BRAF Mutations in Metastatic Melanoma: A Possible Association with Clinical Outcome

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ABSTRACT

Purpose: **The RAS-RAF-mitogen-activated protein kinase pathways mediate the cellular response to growth signals. In melanocytes, BRAF is involved in cAMP-dependent growth signals. Recently, activating mutations in the *BRAF* gene, were reported in a large proportion of melanomas. We have studied mutations in the *BRAF* gene and their association with clinical parameters.**

Experimental Design: **We analyzed exons 1, 11, and 15 of the *BRAF* gene and exons 1 and 2 of the *N-ras* gene for mutations in 38 metastatic melanomas by PCR-single-strand conformation polymorphism and direct sequencing. Kaplan-Meier survival and multivariate analyses were used to correlate mutations with various clinical parameters.**

Results: **Mutations in exon 15 of the *BRAF* gene were detected in 26 (68%) melanomas. In 25 cases, mutation involved the "hot spot" codon 600[2] of the *BRAF* gene. Three melanomas without a *BRAF* mutation carried amino acid substituting base changes at codon 61 of the *N-ras* gene. In a multivariate proportional hazard (Cox) model, BRAF mutation, along with the stage of metastatic melanomas, showed a statistically significant hazard ratio of 2.16 (95% confidence interval 1.02–4.59; χ^2 for the model 6.94, degrees of freedom 2, $P = 0.03$) for diminished duration of response to the treatment. In a Kaplan-Meier survival model, cases with *BRAF* mutation showed longer disease-free survival (median of 12 months) than cases without mutation (median of 5 months), although this association was not statistically significant (Log-rank test $P = 0.13$).**

Conclusions: **Our results, besides confirming the high frequency of *BRAF* mutations in metastatic melanomas, also underline the potential importance of these mutations in disease outcome.**

INTRODUCTION

Cutaneous malignant melanoma is a potentially fatal neoplasm with complex and heterogeneous etiology (1). The sporadic form, which constitutes >90% of all cases, is linked to sunlight exposure (2, 3). The inherited form of melanoma is associated with a melanoma susceptible gene on chromosome 9p21; on this locus, germ-line mutations in the *CDKN2A* gene, which encodes two cell cycle inhibitors $p16^{INK4a}$ and $p14^{ARF}$, are found in a proportion of melanoma prone families (4, 5). However, somatic alterations in sporadic melanoma are heterogeneous. Allelic loss at chromosome 9p21 locus is the most prevalent genetic occurrence in sporadic melanoma; however, mutations and other alterations in the *CDKN2A* gene are rather rare (6).

The predominant oncogenic changes associated with malignant melanoma have been the activating mutations in the *N-ras* gene, most commonly involving codon 61 (7). Recently, a genome-wide mutation detection strategy revealed alterations in a high proportion of melanoma cell lines and primary tumors in the *BRAF* gene (8). An overwhelming proportion of these mutations affected a single residue (V600E; previously V599E[2]) in the kinase activation domain of BRAF. RAF serine/threonine kinases are the key signaling components in the RAS pathways. The key relevance of BRAF in melanoma is caused by its melanocyte-specific activation in cAMP-dependent signaling cascade as a consequence of α-melanocyte-stimulating hormone and related peptide binding to melanocortin receptor 1 (9).

In the present study, we analyzed exons 1, 11, and 15 of the *BRAF* for mutations in 38 metastatic melanomas, and additionally, we also screened for mutations in exons 1 and 2 of the *N-ras* gene. In the *BRAF* gene, we detected mutations in 26 melanomas in exon 15, and most of the mutations involved "hot spot" codon 600[2]. Three melanomas without *BRAF* mutations carried mutations in the codon 61 of the *N-ras* gene. Mutations in the *BRAF* gene showed association with poor treatment response.

MATERIALS AND METHODS

The material for this study consisted of patients treated for progressive metastatic melanoma at the Helsinki University

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[2] In all communications on mutations in the *BRAF* gene, the nucleotide and codon numbers have been based on the NCBI gene bank nucleotide sequence NM_004333. However, according to a sequence given in NCBI gene bank sequence accession no. NT_007914, version 03 Jan. 2003, there is a discrepancy of one codon (three nucleotides) in exon 1 sequence in the sequence NM_004333. The sequence analysis of exon 1 of the *BRAF* gene in this study has shown that the sequence derived from NT_007914 is correct. Because of correctness of the latter, sequence numbering of codons and nucleotides after exon 1 are changed by +1 and +3, respectively. The details are given in the text.

