frequently mutated than any other mutations (3–5, 11). We assessed mutations in exons 15 and 11. Exons 11 and 15 were amplified by using the following primers, respectively: (*a*) 5′-TCCCTCTCAGGCATAAGGTAA-3′ (forward), 5′-CGAACAGTGAATATTTCCTTTGAT-3′ (reverse); and (*b*) 5′-TCATAATGCTTGCTCTGATAGGA-3′ (forward), and 5′-GGCCAAAAATTTAATCAGTGGA-3′ (reverse). Purified PCR products were directly sequenced on both strands. The amplicons were processed with Beckman dye terminator cycle sequencing kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions and analyzed by a CEQ8000XL automated capillary array electrophoresis sequencer (Beckman Coulter). Sequencing was repeated on random positive and negative samples to verify results.

Statistical Analysis. Categorical data were analyzed by χ^2 test unless otherwise specified. Overall and disease-free survival were assessed by Kaplan-Meier survival curve analysis. Proportional hazards test was used to isolate factors influencing the survival. Multivariate analyses considered age, sex, Breslow thickness, presence or absence of lymph node metastasis, site, and presence or absence of *BRAF* mutation (14). All statistical calculations were performed with SAS software version 3.2.1 (SAS Institute, Cary, NC). Data are given as mean ± SE, and a two-tailed test of $P < 0.05$ was considered statistically significant.

RESULTS

We assessed both primary and metastatic melanomas to determine the frequency of *BRAF* mutation. All primary and metastatic melanoma tumors were sequenced at exons 11 and 15. Seventeen T1799A (V600E) mutations and 1 T1799G (V600G) mutation were found in the 59 primary tumors for an overall mutation frequency of 31%. No mutations were detected in exon 11.

BRAF mutations were compared with other known prognostic factors in primary melanomas. Age was the only prognostic factor with correlation to *BRAF* mutation in the primary tumor (Table 1). Although not significant, we observed a trend for greater incident of *BRAF* mutation in female patients (Table 1). The frequency of *BRAF* mutations was significantly higher ($P = 0.001$) in patients <60 years of age (Fig. 1). Mutation rate was >50% in patients <40 years of age compared with <10% in patients ≥70 years of age.

In assessing different primary melanoma sites, we found that the mutation rate was not significant in any particular anatomical site assessed. This offers evidence that *BRAF* mutation is not directly induced by UV exposure. In addition, none of the *BRAF* mutations were of CC→TT or C→T substitutions that are common in UV-induced carcinogenesis (3). The frequency of BRAF mutation in primary melanomas was independent of Breslow thickness (Table 1). There was no significant difference between Breslow thickness ≤1 *versus* >3.5 mm.

Table 1 *BRAF*[a] mutation and clinicopathological factors

	Patients with BRAF mutation/ total patients	*P*
Age		
<60	12/21 (57%)	0.001
≥60	6/38 (16%)	
Gender		
Male	9/39 (23%)	0.087
Female	9/20 (45%)	
Site		
Extremities	5/14 (36%)	0.27
Hand and foot	3/12 (25%)	
Head	3/18 (22%)	
Trunk	7/15 (47%)	
Breslow thickness		
≤0.75 mm[b]	2/7 (29 %)	0.61
≤1.00 mm	3/12 (25%)	
1.01–2.00 mm	6/15 (40%)	
2.01–4.00 mm	6/16 (38%)	
>4.01 mm	3/15 (20%)	
Unknown	0/1	
AJCC stage at primary tumor diagnosis		
I	6/18 (33%)	0.1
II	5/16 (31%)	
III	5/19 (26%)	
IV	2/2 (100%)	

[a] *BRAF*, *B-raf* oncogene; AJCC, American Joint Committee on Cancer.

[b] Breslow thickness is given as a reference.

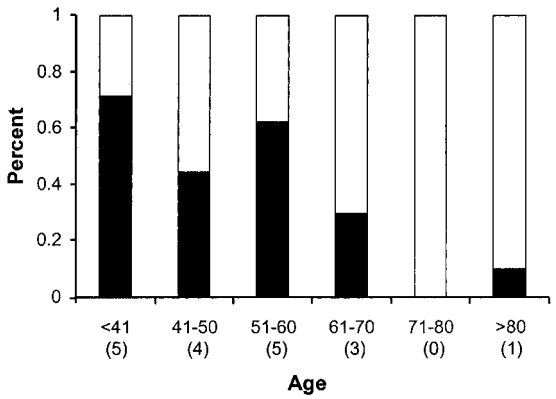

Fig. 1 Correlation between B-raf oncogene (*BRAF*) mutation of primary melanomas ($n = 59$) and age of melanoma patients. *Bars*, *BRAF* mutation (■) *versus* wild type (□). (), the number of patients for each age group.

