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BRAF as a Melanoma Susceptibility Candidate Gene?[1]

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Abstract

A high frequency of activating *BRAF* somatic mutations have been identified recently in malignant melanoma and nevi indicating that BRAF activation could be an early and critical step in the initiation of melanocytic neoplasia. To determine whether *BRAF* mutations could be an earlier event occurring at the germline level, we screened the entire *BRAF* coding region for germline mutations in 80 independent melanoma-prone families or patients with multiple primary melanoma without a familial history. We identified 13 *BRAF* variants, 4 of which were silent mutations in coding regions and 9 nucleotide substitutions in introns. None of these *BRAF* variants segregated with melanoma in the 11 melanoma families studied. Moreover, there was no significant difference in the frequency of heterozygotes for *BRAF* variants between melanoma cases and controls when they were compared. Our data suggest that *BRAF* is unlikely to be a melanoma susceptibility gene.

Introduction

CMM[4] accounts for 5% of skin cancers and 1% of all malignant tumors. CMM is a complex multifactorial disease in which genetic and environmental factors play an important role (reviewed in Ref. 1). Familial melanoma predisposition is associated with germline mutations at the *CDKN2A/ARF* locus (9p21 locus) and CDK4 (12q13 locus; Refs. 2–4). The *CDKN2A/ARF* locus contains two overlapping tumor suppressor genes, *CDKN2A* and *ARF*, that encode two distinct proteins $p16^{INK4A}$ and $p14^{ARF}$ (5). Proteins encoded by melanoma predisposing genes are involved in the regulation of cell growth via the retinoblastoma cell cycle pathway ($p16^{INK4A}$ and CDK4; reviewed in Ref. 6) or in the p53 apoptosis pathway ($p14^{ARF}$; Ref. 7). Mutations in the *CDKN2A* gene have been found in between 20 and 40% of families with multiple melanoma cases (8), whereas germline mutations in *CDK4* (4, 9) and $p14^{ARF}$ (10, 11) have been reported in only very few melanoma-prone families world-wide. Linkage analysis performed with chromosome 9p21 genetic markers clearly showed the existence of unlinked families as well as families linked to the *CDKN2A/ARF* locus where no mutations have yet been identified. In patients with sporadic multiple melanoma, germline mutations in the *CDKN2A* gene have been identified in 10% of cases (12, 13), whereas no mutations have been found in early onset sporadic melanoma cases (<18–25 years of age; Ref. 14) nor in uveal melanoma kindreds (15). Taken together these different observations suggest the existence of other high-risk melanoma-susceptibility genes.

The Ras-RAF-MAP kinase pathway is a membrane-to-nucleus signaling cascade of molecules involved in the regulation of cell proliferation in response to extracellular mitogenic signals (reviewed in Ref. 16). In melanocytes (pigment-producing cells), the binding of α-melanocyte stimulating-hormone and other α-melanocyte stimulating-hormone-related proopiomelanocortin-derived peptides to the melanocortin-1 receptor, induces proliferation and melanogenesis in response to ultraviolet (UV) A/B radiation via the activation of two specific kinases, BRAF and ERK (17). Different observations suggest that this pathway plays a major role in the development of melanoma. In mice, aberrant activation of this pathway appears to be necessary for the development of melanoma (18). Indeed, in a doxycline-inducible ^{V12G}H-RAS mouse melanoma model, null for the tumor suppressor gene *CDKN2A*, *i.e.* both $p16^{INK4A}$- and $p19^{ARF}$-deficient, the genesis and maintenance of melanoma are strictly dependent on the expression of ^{V12G}H-RAS. In humans, mutations of the genes involved in this MAP kinase pathway are detected in melanomas. *RAS* mutations are found in ~25% of primary melanomas and 50% of congenital melanocytic nevi (19). Recently, *BRAF* somatic missense mutations were shown to occur in 66% of malignant melanoma (20, 21). All of the mutations are within the kinase domain, with a hotspot single substitution V599E in exon 15 detected in 80% of nevi (22) and primary melanoma (22), and in 60% of melanoma cell lines (20). Functionally, mutated ^{V599E}BRAF proteins display elevated kinase activity and transform NIH3T3 cells (20). All together, these data indicate that BRAF activation is an early and critical step in the initiation of melanocytic neoplasia. We hypothesized that *BRAF* could be a melanoma susceptibility gene.

