No Evidence for *BRAF* as a Melanoma/Nevus Susceptibility Gene

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Abstract

Somatic mutations of *BRAF* have been identified in both melanoma tumors and benign nevi. Germ line mutations in *BRAF* have not been identified as causal in families predisposed to melanoma. However, a recent study suggested that a *BRAF* haplotype was associated with risk of sporadic melanoma in men. Polymorphisms or other variants in the *BRAF* gene may therefore act as candidate low-penetrance genes for nevus/melanoma susceptibility. We hypothesized that promoter variants would be the most likely candidates for determinants of risk. Using denaturing high-pressure liquid chromatography and sequencing, we screened peripheral blood DNA from 184 familial melanoma cases for *BRAF* promoter variants. We identified a promoter insertion/deletion in linkage disequilibrium with the previously described *BRAF* polymorphism in intron 11 (rs1639679) reported to be associated with melanoma susceptibility in males. We therefore investigated the contribution of this *BRAF* polymorphism to melanoma susceptibility in 581 consecutively recruited incident cases, 258 incident cases in a study of late relapse, 673 female general practitioner controls, and the 184 familial cases. We found no statistically significant difference in either genotype or allele frequencies between cases and controls overall or between male and female cases for the *BRAF* polymorphism in the two incident case series. Our results therefore suggest that the *BRAF* polymorphism is not significantly associated with melanoma and the promoter insertion/deletion linked with the polymorphism is not a causal variant. In addition, we found that there was no association between the *BRAF* genotype and mean total number of banal or atypical nevi in either the cases or controls. (Cancer Epidemiol Biomarkers Prev 2005;14(4):913–8)

Introduction

Much progress has been made in recent years in the understanding of high penetrance susceptibility to melanoma. Three causal genes have been identified, *CDKN2A* (p16; ref. 1), *CDK4* (2), and *CDKN2A* (p14ARF; refs. 3, 4), and linkage evidence of a fourth gene at 1p22 has been reported more recently (5). However, mutations in these genes account for only a very small proportion of melanoma susceptibility in the general population.

Low-penetrance genes are believed to account for a much larger percentage of melanoma susceptibility in the general population. However, much less progress has been made in the identification of such genes. Only one important common low-penetrance gene has been identified, *MC1R*, variants of which play a major role in determining hair color (6), freckles (7), susceptibility to the sun, and melanoma (8, 9). There is also evidence both for (10, 11) and against (12) different polymorphisms in *CDKN2A* acting as low-penetrance genes. In terms of other low-penetrance melanoma susceptibility genes, there are conflicting data on the role of genes coding for detoxifying enzymes *GSTM1* and *CYP2D6* (13-17) and an unconfirmed small effect of the vitamin D receptor gene (18). Suggestions that a polymorphism in the DNA repair gene, *XRCC3*, predisposed to melanoma (19) were not confirmed (20, 21) and there is only one report of the nucleotide excision repair gene *XPD* in association with melanoma (22). A report of a *TP53* polymorphism in association with melanoma remains to be repeated (23), and initial reports of a role for a polymorphism of *EGF* (24) have not been confirmed (25, 26).

Most recently, the identification of somatic mutations in the *BRAF* gene in the majority of melanoma tumors (27, 28), and even precursor nevi (29), suggested that this gene and the RAS-RAF-MEK-ERK-MAP kinase pathway plays a key role in melanoma carcinogenesis. *BRAF* has therefore been considered as a potential susceptibility gene. Germ line mutations in *BRAF* have not been found in melanoma families (30, 31). More recently, however, a study was published which suggested that a *BRAF* haplotype might be associated with sporadic melanoma (32). There was no association between this haplotype and melanoma overall: the observation was made for males only and therefore may be a chance finding. Moreover, this clearly needed to be assessed in another population.

Approximately a third of the promoter variants could alter gene expression to a functionally relevant extent (33). Therefore, if *BRAF*, as part of a signaling pathway, does play a role in susceptibility to melanoma, then we hypothesized that promoter variants would be strong candidates for determinants of risk in populations as inappropriate levels of expression might compromise such a pathway. We have screened approximately 1 kb of sequence upstream of the start codon of *BRAF* (hypothesized to encompass the promoter region), in four sections.

In the study by Meyer et al. (32), the presence of the intron 11 polymorphism, rs1639679, strongly correlated with the H2 haplotype reported to be associated with melanoma in males. We have therefore screened our United Kingdom samples for

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Table 1. Promoter variants in the putative *BRAF* promoter and their frequencies

Putative promoter variant	Allele frequency (%)
g.−1207_−1204delGTTinsTGAATTC	6.8
g.−787C > T	0.3
g.−599A > G	7.1
g.−295G > A	1.4
g.−173G > A	0.8
g.−124C > T	0.8

the intron 11 polymorphism rs1639679 in a series of samples from English melanoma patients and controls. We have also examined the *BRAF* haplotype (for single nucleotide polymorphisms rs1639679, rs1267621, rs1267606, and rs1267646) in relation to a novel promoter variant in a subset of these individuals.