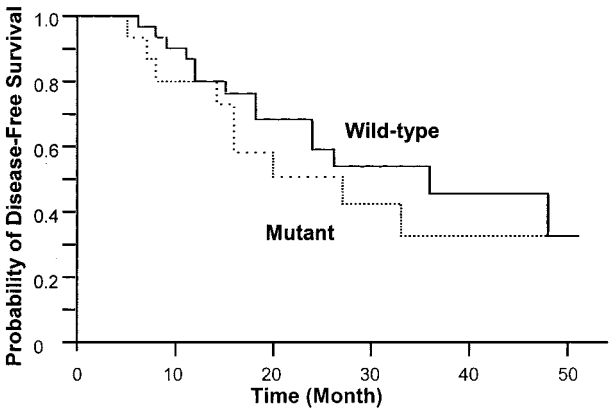

Fig. 2 Disease-free survival Kaplan-Meier curves of B-raf oncogene (*BRAF*) mutation of primary melanomas. Comparison of *BRAF* mutation *versus* wild type.

This suggests that the incidence of *BRAF* mutation does not increase with increasing tumor thickness.

We did not find significant correlation or trend between presence of *BRAF* mutation in primary tumors and overall disease-free survival (Fig. 2). This short-term follow-up analysis suggests that *BRAF*, by itself, is not likely to be a major predictive factor for disease outcome but may complement other major genetic aberrations frequently occurring in melanomas (13).

Primary tumors associated with concurrent lymph node metastasis had a somewhat higher *BRAF* mutation frequency; however, the difference was not statistically significant compared with primaries with no lymph node metastasis (Table 2). The *BRAF* mutation frequency of primary tumors did not significantly correlate to American Joint Committee on Cancer stage at the time of primary tumor diagnosis. We further studied *BRAF* mutation to determine whether it correlated to tumor progression as in metastasis. *BRAF* mutation in exon 15 was studied in 68 melanoma metastases. The *BRAF* mutation rate in metastatic melanomas was 57% ($n = 39$), significantly higher than the 31% in primary melanomas ($P = 0.0024$). The breakdown of metastasis sites were: (*a*) regional lymph node (10 of 20; 50%); (*b*) skin (21 of 35; 60%); (*c*) lung (2 of 4; 50%); (*d*) bowel (4 of 5; 80%); and (*e*) kidney/liver (2 of 4; 50%). These results indicated that frequency of *BRAF* mutation is significantly higher in metastasis than primary melanomas and that it may be frequently acquired during metastasis. In addition, the results suggest that some melanomas may acquire *BRAF* mutation later in the development of metastasis. To examine this further, we assessed 13 pairs of primary melanomas and their respective nodal or distant metastases. In five pairs (38%), the primary tumors had the wild-type gene, and the respective metastasis had a *BRAF* mutation. Four pairs had mutations in both primary tumor and metastasis, and four pairs had wild-type genes in both primary tumor and metastasis. These results suggest that although *BRAF* may be acquired during the development of metastasis, it is not a key factor.

DISCUSSION

In melanoma, ras mutations have been studied extensively. The frequency of *N-ras* mutations can vary from 13 to 23% in primary tumors and >25% in metastasis, depending on the study and method of analysis (15, 16). Demunter *et al.* (15) showed two patterns of *N-ras* mutations in melanoma: (*a*) early stage mutation in nevi or during radial growth; and (*b*) late-stage mutation in metastatic lesions. However, the number of tumors examined in their series was too small to be conclusive. Our findings also indicate two different temporal patterns of *BRAF* mutation. Recently, *BRAF* mutations were reported in 70–80% of congenital, intradermal compound, and dysplastic nevi (5). Unfortunately, neither the size of the nevi nor the incidence of subsequent melanoma in those patients was described. Size of nevi significantly influences the potential development into melanoma. Our studies indicate that *BRAF* mutation is significantly less frequent in primary melanomas than what was reported in both nevi and metastases. If all nevi had a high frequency of *BRAF* mutation (>65%) as reported and develop into primary melanomas, a substantial proportion of *BRAF* mutations would have to be lost during neoplastic transformation. Nevi are often