To date, four oncogenes have been demonstrated to be susceptibility genes for familial cancers: *CDK4* in melanoma, *RET* in multiple endocrine neoplasia type 2, *MET* in papillary renal cell carcinoma, and *KIT* in familial gastrointestinal stromal tumors. As the somatic *BRAF* mutations, including V599E, result in 50-fold lower transforming activity than ^{V12G}HRAS in the NIH3T3 cell line, it is conceivable that *BRAF* germline mutations could predispose to melanoma. Thus, we also postulated that *BRAF* germline mutations could be responsible for dysplastic nevi considered as a precancerous phenotype by analogy with C-cell hyperplasia seen in *RET* oncogene carriers before the occurrence of medullary thyroid carcinoma.

To evaluate the *BRAF* gene as a candidate in melanoma predisposition, we screened the entire *BRAF* coding region (exons 1–18) for germline mutations in 80 independent melanoma families or patients

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[4] The abbreviations used are: CMM, cutaneous malignant melanoma; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; dHPLC, denaturing high-performance liquid chromatography; DNS, dysplasic naevus syndrome; NST, neural system tumor.

Table 1 *Clinical features of 80 melanoma patients tested in the study*

No.	Clinical subgroups	CMM within family	MPM[a]	Uveal melanoma	NSTs within family	DNS within family
1	CMM families at least 3 cases (n = 23)					
	Group 1: family no. 1 to 13	>3	No	No	No	Yes
	Group 2: family no. 14 to 21	>3	No	No	No	No
	Group 3: family no. 22 and 23	>3	No	No	No	ND
2	CMM families with 2 cases and MPM case (n = 12)					
	Group 4: family no. 24 to 31	2	Yes[b]	No	No	Yes
	Group 5: family no. 32 to 34	2	Yes[b]	No	No	No
	Group 6: family no. 35	2	Yes[b]	No	No	ND
3	MPM case (n = 16)					
	Group 7: individual no. 36 to 45	1	Yes[c]	No	No	Yes
	Group 8: individual no. 46 to 49	1	Yes[c]	No	No	No
	Group 9: individual no. 50 and 51	1	Yes[c]	No	No	ND
4	CMM and NSTs (n = 11)					
	Group 10: individual 52 to 62	1	No	No	Yes	ND
5	Uveal melanoma (n = 18)					
	Group 11: family 62 to 75	0	No	Yes (2 cases)	No	ND
	Group 12: family 76 to 79	1	No	Yes (1 case)	No	ND
	Group 13: individual 80	0	Yes	Yes (1 case)	No	ND

[a] One melanoma patient having developed 2 melanomas.
[b] MPM, multiple primary melanoma; ND, not determined.
[c] One patient at least 3 melanomas.

by sequencing analysis or dHPLC analysis. Patients tested were either index cases in melanoma-prone families or multiple melanoma patients. The inclusion criteria were cutaneous melanoma-prone families including families with DNS, patients with multiple cutaneous primary melanoma without a familial history, families with cutaneous melanoma and NSTs, and uveal melanoma-prone families (Table 1). The rationale for inclusion of these last two categories was, respectively: (*a*) BRAF proteins are expressed at high levels in adult mouse neural tissues (23) and 11% of human glioma cell lines present V599E, the hotspot mutation (20); and (*b*) transgenic mice overexpressing H-RAS developed cutaneous but also ocular tumors spontaneously (24), yet no uveal susceptibility gene has been identified to date. Moreover, uveal and skin melanocytes have the same embryonic origin (the neural crest), and cells originating from this lineage are known to express BRAF (16, 17).

Materials and Methods

Patient Selection and Control Group

Multiple cutaneous melanoma-prone family cases or multiple primary melanoma patients were enrolled through the Dermatology Department of the Institut Gustave Roussy and different oncogenetics or dermatology departments from all over France. Uveal melanoma-prone families were collected by the Ophtalmology and Oncogenetics Departments of the Institut Curie.

