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BRAF/NRAS Mutation Frequencies Among Primary Tumors and Metastases in Patients With Melanoma

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Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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ABSTRACT

Purpose
The prevalence of *BRAF*, *NRAS*, and *p16CDKN2A* mutations during melanoma progression remains inconclusive. We investigated the prevalence and distribution of mutations in these genes in different melanoma tissues.

Patients and Methods
In all, 291 tumor tissues from 132 patients with melanoma were screened. Paired samples of primary melanomas (n = 102) and synchronous or asynchronous metastases from the same patients (n = 165) were included. Tissue samples underwent mutation analysis (automated DNA sequencing). Secondary lesions included lymph nodes (n = 84), and skin (n = 36), visceral (n = 25), and brain (n = 44) sites.

Results
BRAF/NRAS mutations were identified in 58% of primary melanomas (43% *BRAF;* 15% *NRAS*); 62% in lymph nodes, 61% subcutaneous, 56% visceral, and 70% in brain sites. Mutations were observed in 63% of metastases (48% *BRAF;* 15% *NRAS*), a nonsignificant increase in mutation frequency after progression from primary melanoma. Of the paired samples, lymph nodes (93% consistency) and visceral metastases (96% consistency) presented a highly similar distribution of *BRAF/NRAS* mutations versus primary melanomas, with a significantly less consistent pattern in brain (80%) and skin metastases (75%). This suggests that independent subclones are generated in some patients. *p16CDKN2A* mutations were identified in 7% and 14% of primary melanomas and metastases, with a low consistency (31%) between secondary and primary tumor samples.

Conclusion
In the era of targeted therapies, assessment of the spectrum and distribution of alterations in molecular targets among patients with melanoma is needed. Our findings about the prevalence of *BRAF/NRAS/p16CDKN2A* mutations in paired tumor lesions from patients with melanoma may be useful in the management of this disease.

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INTRODUCTION

Melanoma is a complex disease that arises through multiple etiologic pathways. Studies of the genetic and molecular characteristics of melanomas are valuable in the development of new treatment strategies. Mutations in the p16 cyclin-dependent kinase inhibitor 2A (*p16CDKN2A*) gene, located on chromosome 9p21, are the most recognized cause of inherited melanoma susceptibility,[1,2] contributing to geographic variations in incidence. In Italy, there is a gradient of melanoma incidence—higher in northern regions and lower in southern regions; such a gradient has been analogously described for the *p16CDKN2A* mutation prevalence.[3,4]

The MAPK-ERK pathway, which includes the cascade of BRAF, NRAS, MEK1/2, and ERK1/2 gene products, also plays a major role in the development and progression of melanoma.[5,6] Mutations of *BRAF* and *NRAS* genes have been identified with high frequency in nevi and cutaneous melanomas, suggesting that they represent early events in the development of melanocytic tumors.[7-9] Furthermore, melanomas on skin that have not been chronically exposed to sun usually carry either a mutated *BRAF* or a mutated *NRAS* (somatic mutations in such genes are mutually exclusive).[8,10-12]

Similar rates of *BRAF* mutations are present in primary and metastatic melanomas, as well as in cultured malignant melanoma cell lines, suggesting that *BRAF* mutations occur before tumor dissemination and that their incidence remains constant during tumor progression.[6] However, prevalence of such mutations during the disease progression phases and among different types of metastasis remains inconclusive. The aim of this study was to investigate the prevalence and distribution of pathogenetic mutations in *BRAF*, *NRAS*, and *p16CDKN2A* genes among primary and metastatic melanoma tissues.

PATIENTS AND METHODS

Patients

Eligible patients had a histologically proven diagnosis of advanced melanoma (disease stages III and IV, according to American Joint Committee on Cancer [AJCC] guidelines)[13] and had primary and metastatic tumor tissue samples available for molecular analysis. Patients were enrolled consecutively between June 2008 and December 2010 from centers in Italy. To avoid bias, patients were included regardless of age of onset, family history of cancer, and disease characteristics. About one tenth of the present cohort (12 patients) had been tested for *BRAF*/*NRAS*/*p16CDKN2A* somatic mutations previously.[4]

Patients were informed about the study aims and limits, and they provided written consent for the molecular analysis of their tissue samples. The study was reviewed and approved by the ethical review boards at both participating centers.

