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 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 p16INK4A and p14ARF (5). Proteins encoded by melanoma predisposing genes are involved in the regulation of cell growth via the retinoblastoma cell cycle pathway (p16INK4A and CDK4; reviewed in Ref. 6) or in the p53 apoptosis pathway (p14ARF; 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 p14ARF (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 doxycycline-inducible V12GH-RAS mouse melanoma model, null for the tumor suppressor gene CDKN2A, i.e. both p16INK4A- and p14ARF-deficient, the genesis and maintenance of melanoma are strictly dependent on the expression of V12GH-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 V12GH-RAS 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; diHPCL, denaturing high-performance liquid chromatography; DNS, dysplastic naevus syndrome; NST, neural system tumor.
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 crest, and cells originating from this lineage are known to express BRAF (16, 17). 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 Ophthalmology 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/p14ARF and CDK4 germine 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 cancer cases or uveal and cutaneous melanoma cases or multiple uveal melanomas; (2 melanoma cases including a multiple case); (b) 12 cutaneous melanoma-prone families (1 melanoma family with DNS; (c) 13 melano-
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/p16INK4a, p14ARF, 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 of 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 through solid-phase extraction through Sephacryl S400-HR (Pharmacia) and subsequently analyzed using an ABI 377 sequencer (Perkin-Elmer, Applied Biosystem).

Statistical Analysis. The χ² test was used to compare the frequency of heterozygotes for each BRAF variant between melanoma cases and control groups.

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To investigate a possible role of the BRAF gene in melanoma genetic susceptibility, we studied 80 melanoma-prone families or
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 3. BRAF variants in melanoma-prone families tested

<table>
<thead>
<tr>
<th>Nucleotide change</th>
<th>AA change</th>
<th>Familial segregation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>−5A&gt;G</td>
<td></td>
<td>Family no. 6: 1/3</td>
</tr>
<tr>
<td>IVS2 +47unA</td>
<td>I155I</td>
<td>Family no. 2: 1/3</td>
</tr>
<tr>
<td>465C&gt;T</td>
<td></td>
<td>Family no. 4: 1/4</td>
</tr>
<tr>
<td>IVS7 −20C&gt;T</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>IVS8 −110G&gt;A</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>1299G&gt;A</td>
<td>R443R</td>
<td>Family no. 16: 1/3</td>
</tr>
<tr>
<td>IVS11−64G&gt;A</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>IVS12 −48C&gt;T</td>
<td></td>
<td>Family no. 4: 3/4, Family no. 7: 4/5, Family no. 5: 2/5</td>
</tr>
<tr>
<td>1578T&gt;C</td>
<td>IVS13−54C&gt;T</td>
<td>Family no. 10: 2/3, Family no. 67: 1/2</td>
</tr>
<tr>
<td>IVS15 +48A&gt;G</td>
<td>G642G</td>
<td></td>
</tr>
<tr>
<td>1926A&gt;G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVS16 +16G&gt;C</td>
<td></td>
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</tbody>
</table>

* Family identification and number of melanoma cases carrying BRAF variant out of number of melanoma cases within the family: ND. Segregation analysis not possible.

** BRAF variants in linkage disequilibrium.

*** BRAF variants described in Ref. 20, frequency < 0.01.

### Table 4. Frequencies of heterozygotes for BRAF germline variants

<table>
<thead>
<tr>
<th>Clonal subgroups</th>
<th>n</th>
<th>BRAF variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMM families at least 2 cases</td>
<td>23</td>
<td>16/49%</td>
</tr>
</tbody>
</table>
| MPM case | 16 | 0%
| MPM case and CNTS | 18 | 0%
| CNTS | 80 | 0%
| Total melanoma case | 94 | 17/54% |

* Not significantly different between melanoma-prone families and control group (Q-analysis).

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.

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Consequently, our data suggest that variants are polymorphisms rather than disease-causing mutations. Variants segregated with disease in melanoma-prone families, and the regions in 80 melanoma-prone families or cases. None of these intronic nucleotide substitutions and 4 silent mutations in coding BRAF

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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References


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