The 80 melanoma families or sporadic cases were (Table 1): (*a*) 23 cutaneous melanoma-prone families (>3 melanoma cases) including 13 melanoma-prone families with DNS; (*b*) 12 cutaneous melanoma-prone families (2 melanoma cases including a multiple case); (*c*) 16 patients with multiple cutaneous primary melanoma (patients who developed at least 3 primary melanomas); (*d*) 11 cutaneous melanoma-prone families with joint proneness to melanoma and NSTs; and (*e*) 18 uveal melanoma families (2 uveal melanoma cases or uveal and cutaneous melanoma cases or multiple uveal melanoma cases). For all of the subjects, the search for *CDKN2A/p16^{INK4A}/p14^{ARF}* and *CDK4* germline mutations was negative. All of the melanoma cases were confirmed by pathological reports. Written informed consent was obtained for all of the subjects before participation in the study under a protocol approved by the internal as well as an external Institutional Review Board (Hospital Necker, Paris, France).

Controls were constituted of lymphoblastoid DNA samples from 91 breast and/or ovarian cancer patients free of melanoma. These DNA samples were considered as waste and used anonymously.

Mutation Analysis of the BRAF Gene

DNA Samples and PCR. Genomic DNA was extracted from peripheral blood lymphocytes, using the QIAamp DNA blood mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. The coding exons and intron-exon junctions of the *BRAF* gene were screened for mutations by direct sequencing of exons 11 and 15, and by dHPLC followed by sequencing of abnormal profiles for the 16 other exons. PCR primers were designed to amplify each exon including at least 50–100 bp of flanking intronic sequences and primers were chosen with the assistance of the computer program Oligo Version 4.0. Primer sequences and the size of the PCR products for the different *BRAF* exons are described in Table 2.

The PCR reactions were performed in a final volume of 25 μl with 25 ng of genomic DNA, 200 μM of each deoxynucleotide triphosphate (Pharmacia LKB Biotechnology, France), 0.5 μM of each primer (MWG, Ebersterg, Germany), 1.25 UI of Taq DNA polymerase Hot Start (Qiagen), and 1× PCR

Table 2 *Primers used for amplification and sequencing of BRAF gene*

Amplified fragment	Primer sequence	Annealing temperature	Product size (bp)
Exon 1	F: CCTCCCCCAGCTCTCCCGC R: CCCCCGCCGCCTCTTTCCAAAATA	56°C	294
Exon 2	F: ACTGGCAGTTACTGTGATGTAG R: CCACCTCCTAAAATAATCAAGA	55°C	294
Exon 3	F: CCGGATTGAATATAAGTCTG R: TGCCACCAAATAATTACATA	55°C	455
Exon 4	F: CCCATTTGACTTTTTAAAGATGA R: TGGCCTACAGTATTTCTTCA	55°C	368
Exon 5	F: GCCCCTCGATAACCAATTTTCA R: CCAAAATTACTCATCCATATTTCA	55°C	297
Exon 6	F: CCCCCGGTTTTCATTTTATAATA R: CCAGCATTACAATTTGGGAGAGA	55°C	494
Exon 7	F: CTGGGTTTTGCACAAGTTAGGTT R: CGCCCAAGCAGAAGTCAAA	60°C	190
Exon 8	F: GGCAGTATTGGATTTTTAAATTAA R: ATGGCACTTATTTCTGATCTA	55°C	428
Exon 9	F: CCAAATTGTTTTGTGTAATAGTTA R: GGGTTTCTCTACACATTTTTCTCT	55°C	303
Exon 10	F: CCCCTGGATAAATTAACATACTT R: GCCGTAGAAATATGCTTTAA	55°C	367
Exon 11	F: CCCTCTCAGGCATAAGGTAA R: GCGAACAGTGAATATTTCCT	50°C	313
Exon 12	F: GGGCTATGATAATTAGTGAAAA R: GGGAACCAGGAGCTAATAAA	55°C	241
Exon 13	F: CCGACAGACTACTTTGGTTCT R: CAGCCAAAACCTTTAAAACA	55°C	334
Exon 14	F: CCCCCAAGTATGTTCTGTAGA R: GCATGCACAATCCTTTATTAA	55°C	265
Exon 15	F: TCTAAGAGGAAAGATGAAGTACTATG R: AGACCTTCAATGACTTTCTAGTAA	50°C	346
Exon 16	F: GCATTGCTCTAGGAATTATAGT R: GCCTTCGTATATAGACGGTAAAA	55°C	267
Exon 17	F: GTGGCATTGGTTTTTTAAAACT R: GCCCAAAAAAGTGCTCAGAA	55°C	360
Exon 18	F: GATGGGACTCTTAAAGATTTATA R: GGGGAAAAATTATATCTAGTCTT	55°C	453