Samples

Paired samples of primary melanomas and synchronous or asynchronous metastases from the same patient were collected. Formalin-fixed, paraffin-embedded (FFPE) tumor tissues were taken from pathologic archives. By using light microscopy, the neoplastic portion of each tissue section was isolated to obtain tumor samples with at least 80% neoplastic cells (improving the sensitivity of nucleotide sequencing, which may detect a mutation when the mutant alleles are at least 15% to 20% of the analyzed DNA sample). Histologic classification, including Breslow thickness and disease stage at diagnosis, was confirmed by medical records, pathology reports, and/or review of pathologic material.

For reference, 29 melanoma cell lines cultured from primary and metastatic excised tumors were obtained from Istituto Dermopatico dell'Immacolata of Rome, the National Cancer Institute of Naples, and the publicly available American Type Culture Collection (ATCC). The cell line controls were obtained from primary tumors (n = 6), lymph node metastases (n = 7), subcutaneous metastases (n = 9), visceral metastases (n = 2), and the ATCC catalog (n = 5). These cell lines were established as primary cell cultures from tumor samples from donor patients with documented diagnosis of melanoma, after informed consent.

Tissue sections of brain metastases were obtained from 24 patients surgically treated in other Italian clinical centers (after informed consent). An additional cohort of patients with brain metastasis was included to better assess the distribution of *BRAF*/*NRAS* mutations in such secondary tumors.

Mutation Analysis

Genomic DNA was isolated from tumor tissues or melanoma cell lines.[14] For paraffin-embedded samples, paraffin was removed by xylene treatment (Pisano et al[14]), and DNA was purified by using the QIAamp DNA FFPE tissue kit (QIAGEN, Valencia, CA). Polymerase chain reaction (PCR) was performed on 25 to 50 ng of isolated genomic DNA in a 9700 thermal cycler (Applied Biosystems, Foster City, CA); all PCR-amplified products were directly sequenced by using an automated fluorescent cycle sequencer (ABI PRISM 3130, Applied Biosystems), as previously described.[4]

Sequencing analysis was conducted in duplicate—starting from two different tumor sections and performing two different PCR-based amplifications—and in both directions (forward and reverse) for all samples. For discordant tumors, the sequence analysis was performed in triplicate—three different tumor sections and three different PCR-based amplifications—to avoid any chance of PCR artifacts. A nucleotide sequence was considered as valid when the quality value was higher than 20 (< 1/100 error probability); in this study, the quality value average was 35 (range, 30 to 45; < 1/1,000 to 1/10,000 error probability).

Mutation screening was conducted to analyze the full coding sequences and splice junctions of *p16CDKN2A* (exons 1α, 2, and 3) and *NRAS* (exons 2 and 3) genes, and the entire sequence of the *BRAF* exons 11 and 15 (because

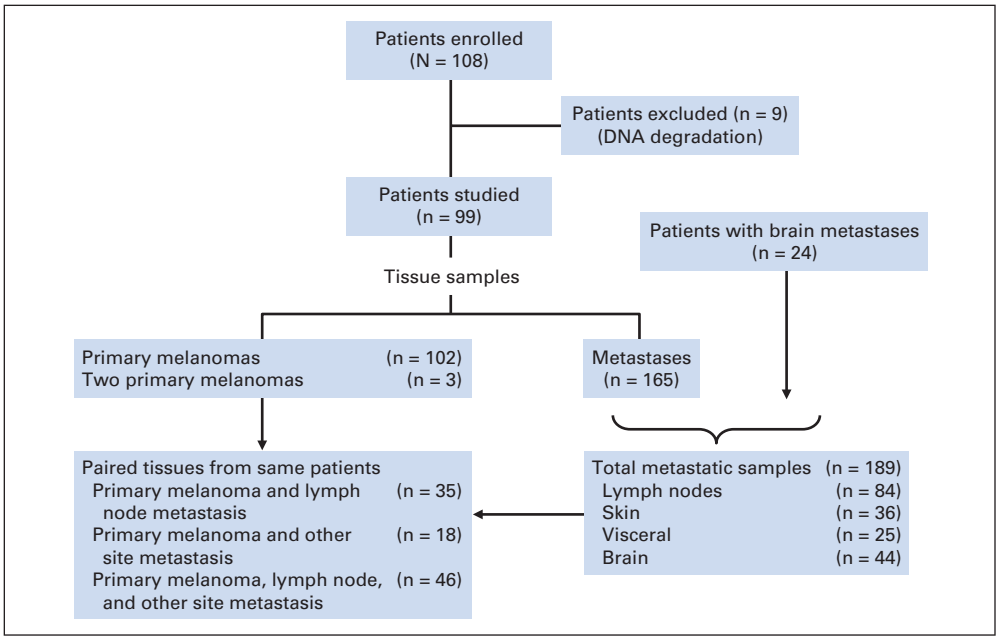

Fig 1. Patients and tissues included in the study.