Central Hospital between 1988 and 1996 and which have been described earlier (10). In 38 cases, tumor material was available for the present study. All of the cases included in the study had entered into a chemoimmunotherapy trial, consisting of dacarbazine, vincristine, bleomycin, and lomustine in combination with human leukocyte IFN. Twenty-three of these cases were males, and 15 were females; the median age of patients was 54 (range 33–75 years). Clinical stage described for the patients was assessed at the diagnosis of metastatic disease and at time of initiation of chemoimmunotherapy according to the TNM[3] classification by International Union against Cancer (11–13). The localization of metastasized melanomas in the cases included in this study was cutaneous, lymphnode, lung, liver, brain, bone, and spleen. The mean disease-free survival (progression time from the diagnosis of primary melanoma to the appearance of first metastasis) of the patients was 35.5 months (range 0–200 months). Response to chemoimmunotherapy has been assessed previously according to WHO (12–14). Among the cases presented here, 6 (16%) achieved CR, 15 (39%) patients experienced PR, 6 (16%) presented SD, and 11 (29%) had PD. The duration of response varied from 1 to 114+ months (median 4.9 months), which included 11 cases that did not respond to the treatment at all (response time 0 month; Table 1). The duration of CR was determined from the date CR was first recorded to the date PD was noted. In those who achieved PR, the duration of overall response was calculated from the 1st day of the treatment to the date of first observation of PD (14).

DNA Extraction and SSCP Analysis. DNA was extracted from frozen tissues by incubating the sections removed from the slides with sterile scalpels in a digestion buffer containing 200 μg/ml Proteinase K. Control DNA samples were extracted from total WBCs obtained from two healthy individual donors. Exons 1, 11, and 15 of the *BRAF* gene and exons 1 and 2 of the *N-ras* gene were amplified for SSCP analysis using primers described in Table 2. PCR was carried in 10-μl volume reactions containing 50 mM KCl, 0.11 mM each deoxynucleotide triphosphate, 1 μCi [α-^{32}P]dCTP, 0.3 units of TaqDNA polymerase, 1–2 mM $MgCl_2$, and 0.15–0.3 μM each primer. For the amplification of exon 1 of the *BRAF* gene, 10% DMSO was included as cosolvent. The temperature for PCR was set as denaturation at 95°C for 1 min, annealing (at temperatures specific for each exon as given in Table 2) for 1 min, and polymerization at 72°C for 1 min for three cycles, followed by 27–33 cycles at same temperatures with the segment time of 30 s each. Amplified products were electrophoresed on a 0.5 × mutation detection enhancement gel in at least three different conditions.

Sequence Analysis. Mutations detected by SSCP in different exons of the *BRAF* and *N-ras* genes were identified and confirmed by direct sequencing using Rhodamine dye terminator cycle sequencing kit (Big Dye; Applied Biosystems). DNA samples from melanomas containing mutations in different exons were amplified by PCR. Different fragments (exons 1, 11, and 15 of the BRAF gene and exons 1 and 2 of the *N-ras* gene) were also amplified using DNA from healthy controls as templates and were sequenced as controls. The amplified products were purified using Sephadex micro-spin columns (Amersham-Pharmacia) and subjected to 26 cycles of sequencing reaction using forward or reverse primers separately (Table 2). The precipitated sequencing reaction products were electrophoresed on a denaturing polyacrylamide gel in an automated sequencer (ABI 377; Applied Biosystems) and analyzed using Prism and Edit View 1.0.1 software. The sequencing data were analyzed using Align software in DNA star package.

Statistical Analysis. The cumulative survival curves for two outcomes, disease-free survival and duration of response (in months), in cases with and without *BRAF* mutations were drawn with Kaplan-Meier method. The differences between the curves were analyzed with Log-rank test. The association between *BRAF* mutations, duration of response to the treatment in months, and clinical stage of the disease was carried out using proportional hazard regression (Cox) model. For the latter analysis, the clinical stage at the initiation of chemoimmunotherapy was used, because the analysis refers to post-treatment variables. All of the statistical tests were carried out using Statistica software.