Hot Start buffer (Qiagen). To amplify exon 1 (which had not been investigated in the study by Davies *et al.*; Ref. 20), 1.25 M of Betain (Sigma, Saint Quentin Fallasier, France) were added to the PCR reaction mix, and PCR products were purified through Sephadex G50 to eliminate Betain molecules. Amplification reactions were performed using a MWG thermocycler with the following cycling profile: denaturation step at 94°C for 5 min, 35 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min) and a final extension step at 72°C for 10 min. The annealing temperature were, respectively, 56°C and 60°C for exons 1 and 7.

Sequencing Analysis. To screen *BRAF* exons 11 and 15 for germline mutations, PCR products were bidirectionally sequenced with the Big Dye Terminator sequencing kit, using the same primers as those used for PCR. PCR products were purified by solid-phase extraction through Sephacryl S400-HR (Pharmacia) and subsequently analyzed using an ABI 377 sequencer (Perkin-Elmer, Applied Biosystem).

dHPLC Analysis. Screening for germline mutations in exons 1–18 excluding exons 11 and 15 was performed by dHPLC analysis, an automated method for heteroduplex detection. To obtain the heteroduplex, the PCR products were heat-denatured at 95°C for 5 min followed by gradual cooling from 95°C down to 25°C (0.1°C/s) to allow reannealing. Analyses were carried out on an automated dHPLC (Wave; Transgenomic) instrument. The PCR products were eluted from the column using an acetonitrile gradient in a 0.1 M triethylamine acetate buffer (pH 7.0), at the constant flow rate of 1.5 ml/min. Samples displaying abnormal profiles were subsequently sequenced with the Big Dye Terminator sequencing kit, as described previously. This method allows heterozygote patients to be detected but cannot discriminate wild-type/wild-type and mutant/mutant homozygotes.

Statistical Analysis. The χ^2 test was used to compare the frequency of heterozygotes for each *BRAF* variant between melanoma cases and control groups.

Results and Discussion

To investigate a possible role of the *BRAF* gene in melanoma genetic susceptibility, we studied 80 melanoma-prone families or multiple primary melanoma selected according to various criteria (Table 1), having ascertained the absence of germline mutations in the known melanoma susceptibility genes, *i.e.*, *CDKN2A/p16^{INK4A}/p14^{ARF}* and *CDK4*.

BRAF somatic missense mutations in melanoma and nevi were detected in exons 11 and 15 within the kinase domain of the *BRAF* gene (CR3 domain; Fig. 1). The most frequent mutations involved either codon 599 with a mutational hotspot, V599E, located in exon 15 within the kinase activation loop or codons 463 (G463E and G463V) and 465 (G465A, G465E, and G465V) that participate in the G-loop and codon 438 (K438Q) located in exon 11 (20, 21). We first sequenced *BRAF* exons 11 and 15 for each index case in the 80 melanoma-prone families or individuals selected. No mutation was detected in *BRAF* exon 15 in these patients. In *BRAF* exon 11, we detected a silent germline single-base substitution G1299A that did not change amino acids at position 443 (R443R), in 1 patient. This variant was not detected in the other 2 melanoma patients in these 3 melanoma kindred cases. No germline mutations were detected in this study at the molecular hotspots described in nevi, primary melanoma, and melanoma cell lines (20–22).