almost all pathogenetic mutations of *BRAF* have been detected at either the kinase domain at exon 15 or the adenosine triphosphate–binding pocket at exon 11).[5,6] Primer sets and PCR assay protocols were as previously described.[4] To confirm that each *CDKN2A* gene variant detected by sequencing was a real mutation or a polymorphism, 105 unrelated healthy individuals (corresponding to 210 control chromosomes), originating from the same geographic area and with no family history of cancer, were used as controls and screened for candidate sequence variations.

Statistical Analysis

Univariate analysis of the presence of *BRAF, NRAS,* or *p16CDKN2A* mutations versus the number and type of metastatic sites and primary melanoma locations was performed by using Pearson's χ^2 test with the statistical package SPSS version 7.5 for Windows (SPSS, Chicago, IL).

RESULTS

Patients and Samples

A total of 108 patients with advanced (AJCC stages III and IV[13]) melanoma were enrolled, of whom nine were excluded (tissue DNA degradation). Among the remaining 99 patients, paired samples of primary melanomas (n = 102; three patients had two primary tumors each) and synchronous or asynchronous metastases (n = 165) were collected (Fig 1; Appendix Table A1, online only). Median age of the 99 enrolled patients was 52 years (range, 27 to 84 years); 58 (58%) were women. Considering the 102 primary melanomas, trunk was the most frequent location (trunk, 47 [46%]; limbs, 38 [37%]; head and neck, 17 [17%]); median Breslow thickness was 2.2 mm (range, 0.85 to 8.0 mm). In addition, 24 patients surgically treated in other Italian centers provided tissue sections of brain metastases. Overall, a total of 291 tumor samples were screened for gene mutations from the sites shown in Figure 1.

All mutations detected in this study have been reported previously in the Human Gene Mutation Database[15] and in the Catalogue of Somatic Mutations in Cancer (COSMIC).[16]

BRAF/NRAS *Mutation Frequencies*

BRAF or *NRAS* mutations were detected in 59 (58%) of 102 primary tumors: 44 (43%) *BRAF* and 15 (15%) *NRAS*. Among the 189 metastatic tissue samples, 119 (63%) carried mutations: 91 (48%) *BRAF* and 28 (15%) *NRAS*. Similar frequencies of *BRAF* and *NRAS* mutations were seen across metastatic sites (Table 1). In the control melanoma cell lines, the equivalent rate of *BRAF/NRAS* mutations was 21 (72%) of 29, including a *BRAF* mutation frequency of 17 (59%) of 29 and *NRAS* mutation prevalence of four (14%) of 29 (Table 1). In our series, no concomitant mutations of *BRAF* and *NRAS* genes were detected.

All but one of the BRAF mutations across samples was of the BRAF V600 subtype. Of these 135 mutations, 123 (91%) were BRAF V600E, occurring in 42% of all patient samples (39% of primary tumors and 44% of metastatic sites [range, 40% to 53%]). Likewise, 13 (45%) of the 29 control melanoma cell lines exhibited BRAF V600E (76% of the 17 BRAF V600 mutations). Other V600 subtypes identified were V600K, V600D, and V600R (Table 1).

Among the 99 patients who had paired samples of primary and secondary melanomas, 84 (85%) showed consistent mutation patterns between primary tumors and metastatic lesions. In particular, the frequency of *BRAF/NRAS* mutations was highly consistent between the primary tumor and metastases in the lymph nodes and visceral sites. Of 84 patients with lymph node metastases, 78 (93%) had paired primary and secondary tumor samples that had the same *BRAF/NRAS* mutations. Similarly, for visceral metastases, 24 (96%) of 25 patients showed similar *BRAF/NRAS* mutation status between primary and secondary tumors (Table 2). However, in patients with data available for brain and skin metastases, rates of consistency in *BRAF/NRAS* mutations between primary and secondary samples were significantly lower than for lymph and visceral metastases: 16 (80%) of 20 (χ^2 P = .0323) brain lesion samples and 27 (75%) of 36 (χ^2 P < .001) skin secondary tumors exhibited the same *BRAF/NRAS* mutation status as the paired primary tumor (Table 2).