RESULTS

Mutations in the *BRAF* Gene. Exons 1, 11, and 15 were analyzed for mutations in the *BRAF* gene using the PCR-SSCP technique. Exon 1 was included in the analysis because in the original report on mutations in the *BRAF* gene, it was reported that this exon could not be amplified (8). However, we amplified a 276-bp fragment containing exon 1 in DNA from metastatic melanomas and blood samples drawn from healthy controls that were included in this study. No mutational bandshift in SSCP was detected in any of the samples in this fragment. DNA samples from three melanomas (cases 1, 2, and 10) and two healthy controls were sequenced to check that we have amplified the correct fragment. Alignment of the obtained sequence with the reported sequence in the NCBI gene databank (accession number NT_007914; version 03 Jan. 2003) showed that the sequence of the amplified fragment was correct. However, alignment of NT_007914 *BRAF* sequence with mRNA sequence in the databank with accession no. NM_004333 showed a discrepancy of three nucleotides in exon 1 sequence (Fig. 1). The codon and nucleotide numbering in the *BRAF* gene reported thus far in all publications has been based on the NM_004333 mRNA sequence, which originates from an earlier study and has not been subjected to review by NCBI (15, 16). Our sequencing results show that the correct version of the sequence is given in the NCBI databank sequence with accession no. NT_007914. Therefore, the exon 1 of the *BRAF* gene comprises of 46 codons instead of 45, and the total number of amino acid residues in the *BRAF* gene is 766 instead of 765. As a consequence, we propose a change in nucleotide numbering after nucleotide 94 (starting from ATG codon) by +3 and consequently the codon numbers correspondingly change by +1. This numbering will reflect the correct sequence of the *BRAF* gene. The amino acid sequence

[3] The abbreviations used are: TNM, Tumor-Node-Metastasis; CR, complete response; SD, stable disease; PD, progressive disease; PR, partial response; SSCP, single-strand conformation polymorphism; MAP, mitogen-activated protein; NCBI, National Center for Biotechnology Information; ERK, extracellular signal-regulated kinase.

Table 1 *BRAF* and *N-ras* mutations in metastatic melanomas

Case	Sex[a]	Age	Stage at dg[b]	Stage at start[c]	Location[d]	Response[e]	Duration of response[f]	DFS[g]	*BRAF*	*N-ras*
1	2	45	3	4	ln, lu, li	PD	0	141	WT	WT
2	1	47	4	4	ln	PR	7	105	T>A[h]	WT
3	2	42	3	4	sc, bo	PR	8	8	T>A	WT
4	2	68	4	4	sc, lu	SD	3	13	T>A	WT
5	2	42	3	3	sc	CR	60	27	T>A	WT
6	1	46	3	4	sc, lu	PD	0	17	WT	A>G[i]
7	1	56	3	3	sc	PR	21	5	WT	WT
8	1	44	4	4	lu	PD	0	17	T>A	WT
9	1	67	4	4	sc, lu	SD	10	94	WT	WT
10	1	73	3	4	li	PR	6	8	T>A	WT
11	2	67	3	3	sc	PR	5	10	T>A	WT
12	2	36	3	3	sc, ln	PD	0	18	T>A	WT
13	1	54	4	4	sc, lu	SD	3	200	T>A	WT
14	1	53	3	4	lu	PD	0	11	T>A	WT
15	1	51	4	4	sc	PR	28	14	WT	C>A[j]
16	2	58	3	3	sc	CR	1	6	T>A	WT
17	2	50	4	4	sc, ln, br	SD	4	164	A>G[k]	WT
18	2	71	4	4	sc, ln, lu, li, bo	PD	0	2	WT	WT
19	2	65	3	3	sc	CR	114	7	WT	WT
20	1	45	3	4	ln, lu	PD	0	22	T>A	WT
21	1	54	4	4	sc, ln	SD	2	0	GT>AA[l]	WT
22	1	65	3	3	sc	CR	5	7	WT	A>G[i]
23	1	60	3	3	ln	PR	5	1	T>A	WT
24	1	33	3	4	ln, li	PR	18	1	WT	WT
25	1	60	3	3	sc	CR	3	11	T>A	WT
26	1	51	3	3	sc, ln	PR	5	10	T>A	WT
27[m]	1	68	4	4	ln, lu	PR	11	181	T>A	WT
28	2	52	3	4	sc, lu, li	PD	0	114	GT>AA	WT
29	2	39	3	4	li	PD	0	24	T>A	WT
30	2	75	3	4	lu, sp	PR	6	4	WT	WT
31	1	71	3	3	sc, ln	PR	7	13	T>A	WT
32	1	59	3	4	ln	PR	11	60	T>A	WT
33	2	72	3	3	sc, ln	PR	14	0	WT	WT
34	1	41	3	4	sc, br	SD	10	0	WT	WT
35	1	52	4	4	sc, lu	PD	0	12	T>A	WT
36	1	33	3	3	sc	PR	7	7	T>A	WT
37	2	64	3	3	sc, ln	CR	27	1	GT>AA	WT
38	1	63	3	3	sc	PD	0	12	T>A	WT