Whereas the somatic mutations described in malignant melanoma are located within the BRAF kinase domain, two other regions that are well conserved among members of the Raf family (CR1 and CR2) are also known to play an essential role in the regulation of BRAF protein activity; the CR1 domain, encoded by exons 3–6, is involved in binding to the RAS protein, and the CR2 domain, encoded by exon 8, contains a phosphorylation site at Ser364 that regulates BRAF kinase activity (16, 25; Fig. 1). We hypothesized that germline mutations in other BRAF domains outside the CR3 domain could also alter its function. We screened the entire coding sequence (excepted exons 11 and 15) including exon 1 that was not verified in the study by Davies

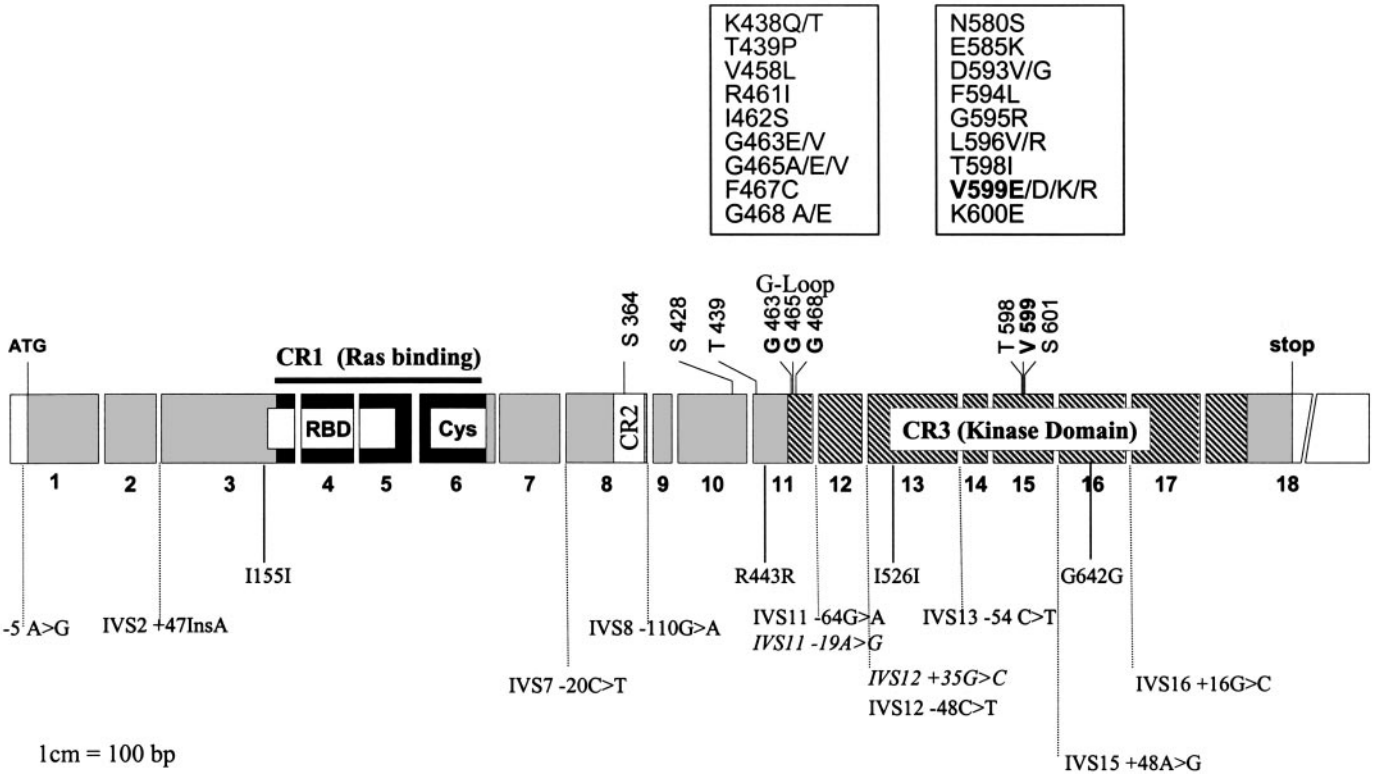

Fig. 1. Schematic representation of *BRAF* gene structure indicating published somatic mutations identified in human cancers, which are located in exons 11 and 15 (*two top boxes*), the V599E hot spot mutation detected in malignant melanomas (*highlighted in bold*), the location of the G-loop (ATP-binding site), the three conserved regions (*CR1, CR2,* and *CR3*), and the major phosphorylation sites in BRAF protein (*S364, S428, T439, T598,* and *S601*). *BRAF* germline variants identified in the 80 melanoma-prone families or in the 91 controls are indicated in the *bottom part*. The *BRAF* germ-line variants identified only in the 91 controls are indicated in *italic*. *RBD,* Ras binding domain; *Cys,* cysteine-rich sequence.

Table 3 *BRAF variants in melanoma-prone families tested*

Nucleotide change	AA change	Familial segregation[a]
−5A>G		Family no. 6: 1/3
IVS2 +47InsA		Family no. 2: 1/3
465C>T	I155I	Family no. 4: 1/4
IVS7 −20C>T		ND
IVS8 −110G>A		ND
1299G>A	R443R	Family no. 16: 1/3
IVS11 −64G>A		ND
IVS12 −48C>T		Family no. 4: 3/4, Family no. 7: 4/5, Family no. 7: 2/5
1578T>C	I526I	
IVS13 −54C>T		Family no. 10: 2/3, Family no. 67: 1/2
IVS15 +48A>G		ND
1926A>G[b,c]	G642G	Family no. 7: 4/5, Family no. 4: 3/4, Family no. 12: 2/3, Family no. 23: 2/2, Family no. 5: 1/2, Family no. 6: 1/3, Family no. 69: 1/2
IVS16 +16G>C[b]		Family no. 7: 4/5, Family no. 4: 3/4, Family no. 12: 2/3, Family no. 23: 2/2, Family no. 5: 1/2, Family no. 6: 1/3, Family no. 69: 1/2

[a] Family identification and number of melanoma cases carrying *BRAF* variant out of number of melanoma cases within the family; ND: Segregation analysis not possible.