Among the 20 paired samples (15 [15%] of 99 patients) with discrepancies in *BRAF/NRAS* mutation patterns between primary and secondary tumors, 10 (50%) displayed a wild-type primary tumor and a mutated metastasis (six in *BRAF* and four in *NRAS*), eight presented with a mutated primary tumor and a wild-type metastasis (seven in *BRAF* and one in *NRAS*), and two carried a change in mutation

Table 1. Somatic Mutations Detected in *BRAF* and *NRAS* Genes Among In Vivo (primary and secondary tumor sites from patients with melanoma) and In Vitro (melanoma cell lines) Samples

Sample	No. of Samples	Frequency of Mutations and Subtypes							
		BRAF Mutation			*NRAS* Mutation			*BRAF* or *NRAS* Mutation	
		Subtype	No.	%	Subtype	No.	%	No.	%
Primary tumor	102		44	43		15	15	59	58
		V600E	40		Q61R	10			
		V600K	3		Q61L	3			
		V600D	1		Q61K	2			
All metastatic sites	189	91		48		28	15	119	63
		V600E	83		Q61R	17			
		V600K	6		Q61L	8			
		V600D	1		Q61K	3			
		L597R	1						
Lymph node metastases	84		40	48		12	14	52	62
		V600E	36		Q61R	9			
		V600K	3		Q61K	2			
		L597R	1		Q61L	1			
Brain metastases	44		21	48		10	23	31	70
		V600E	18		Q61R	4			
		V600K	2		Q61L	6			
		V600D	1						
Skin metastases	36		19	53		3	8	22	61
Locoregional	22		11	50		2	9	13	59
Distant	14		8	57		1	7	9	64
		V600E	19		Q61R	2			
					Q61L	1			
Visceral metastases	25		11	44		3	12	14	56
Liver	20		9	45		2	10	11	55
Lung	5		2	40		1	20	3	60
		V600E	10		Q61R	2			
		V600K	1		Q61K	1			
Cell lines	29	17		59		4	14	21	72
		V600E	13		Q61L	2			
		V600R	3		Q61K	1			
		V600D	1		Q61R	1			

Table 2. Consistency Between *BRAF/NRAS* Mutation Status in Primary and Secondary Lesions in Patients With Melanoma and Mutation Patterns in Those in Whom There Were Discrepancies

Tissue Type	No. of Samples	Patients With Consistent Mutation Patterns (secondary *v* primary melanoma samples)		Mutation Patterns Among Discrepant Paired Samples			
				BRAF		*NRAS*	
		No.	%	Primary Tumor	Metastasis	Primary Tumor	Metastasis
Lymph node metastases	84	78	93	V600K	wt	wt	wt
				wt	L597R	wt	wt
				wt	V600E	wt	wt
				wt	V600E	wt	wt
				wt	V600E	wt	wt
				V600E	wt	wt	wt
Visceral metastases	25	24	96	V600E	wt	wt	wt
Brain metastases	20	16	80	V600E	wt	wt	wt
				wt	wt	wt	Q61L
				wt	wt	wt	Q61L
				wt	wt	wt	Q61R
Skin metastases	36	27	75	wt	wt	wt	Q61L
				V600E	wt	wt	wt
				V600E	wt	wt	wt
				wt	wt	Q61R	wt
				wt	V600E	Q61R	wt
				wt	V600E	Q61R	wt
				wt	V600E	wt	wt
				wt	V600E	wt	wt
				V600E	wt	wt	wt

Abbreviation: wt, wild-type.

pattern between the two tumor lesions (an *NRAS* mutation in primary melanoma and a *BRAF* mutation in melanoma metastasis; Table 2). Overall, nine of the 20 discrepant metastatic lesions occurred in only four patients, whereas each of the remaining 11 patients carried a single discrepant metastatic lesion (Table 3).