[a] 1, male; 2, female.
[b] Clinical stage according to TNM classification at the diagnosis of metastatic disease.
[c] Clinical stage according to TNM classification at the initiation of chemoimmunotherapy, used for the analyses on post-treatment time variables (see "Materials and Methods").
[d] Location of the metastases at the initiation of chemoimmunotherapy; ln, lymph node; lu, lung; li, liver; bo, bone; br, brain; sp, spleen.
[e] Response to chemoimmunotherapy according to WHO criteria.
[f] Duration of response indicates the duration of response to chemoimmunotherapy, before relapse of the disease, and is given in months (see "Materials and Methods"). Zero months indicates no response.
[g] DFS is disease-free survival that indicates time in months from the diagnosis of primary melanoma to the appearance of first metastasis.
[h] T1799A (V600E) mutation in exon 15.
[i] CAA>CGA mutation in codon 61 of the *N-ras* gene.
[j] CAA>AAA mutation in codon 61 of the *N-ras* gene.
[k] A1801G (K601E) mutation in exon 15.
[l] GT1798-99AA (V600K) tandem mutation in exon 15.
[m] Case 27 also carried single base change in exon 11 of the *BRAF* gene at codon 444 (CGG>CGA), which did not result in any change in amino acid residue.

from codon 30–35, according to the corrected version, is Ala-Gly-Ala-Ala-Ala-Ser, whereas in the incorrect version, the sequence from codon 30–34 is Arg-Pro-Ala-Ala-Ser. Therefore, in the correct version, the Ser residue is at codon 35 and not 34, and numbering of all residues, thereafter, changes by +1. Accordingly, in this study, we have used the corrected nucleotide and codon numbers throughout, *e.g.*, the mutational hot spot codon and nucleotide reported in earlier reports as 599 and 1796, respectively, are codon 600 and nucleotide 1799 in the correct version (8, 17–28).

In exon 11 of the *BRAF* gene, one base change in codon 444 was detected in one melanoma (case 27), which did not result in any amino acid change (CGG>CGA). In exon 15, we detected mutations in 26 of 38 cases (Table 1; Fig. 2*A*). In 25 melanomas, mutation involved hot spot codon 600 (previously 599). Twenty-two melanomas carried T1799A (V600E; previ-

Table 2 Primers used for the amplification of the *BRAF* and *N-ras* genes

Exon	Primer	Sequence	Annealing temp.	Size (bp)
BRAF gene				
1	Forward	5′TTC CCC CTC CCC GCC CGA CAG	68	279
	Reverse	5′GCC CCC ACC GCC GCC TCT TTC		
11	Forward	5′CTC TCA GGC ATA AGG TAA TG	53	204
	Reverse	5′CAC TTT CCC TTG TAG ACT GTT		
15	Forward	5′CCT AAA CTC TTC ATA ATG CTT	52	209
	Reverse	5′ATA GCC TCA ATT CTT ACC AT		
N-ras gene				
1	Forward	5′CGC CAA TTA ACC CTG ATT ACT	56	174
	Reverse	5′CAC TGG GCC TCA CCT CTA		
2	Forward	5′CCC CTT ACC CTC CAC AC	55	196
	Reverse	5′AGG TTA ATA TCC GCA AAT GAC		