[b] *BRAF* variants in linkage desequilibrium.
[c] *BRAF* variants described in Ref. 20, frequency < 0.01.

et al. (20), and intron-exon junctions for mutations in the *BRAF* gene using the dHPLC method in the same sample set of 80 index cases. We identified 12 *BRAF* variants, 10 of which were novel (Fig. 1; Table 2). DNA sequence analysis revealed 3 single-base nucleotide substitutions in the coding sequence (exons 3, 13, and 16) and 9 in intronic regions. The 3 single nucleotide changes in exon 3, (465C>T), 13 (1578T>C), and 16 (1926A>G), did not affect the amino acid sequence (respectively, I155I, I526I, and G642G) of the BRAF protein. In intron 2, one nucleotide insertion of an adenine at position +47 was detected in 1 patient. We also detected a single substitution (A>G) in the noncoding sequence of exon 1 (5′-untranslated region), 5 bp upstream of the ATG translation initiation codon. Among the 12 different *BRAF* germline variants detected, two variants located, respectively, in intron 13 (IVS12 + 35G>C) and in exon 16 (G642G) were reported previously as polymorphisms in the initial report by Davies *et al.* (20). We observed that two variants located at the 3′ end of the *BRAF* gene, G642G and IVS16 + 16G>C, exhibited complete linkage disequilibrium.

The potential pathogenicity of each *BRAF* variant was assessed by studying segregation with melanoma in 11 families through sequencing analysis of all of the available family members. None of the *BRAF* variants cosegregated fully with melanoma in the families tested (Table 3). In addition, no specific *BRAF* variant segregated in families with patients affected by both melanoma and DNS (data not shown). Moreover, no variant was specifically associated with any clinical subgroups, *i.e.*, CMM families, multiple primary melanoma cases, CMM and NSTs families, or uveal melanoma families. The absence of the two most frequent *BRAF* variants exhibiting linkage disequilibrium (G642G and IVS16 + 16G>C) in clinical subgroups 2 and 4 was probably because these groups were small, 12 and 11 patients, respectively (Table 4). These observations suggest that the different variants detected in 80 melanoma-prone index cases are probably not germ-line mutations conferring a high risk of developing melanoma in carriers.