Concordance in *BRAF/NRAS* mutation status among metastatic samples was then evaluated in the subset of 46 patients with paired multiple metastases (one lymph node and at least one other site lesion; Fig 1). Rates of consistency in *BRAF/NRAS* mutations between lymph node and other site metastases were quite similar to those observed between primary and secondary tissues: 21 (91%) of 23 visceral lesions, five (83%) of six brain metastases, and 25 (76%) of 33 skin secondary tumors exhibited the same *BRAF/NRAS* mutation status as the paired lymph node sample (Appendix Table A2, online only). The *BRAF/NRAS* mutation status was not evaluated for association with clinical outcome in our series.

p16CDKN2A *Mutation Frequencies*

Sixteen (16%) of the 99 patients had *p16CDKN2A* gene mutations. Among available DNA samples, the rate of mutations was much higher in melanoma metastases (21 [14%] of 151) versus primary melanomas (five [7%] of 74). The rate of consistency between secondary and primary tumor samples was five (31%) of 16. The highest prevalence of *p16CDKN2A* alterations was observed in our series of 29 melanoma control cell lines (62%; Table 4).

Table 5 shows the distribution of mutations in the *p16CDKN2A* gene among the 16 patients identified as having this mutation, showing that in most of these patients (11; 69%) mutations existed only in metastatic sites, although the primary tumor exhibited wild-type status.

Finally, no correlation was inferred between *p16CDKN2A* and *BRAF/NRAS* mutations from either primary or secondary melanomas (Appendix Table A3, online only). Regardless of *p16CDKN2A* mutation status, approximately 60% of samples had *BRAF* or *NRAS* mutations in both primary and metastatic sites.

DISCUSSION

Melanoma is a complex disease influenced by alterations in several genes and metabolic pathways that continue to evolve through the course of the disease. There is increasing evidence that melanoma develops as a result of accumulated genetic abnormalities within melanocytes.[17] The MAPK-ERK pathway, which includes the cascade of NRAS, BRAF, MEK1/2, and ERK1/2 proteins, is involved in the control of cell growth, proliferation, and migration. Mutations in this pathway may play a major role in the development and progression of melanoma.[5] In addition, the p16CDKN2A protein acts as a suppressor of cell proliferation, and dysfunction in this pathway is observed in many types of cancer.[18] In our study, we explored the relative frequency of genetic factors known to play a significant role in melanocyte development and their distribution among different melanoma tissues and disease progression sites.

As expected, a high prevalence of somatic mutations of *BRAF* and *NRAS* genes was detected in primary and secondary melanomas. The frequency of *BRAF/NRAS* mutations in primary tumors (43%/15%) was consistent with that reported in a meta-analysis in which *BRAF* mutation was present in 41% of cutaneous melanomas (n = 2,521 patients) and *NRAS* mutation in 18% (n = 1,972

Table 3. Patients With Discrepancies Between Primary Melanoma and Paired Metastases in *BRAF* and *NRAS* Genes

Gene and Sample No.	Primary Melanoma	Metastasis Stage I	II	III	IV	V
BRAF						
1	wt	**L: L597R**	S: wt			
2	wt	L: wt	S: wt	**S: V600E**	S: wt	**S: V600E**
3	wt	L: wt	S: wt	**S: V600E**	S: wt	
4	wt	**L: V600E**	**S: V600E**			
5	wt	**L: V600E**				
6	wt	**L: V600E**	V: wt			
7	wt	B: wt				
8	wt	B: wt				
9	wt	B: wt				
10	V600E	L: V600E	**S: wt**	**S: wt**	S: V600E	S: V600E
11	V600E	L: V600E	**V: wt**			
12	V600E	**L: wt**				
13	V600E	**S: wt**				
14	V600E	**B: wt**				
15	V600K	**L: wt**	V: V600K			
NRAS						
1	wt	L: wt	S: wt			
2	Q61R	L: Q61R	S: Q61R	**S: wt**	**S: wt**	**S: wt**
3	wt	L: wt	**S: Q61L**	S: wt	S: wt	
4	wt	L: wt	S: wt			
5	wt	L: wt				
6	wt	L: wt	V: wt			
7	wt	**B: Q61L**				
8	wt	**B: Q61L**				
9	wt	**B: Q61R**				
10	wt	L: wt	S: wt	S: wt	S: wt	S: wt
11	wt	L: wt	V: wt			
12	wt	L: wt				
13	wt	S: wt				
14	wt	B: wt				
15	wt	L: wt	V: wt			

NOTE. Discrepant results are shown in bold.
Abbreviations: B, brain; L, lymph node; S, skin; V, visceral; wt, wild-type.