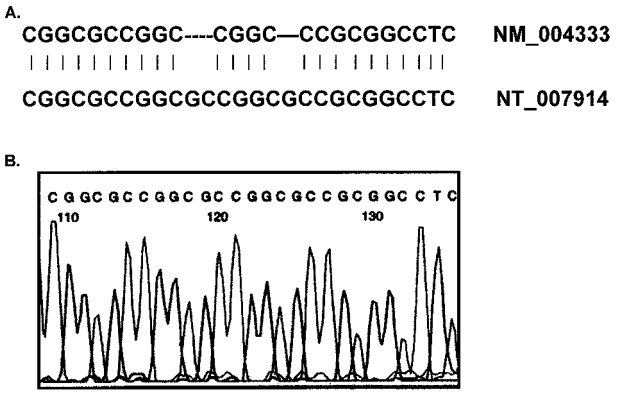

Fig. 1 *A*, alignment of partial coding part of exon 1 of *BRAF* sequences from the NCBI gene bank NM_004333 and NT_007914 shows three extra nucleotides in the latter sequence. *B*, part of exon 1 sequence of the *BRAF* gene obtained from a control DNA isolated from whole blood drawn from a healthy donor. This analysis shows that the *BRAF* sequence reported in the NCBI gene bank database with accession no. NT_007914 is the correct version. Consequently, the nucleotide and codon numbering used in various studies thus far requires a revision by +3 and +1, respectively. In this study, we have, accordingly, used the corrected codon and nucleotide numbers.

ously called T1796A; V599E) mutation (Fig. 2*B*), whereas in three cases, the same codon carried a tandem mutation GT1798–99AA (previously GT1795–96AA), causing valine to lysine change in the amino acid (Fig. 2*C*). This tandem mutation has been reported in melanocytic nevi and is different from the TG1799–1800AT (previously TG1796–97AT) mutation reported earlier (8, 22). Additionally, in one melanoma, we detected A1801G (K601E; previously A1798G; K600E) mutation (data not shown), similar to one reported in a case of colon cancer (17).

Association of BRAF Mutations with Clinical Parameters. In Kaplan-Meier survival model, cases with *BRAF* mutations showed median disease-free survival of 12 months, whereas cases without mutation had median survival of 5 months, although this difference between the two groups was not statistically significant (Log-rank test $P = 0.13$; Fig. 2*E*). The inclusion of data on stage at diagnosis of metastatic disease in a multivariate model did not affect the outcome on association between BRAF mutations and disease-free survival (data not shown). The cases with *BRAF* mutations showed a shorter duration of the response to the treatment than cases without any *BRAF* mutations (median 3.4 *versus* 9.8 months; Log-rank test $P = 0.03$; Fig. 2*F*). In a multivariate proportional hazard (Cox) model, where the effect of *BRAF* mutations along with clinical stage of the melanomas on the duration of response to the treatment was determined, cases with *BRAF* mutations showed a statistically significant hazard ratio 2.16 (95% confidence interval 1.02–4.59; χ^2 for the model 6.94, degrees of freedom 2, $P = 0.03$) for diminished duration of response to the treatment. In another model, where values for age, p53, MDM2, MIB expression, and metastatic stage were included, cases with *BRAF* mutations retained significance for shorter response period to the treatment than cases without *BRAF* mutations (data not shown). We could not assess association between duration of response to the treatment and location of metastasized tumors because of the small number of cases in the subgroups (Table 1). However, when melanomas were grouped into two, those with only s.c. metastasis (10 cases) and all others (28 cases), the location had no effect on the association between mutations in *BRAF* gene and duration of response to the treatment (data not shown).

DISCUSSION

In the present study on metastatic melanomas, we searched for mutations in exon 1, 11, and 15 of the *BRAF* gene. The earlier study had revealed distribution of mutations in regions of BRAF kinase domain that are encoded by exons 11 and 15. Exon 1 was included for the reason that it had not been screened in earlier studies, and sequencing analysis showed that the correct version of the sequence has an additional three nucleotides and, therefore, one additional codon in this exon. Consequently, on the basis of our sequencing results, we have proposed and used a revised numbering of nucleotides (changed by +3 after nucleotide 94 in exon 1) and codons (changed by +1) of the *BRAF* gene, which is based on the correct version of the sequence given in the NCBI gene bank database (accession no. NT_007914, version 03 Jan. 2003).