Finally, we determined the frequency of *BRAF* variants in a control population. We screened the entire coding sequence and intron-exon junctions in the *BRAF* gene by dHPLC in 91 unrelated individuals without a history of melanoma. In this control group, 7 of 15 *BRAF* variants were identified, 2 of which were new (IVS11–19A>G and IVS12 + 35G>C). The 3 most frequent variants (IVS12–48C>T, G642G, and IVS16 + 16G>C) and the 2 rare variants (R443R and

Table 4 *Frequencies of heterozygotes for BRAF germline variants*

No.	Clinical subgroups	*n*	−5A>G	IVS2 +47InsA	I155I	IVS7 −20C>T	IVS8 −110G>A	R443R	IVS11 −19A>G	IVS11 −64G>A	IVS12 +35G>C	IVS12 −48C>T	I526I	IVS13 −54C>T	IVS15 +48A>G	G642G	IVS16 +16G>C
1	CMM families at least 3 cases	23	1 (4%)[a]	1 (4%)[a]	1 (4%)[a]	0	0	1 (4%)[a]	0	0	0	3 (13%)[a]	1 (4%)[a]	1 (4%)[a]	0	8 (35%)[a]	8 (35%)[a]
2	CMM families with 2 cases and MPM case	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	MPM case	16	0	0	0	0	0	0	0	1 (6%)[a]	0	3 (19%)[a]	0	0	7 (44%)[a]	7 (44%)[a]	
4	CMM and CNTS	11	0	0	0	1 (9%)[a]	0	0	0	0	0	0	0	0	1 (9%)[a]	0	0
5	Uveal melanoma	18	0	0	0	0	1 (5.5%)[a]	0	0	1 (5.5%)[a]	0	1 (5.5%)[a]	0	1 (5.5%)[a]	0	3 (17%)[a]	3 (17%)[a]
	Total melanoma case	80	1 (1%)[a]	1 (1%)[a]	1 (1%)[a]	1 (1%)[a]	1 (1%)[a]	1 (1%)[a]	0	2 (2.5%)[a]	0	7 (9%)[a]	1 (1%)	2 (2.5%)[a]	1 (1%)	18 (22.5)[a]	18 (22.5)[a]
	Control	91	0	0	0	0	0	1 (1%)	1 (1%)	0	3 (3%)	13 (14%)	0	1 (1%)	0	27 (30%)	27 (30%)

[a] Not significantly different between melanoma-prone families and control group (χ^2 analysis).

IVS13–54C>T) detected in melanoma-prone families were also found in controls. The frequency of heterozygotes for the 15 *BRAF* variants detected in melanoma-prone families and controls is indicated in Table 4. The χ^2 test was used to compare the frequency of heterozygotes for each *BRAF* variant between individuals from melanoma-prone families and controls. No statistically significant difference was found, suggesting that *BRAF* germline variants are germline polymorphisms and are not low or moderate risk melanoma susceptibility alleles. Studies based on allelic frequencies rather than heterozygote frequencies may be required to corroborate our study.

In conclusion, by screening the entire *BRAF* gene in 80 melanoma-prone individuals, we found 13 variants within the *BRAF* gene, 9 intronic nucleotide substitutions and 4 silent mutations in coding regions in 80 melanoma-prone families or cases. None of these variants segregated with disease in melanoma-prone families, and the frequency of heterozygotes for these variants did not differ significantly between melanoma cases and controls suggesting that *BRAF* variants are polymorphisms rather than disease-causing mutations. Consequently, our data suggest that *BRAF* is not a melanoma susceptibility gene. However, detection of *BRAF* somatic mutations in nevi does suggest that the *BRAF* mutations occur at a very early stage in melanoma pathogenesis.

Although *BRAF* somatic missense mutations have been reported at a very high frequency in nevi and melanoma, and at a lower frequency in many human cancers, our study shows that the *BRAF* gene does not seem to play any role in melanoma susceptibility. However, our negative results may suggest that other genes in the RAS-RAF-MAP kinase pathway play a role in melanoma susceptibility and should be tested for germline mutation in melanoma-prone families.

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