patients).[19] Confirming previous data,[20] *BRAF* and *NRAS* mutations were mutually exclusive in our tissue sample collection. Overall, slightly higher rates of *BRAF/NRAS* mutation in metastatic (63%) versus primary site samples (58%) were observed in our series. A markedly higher rate of *BRAF/NRAS* mutations (72%) was detected in the control melanoma cell lines, mostly due to a higher *BRAF* mutation frequency (59% *v* 43% in primary tumors and 48% in metastatic sites). Since cultured melanomas are thought to represent cells with the most malignant phenotype, our observations support previous findings[21] in which selection of *BRAF* mutant alleles may occur during tumor progression. In this regard, the demonstration of a sequential increase in mutation rates for both *BRAF* and *NRAS* genes during melanoma progression—from in situ melanomas to the radial and vertical growth phases of invasive melanomas[22]—strongly suggests that *BRAF/NRAS* somatic mutations may not act as founder events in melanomagenesis.

Twenty paired samples from 15 patients (15%) demonstrated discrepancies in *BRAF/NRAS* mutation patterns between primary and secondary tumors, the highest frequency of these discrepancies being in patients with subcutaneous (25%; $P < .001$) or cerebral (20%; $P = .0323$) metastases. In half the discrepant cases, we found a wild-type primary tumor and a mutated metastasis (60% *BRAF* and 40% *NRAS*). Again, this may represent a further indication that mutations in two such genes might be acquired and become prevalent during disease dissemination in a fraction of patients with melanoma. However, the most intriguing data were represented by the observation of wild-type metastases in cases with mutated primary tumors (nearly all in *BRAF*) or, to a lesser extent, a different mutation pattern between melanoma lesions (*NRAS* mutation in primary and *BRAF* mutation in secondary tumors) in the remaining half of the discrepant cases. These observations provide additional evidence that molecularly heterogeneous cell types may coexist in primary melanoma (presence of both *BRAF*-wild-type and *BRAF*-mutant as well as differently mutated tumor cells has indeed been described[21-23]). However, it is still unclear

Table 4. Number of Somatic *p16CDKN2A* Mutations Detected in Tissue Samples From Patients With Melanoma and Melanoma Cell Lines

Sample	No. of Samples	Frequency of *p16CDKN2A* Mutation No.	%
Primary tumors	74	5	7
Metastatic sites	151	21	14
Lymph nodes	64	9	14
Other sites	87	12	14
Melanoma cell lines	29	18	62
Gene mutations		8	28
Deletions/rearrangements		10	34

Table 5. Patients With Melanoma Who Are Positive for *p16CDKN2A* Mutations

Patients	Primary Melanoma No.	%	Lymph Node Metastasis No.	%	Other Site Metastasis No.	%
No.	16		15		10	
Positive for *p16CDKN2A* mutation	5	31	12	80	9	90
p16CDKN2A subtype						
	wt		IVS1 + 1G>A		wt	
	Arg24Pro		Arg24Pro		Arg24Pro	
	wt		Arg80term		Arg80term	
	Ala36Thr		Ala36Thr		—	
	Ala109Val		Ala109Val		—	
	Trp110term		Trp110term		Trp110term	
	wt		Arg80term		Arg80term	
	Arg24Pro		Arg24Pro		—	
	wt		wt		Arg24Pro	
	wt		Trp110term		Trp110term	
	wt		Arg24Pro		—	
	wt		—		Arg24Pro	
	wt		Ala36Thr		—	
	wt		Val59Gly		—	
	wt		wt		Arg24Pro	
	wt		wt		Trp110term	

Abbreviation: wt, wild-type.

what selective pressure induces the migration of a *BRAF*-wild-type subclone instead of an expected more aggressive *BRAF*-mutant subclone. One could speculate that cells with *BRAF* mutation might undergo activation of the senescence pathways[24] or downregulation of the BRAF-NRAS-MEK-ERK cascade[25]; both events may determine the proliferation arrest of BRAF/NRAS mutated cells. Alternatively, one could hypothesize that the mutated metastasis may derive from another unidentified primary melanoma (in a fraction of patients, no known primary tumor is indeed found among those with metastatic disease).