Furthermore, our results confirm the presence of mutations in the *BRAF* gene in sporadic melanomas. The level of *BRAF* mutations detected in the metastatic melanomas in this study corresponds to that reported earlier, and in all cases, except one, the mutations involved hot spot codon 600 (reported as 599 in

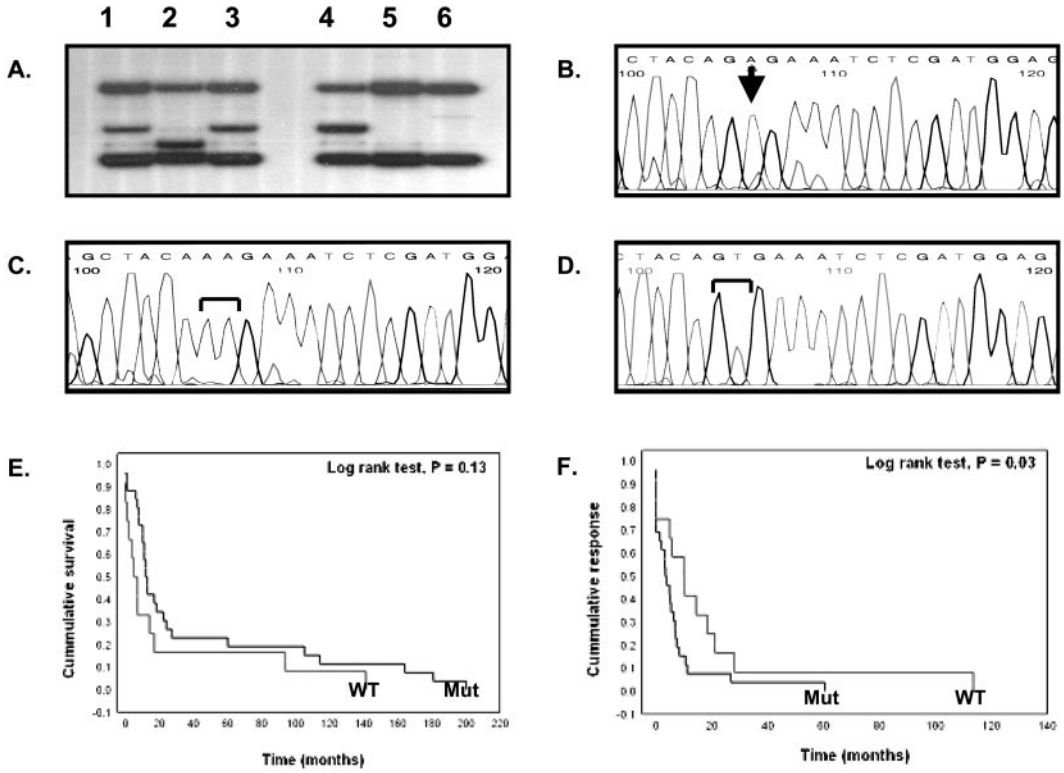

Fig. 2 Results of SSCP and sequencing analysis of exon 15 of the *BRAF* gene are shown. *A*, a representative SSCP analysis of exon 15 of the *BRAF* gene. Samples in *Lanes 1* (case 38), *3* (case 36), and *4* (case 35) show the aberrant bands caused by T1799A ($V600E^2$) mutation; aberrant band in *Lane 2* (case 37) is caused by GT1798–99AA (V600K) tandem mutation. *Lanes 5* and *6* contained DNA from melanoma cases 34 and 33. *B*, part of exon 15 of the *BRAF* gene sequence showing T1799A (V600E) mutation in case 38 of metastatic melanoma (shown by an *arrow*). *C*, part of exon 15 of the *BRAF* gene sequence obtained from DNA extracted from case 37 of melanoma showing GT1798–99AA (V600K) sequence change (shown by a *thick line*). *D*, corresponding wild-type sequence of exon 15 of the *BRAF* gene obtained from a control DNA sample (from a healthy donor). *Thick line*, the position of mutated bases shown in two preceding cases. *E*, Kaplan-Meier survival analysis showing difference in disease-free survival between melanoma cases with and without *BRAF* mutations. Median survival in cases with *BRAF* mutations was 12 months against 5 months without mutations, although this difference was not statistically significant. *F*, Kaplan-Meier curves showing difference in response time to the treatment in cases with and without *BRAF* mutations. Median response period in months was 3.4 against 9.8 in cases with and without *BRAF* mutations, respectively.