Table 2 *BRAF*[a] mutation status of paired primary tumor and metastasis

Patient	*BRAF* mutation of primary tumor	*BRAF* mutation in metastasis
1	Mutant	Mutant
2	Mutant	Mutant
3	Mutant	Mutant
4	Mutant	Mutant
5	Wild type	Mutant
6	Wild type	Mutant
7	Wild type	Mutant
8	Wild type	Mutant
9	Wild type	Mutant
10	Wild type	Wild type
11	Wild type	Wild type
12	Wild type	Wild type
13	Wild type	Wild type

[a] *BRAF*, *B-raf* oncogene.

indolent for a long period before developing into melanomas, and not all nevi develop into melanomas. Because mutation of the *BRAF* kinase domain elevates its kinase activity levels, it would then appear to be nonfunctional for the most part in nevi. Nevi in general are relatively dormant in growth with low metabolic activity. Recent studies on familial melanoma ($n = 42$) have demonstrated that there was no evidence of germ-line *BRAF* mutations in exon 15 (17). *BRAF* is unlikely to be a susceptibility gene for melanoma.

In the present study, there was a strikingly higher frequency of *BRAF* mutation in patients < 40 years old. Older patients who develop melanoma are more likely to have a history of prolonged sun exposure and primary lesions in high UV exposure anatomical sites. The mean age of melanoma diagnosis in the United States is 50–58 years; increased age is inversely related to survival and an independent prognostic factor (18). Melanoma is diagnosed more frequently in females < 40 years of age and males > 45 years of age. Younger women tended to have a higher frequency of *BRAF* mutation in our study. Larger studies will be needed to validate whether age and gender play a significant role in *BRAF* mutation acquisition in primary melanomas. Melanoma in younger patients is often thought to be caused by genetic and intrinsic factors (18). Whether *BRAF* mutation in conjunction with other molecular changes in melanoma can contribute to disease progression remains to be determined.

We demonstrated that 31% of the primary melanomas have *BRAF* mutation, which was much lower than that in studies published previously. The *BRAF* mutation in metastatic tumors was 57%, also slightly lower than what other studies have reported (3, 4). Our studies suggest that *BRAF* mutation is not likely to be a key factor in melanoma tumorigenesis or early tumor progression.

We conclude that *BRAF* mutation may be spontaneous and does not appear as a major genetic prognostic factor. The lack of correlation between *BRAF* mutation and Breslow thickness could be suggestive of the unrelatedness of *BRAF* mutation to genetic instability during early stages of primary tumor growth. Breslow thickness is known as one of the strongest prognostic factors in early stage melanoma (14, 19). *BRAF* mutation acquisition is unlikely attributable to primary tumor growth. In a recent reported study on *BRAF* mutation of radial growth phase, primary melanomas were shown to be less frequent (10%; $n = 20$) than vertical growth phase primary melanomas (63%; $n = 8$; Ref. 20). This study indicated that *BRAF* mutation in early stage primary melanomas was very low and contradicts the high incidence found in nevi. However, the study sample size may be too small to allow accurate clinicopathological correlations. There was no indication of the primary lesion site having a correlation to Breslow thickness. To date, there is no evidence of whether benign nevi harboring *BRAF* mutation(s) facilitates their progress to a malignant state.

Additional studies are needed to determine whether *BRAF* mutation has any pathological role in cutaneous melanoma development. A study reported by Zhu *et al.* (21) suggests that Raf gene activation induces cell senescence and may potentially be a protective mechanism against neoplastic transformation. This study is disparate to recent findings of *BRAF* role as an oncogenic factor. At present, the findings are provocative, but unlike other known genetic changes in melanoma, the correlations with early stage tumor progression are unclear. However, the frequency of *BRAF* mutations is higher in metastastatic tumors, and they are found, in both cutaneous primary and metastatic melanomas, in significantly higher frequency than other known gene mutations to date. Future studies are needed to determine whether *BRAF* mutation in conjunction with other genetic aberrations found in melanomas, such as methylation of tumor suppressor gene promoter regions and/or allelic imbalance, contributes to tumor progression.

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