The BRAF V600E mutation was identified in 42% of tissue samples and 45% of control melanoma cell lines, consistent with the frequency observed in patients screened for inclusion in A Study of Vemurafenib (RO5185426) in Comparison With Dacarbazine in Previously Untreated Patients With Metastatic Melanoma (BRIM 3), in which 47% of patients tested positive for the BRAF V600 variants following *BRAF* mutation analysis with the cobas-4800 BRAF-V600 Mutation Test.[26] Although it remains the most prevalent sequence variant reported in subsets of patients with melanoma, the incidence of the BRAF V600E mutation varies worldwide, from 23% in Chinese patients with melanoma[27] to 45% in Australian patients,[28] in which it appears to be associated with various clinical features such as an inverse association with cumulative sun exposure and a lower rate of tumor proliferation. In our series, other BRAF V600 mutation subtypes occurred in less than 10% of all samples (with V600K being the second most frequent variant); lower than the rate of 26% recently described by Long et al[29] in Australian patients. All known mutations at position V600 of *BRAF* result in constitutive activation of BRAF kinase, causing deregulated downstream signaling via MEK and ERK.[30-32]

A more striking difference in the mutation frequency between primary melanoma, metastases, and cell lines was observed for *p16CDKN2A* (7%, 14%, and 28%, respectively). Inactivation by mutation of this gene seems to be selected during tumor progression; this is consistent with the finding that *p16CDKN2A* silencing promotes uncontrolled cell proliferation, tumor growth, and increased aggressiveness of tumor cells.[33,34] Nevertheless, a functional relationship between *p16CDKN2A* inactivation and *BRAF* activation has been demonstrated. Oncogenic *BRAF* mutations constitutively induce upregulation of *p16CDKN2A* in melanocytic cells and, conversely, any genetic or epigenetic inactivation of *p16CDKN2A* may contribute to malignant progression of BRAF mutant cells.[24,33] Consistent with these findings, *BRAF*/*NRAS* and *p16CDKN2A* mutations were found to coexist (being detected at similar rates of around 60%) in our series of primary tumors and corresponding metastases.

Although *p16CDKN2A* remains a high penetrance melanoma susceptibility gene, oncogenic *BRAF* now represents an identifiable and proven target for cancer therapies.[35,36] In melanoma, a phase III study of oral vemurafenib, a potent inhibitor of BRAF V600 mutations, demonstrated a relative reduction of 63% in the risk of death and 74% in the risk of either death or disease progression compared with dacarbazine chemotherapy in 675 patients carrying the BRAF V600E mutation.[26] Another inhibitor of mutated BRAF, GSK2118436 (GSK436), is under phase III evaluation in comparison with dacarbazine among *BRAF*-mutation–positive patients with stage III to IV melanoma (NCT01227889). A phase I/II study indicated a tumor response at 8 to 9 weeks in 60% of patients with metastatic melanoma or other solid tumors.[37] Despite evidence implicating *NRAS* in melanoma pathogenesis,[17] this gene has not yet become an effective target for melanoma treatment.

Together, these findings indicate that the future of melanoma therapy is likely to focus on targeting multiple pathways. However, the complexity of the molecular events underlying development and progression of melanoma suggests that a better comprehension of both the spectrum and distribution of alterations in molecular targets among patients with such a disease is crucial. In our study, we contributed to provide additional clues about the prevalence of *BRAF*/*NRAS*/*p16CDKN2A* mutations in synchronous or asynchronous paired tumor lesions from a large series of patients with melanoma. The observation of a high consistency between primary melanomas and lymph node or visceral metastases, in contrast with a significantly lower consistency between primary tumors and brain or skin metastases, may have implications in clinical practice. Starting from these findings, the prognostic value of such genetic alterations should be evaluated in a large cohort to assess whether the different distribution of *BRAF*/*NRAS*/*p16CDKN2A* mutations in tumor lesions may have an impact on disease outcome among patients with melanoma.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Appendix

Table A1. Melanoma Patients Eligible for Tumor Tissue Mutation Analysis (n = 123)

No. of Patients	Types of Available Tissue Samples
35*	Paired primary melanoma and lymph node metastasis from same patients
18	Paired primary melanoma and other site (subcutaneous, visceral, and/or cerebral) metastasis from same patients
46†	Paired primary melanoma, lymph node metastasis, and other site (subcutaneous, visceral, and/or cerebral) metastasis from same patients
24	Brain metastasis only

NOTE. In all cases, we considered the second most recent lesion for comparison with metastases.
*One patient had two primary melanomas. First lesion, 2005: *BRAF* and *NRAS* wild-type (wt); second lesion, 2008: *BRAF* and *NRAS* wt.
†Two patients had two primary melanomas. Patient 1, first lesion, 1998: *BRAF* and *NRAS* wt; second lesion, 2009: *BRAF* V600E and *NRAS* wt. Patient 2, first lesion, 2008: *BRAF* and *NRAS* wt; second lesion, 2010: *BRAF* and *NRAS* wt.