all earlier studies) in the kinase domain. The V600E *BRAF* mutant has been reported to possess 10-fold greater basal activity, and it induces focus formation in NIH3T3 cells with much higher efficiency than the wild-type BRAF (8). Our detection of a V600K (GT1798–1799AA) amino acid substitution, found in melanocytic nevi in an earlier study (reported as V599K; GT1795–96AA), confirms the possibility of BRAF activation through substitution with both positively and negatively charged residues (22). The residue 600 (previously 599), which accounts for 25 out of 26 mutations in the *BRAF* gene in this study and >90% of all mutations in all reported studies, is identical at corresponding positions in RAF1 and ARAF1, and it is conserved through the evolution with a single exception of *Drosophila Raf* homologue (8, 21). The hypothesis that in melanocytes, the activation of RAS or BRAF leads to the same phenotype was further strengthened by the mutual exclusivity of mutations. *N-ras* mutations were detected only in melanomas that had wild-type *BRAF*, which is in conformation with earlier data on colorectal cancer and melanomas (17, 21, 22).

The recent discovery in melanocytic nevi of *BRAF* mutations supports their potential role as the initiating somatic changes, which require additional genetic changes for melanoma development (22). A majority but not all melanomas carry mutations in the *BRAF* gene; however, not all melanomas arise from melanocytic nevi. Nevertheless, the mutations in the *BRAF* gene (and also *N-ras* gene) persist from primary through metastatic melanomas, which suggests that probably these mutations are early events but may also have a role in tumor maintenance (29).[4] Association of a diminished response time to the treatment, detected here in patients with *BRAF* mutations, therefore, indicates the involvement of activated BRAF beyond the initiation. However, an independent effect of *BRAF* mutations on response to the treatment would require further evaluation because we found that this association was dependent on the clinical stage.

We also found a nonsignificant association between *BRAF*

[4] R. Kumar *et al.*, unpublished results.

mutations and longer disease-free survival. These associations between cases with *BRAF* mutations and clinical parameters may be seemingly contradictory, but the plausible explanation could be that the effect of *BRAF* mutations can potentially override the effect of widespread genomic destabilization and thus prolong the disease progression period. Thus, it is possible that the subset of melanomas with *BRAF* mutations differ in their biology from tumors without *BRAF* mutations. Activating *BRAF* mutations could overcome one or several extra and intracellular growth inhibitory signals produced by cytotoxic drugs and IFNs. Indeed, it has been shown in cell lines and severe combined immune deficiency mice that constitutive activation of the MAP/ERK kinase/ERK/MAP kinase pathway induces resistance to cisplatin and radiotherapy. In addition, it has been shown that ERK1/2 inhibitor U0126 decreases proliferation and invasion of melanoma cell lines with *BRAF* mutations (18, 27, 28). These findings, taken together with ours, suggest that the role of BRAF and other MAP kinase pathway oncogenes should be further studied in larger patient populations, in reference to treatment outcome. This, together with *in vitro* studies, would produce vital information on whether specific inhibitors of these oncogenes could be used for targeted therapy of some very chemoresistant malignancies, such as melanoma and non-small cell lung carcinoma.

The discovery of mutations in the *BRAF* gene in melanomas in high proportion has already led to the suggestions for the potential role of kinase inhibitors in treatment, which can counter the kinase activity of the activated BRAF (8). The possible association of these mutations with clinical parameters as indicated in this study further augments the potential role for such treatment. However, any such strategy would require consideration of other complexities in advanced tumors. In conclusion, our study on metastatic melanomas confirms the presence of somatic mutations in the *BRAF* gene in melanoma, which functions in the same pathways as RAS, indicated by mutual exclusivity of *BRAF* and *N-ras* mutations. Furthermore, our results show a possible role of these mutations in the disease outcome.

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