Table A2. Consistency Between *BRAF/NRAS* Mutation Status in Lymph Nodes and Other Site Lesions Among 46 Melanoma Patients With Multiple Metastases

			Patients With Consistent Mutation Patterns (other site *v* lymph node samples)	
Tissue Type	No. of Patients	No. of Samples	No.	%
Visceral metastasis	21	23	21	91
Brain metastasis	6	6	5	83
Skin metastasis	19	33	25	76

Table A3. Comparison Between Somatic Mutations in *BRAF/NRAS* and *P16CDKN2A* Genes Among Primary and Secondary Tumor Sites From Patients With Melanoma

	p16CDKN2A Mutant		*p16CDKN2A* wt	
Sample and *BRAF/NRAS* Status	No.	%	No.	%
Primary tumors	(n = 5)		(n = 68)	
BRAF/NRAS mutant	3	60	42	62
BRAF/NRAS wt	2	40	26	38
Metastatic sites	(n = 18)		(n = 126)	
BRAF/NRAS mutant	11	61	80	63
BRAF/NRAS wt	7	39	46	37

Abbreviation: wt, wild-type.

GLOSSARY TERMS

BRAF: BRAF is an isoform of RAF. Raf proteins (Raf-1, A-Raf, B-Raf) are intermediate to Ras and MAPK in the cellular proliferative pathway. Raf proteins are typically activated by Ras via phosphorylation, and activated Raf proteins in turn activate MAPK via phosphorylation. However, Raf proteins may also be independently activated by other kinases.

ERK (extracellular receptor kinase): A second messenger kinase (an enzyme adding phosphate groups from ATP), ERK belongs to the MAPK family and is responsible for transmitting signals from the cellular surface to the nucleus by the activation of transcription factors, including NF-κB. It belongs to the proliferative/mitogenic signal transduction pathway activated by tyrosine kinase receptors.

Formalin-fixed, paraffin-embedded: Formalin-fixed, paraffin-embedded (FFPE) tissue is the standard for tissue preparation in anatomic pathology. The processing of tissue historically has included cutting into thin (5-mm) sections, then placing a cassette for fixation in formalin in a tissue processor, followed by infusion of paraffin and embedding on the block for subsequent sectioning for histologic evaluation or immunohistochemistry.

MAPK (mitogen-activated protein kinase): MAPKs are a family of enzymes that form an integrated network influencing cellular functions such as differentiation, proliferation, and cell death. These cytoplasmic proteins modulate the activities of other intracellular proteins by adding phosphate groups to their serine/threonine amino acids.

NRAS: NRAS represents one of the three members of the Ras gene family (HRAS and KRAS are the remaining family members). The Ras proteins are typically small triphosphate-binding proteins, and are the common upstream molecule of several signaling pathways that play a key role in signal transduction, which results in cellular proliferation and transformation.

p16CDKN2A: Also known as p16, it binds to cyclin-dependent kinase 4 and 6, thereby preventing their interaction with cyclin D. It thus behaves as a negative regulator of proliferation and arrests cells in the G0/G1 phase of the cell cycle.

PCR (polymerase chain reaction): PCR is a method that allows logarithmic amplification of short DNA sequences within a longer DNA molecule.

Polymorphism: Genetic polymorphisms are natural variations in the genomic DNA sequence present in greater than 1% of the population, with SNP (single nucleotide polymorphism) representing DNA variations in a single nucleotide. SNPs are being widely used to better understand disease processes, thereby paving the way for genetic-based diagnostics and therapeutics.

Sequencing: A laboratory process that determines the nucleotide sequence of DNA (can involve the whole genome or whole exome or be targeted to as little as one coding sequence). Unlike somatic mutation genotyping, sequencing can detect previously unknown somatic mutations.

Somatic mutation: A change in the genotype of a cancer cell. This is distinguished from a germline mutation, which is a change in the genotype of all the normal cells in a patient's body. Germline mutations may be passed to offspring, but somatic mutations may not.