The presence of many nevi is the most potent phenotypic factor for melanoma (34, 35) and putative nevus genes are postulated to be low-penetrance melanoma susceptibility genes. We have therefore also explored the relationship between *BRAF* promoter variants identified during screening, the intron 11 polymorphism rs1639679 and nevus phenotype.

Materials and Methods

Study Populations. Patients with melanoma and controls recruited from four different studies were investigated. The first group consisted of 581 consecutively recruited individuals with newly diagnosed melanoma enrolled into the Melanoma Follow-up and Case-control Family Study (henceforth referred to as the cohort sample) in the period from November 2000 until January 2004. These patients are a population-based sample from Yorkshire, United Kingdom. Ages ranged from 18 to 78 with a mean age of 51.5 years. The second case series included 258 United Kingdom melanoma patients taking part in a study of late relapse: these samples were therefore comprised of both late relapsing patients and melanoma patients surviving with no evidence of relapse. Ages in this group ranged from 19 to 79 with a mean of 49.3 years. The third series of cases was comprised of samples from familial melanoma cases: from pedigrees with two or more cases of melanoma. Their ages ranged from 12 to 81 with a mean of 40.7 years. This case series was chosen to screen for variants in the putative promoter region of *BRAF* as a more likely source of significant low- to medium-penetrance mutations. Finally, 673 female controls were recruited from the general population via general practitioners from Yorkshire and Hertfordshire (the general practitioner control group), as previously described (12). Their ages ranged from 19 to 46 years with a mean of 36.5 years.

Nevus phenotype information was available for the first 365 melanoma patients recruited to the cohort study and 628 general practitioner controls. All nevus counting was carried out by nurse examiners according to a protocol (12). Nevi of 2 mm or greater in size were counted and the number of atypical nevi recorded. The atypical mole syndrome score, as a measure of the overall phenotype, was calculated as previously described (36).

Blood samples were taken and DNA extracted using the BACC 2 DNA extraction kit (Tepnel Life Sciences, Manchester, United Kingdom) according to the supplied protocol. Written informed consent was obtained from all participating individuals and institutional and regional ethical committee approval was obtained.

Screening of the Promoter. Approximately 2 kb of sequence upstream of the *BRAF* transcription start site was assessed using Gene2Promoter prediction software (Genomatix Software GmBH, www.genomatix.de) and AliBaba2 transcription factor binding site prediction software (37). A 1 kb region was identified which was predicted to contain the active promoter of the *BRAF* gene. We therefore screened this putative *BRAF* promoter for possible variants; 1 kb of the promoter was screened in the following fragments: 1, 2, 3A, and 3B. The initial screen of fragments 1, 2, and 3A were carried out by denaturing high-pressure liquid chromatography (DHPLC). Samples with aberrant DHPLC profiles were then sequenced to identify the underlying variant. In the case of fragment 3B, samples were screened by sequencing, as the PCR method used to amplify this GC-rich fragment precluded DHPLC analysis.

Polymerase Chain Reaction. PCR amplification of fragments 1, 2, and 3A of the *BRAF* putative promoter region was carried out in a final volume of 25 μL using Amplitaq Gold polymerase, 1× PCR buffer 1.5 mmol/L $MgCl_2$ (Applied Biosystems, Warrington, United Kingdom), 200 μmol/L of each deoxynucleotide triphosphate (Amersham Pharmacia, Buckinghamshire, United Kingdom), 1 pmol/μL of primer, and 1 ng/μL genomic DNA. Amplification reactions were done using a Geneamp PCR system 9700 using standard cycling conditions. The GC-rich PCR system (Roche, East Sussex, United Kingdom) was used for the amplification of promoter fragment 3B. The reactions were carried out in a final volume of 25 μL according to the manufacturer's instructions also using a Geneamp PCR system 9700. Primer pairs used for PCR amplification are as follows. Fragment 1, forward-TGG GAA GTA CTA AAA GGC AGA ATC, reverse-CCT CAA CAA TGA AGA AAA AGC TT; fragment 2, forward-GCA TGG AAA GTG CTC AAC TG, reverse-CAA AGT GTG AGA GCG CGG; fragment 3A, forward-CTG AGG ACG GAG GAG ACA AGA G, reverse-GCG GAG ACG ACG AGA AGT CGC CG; and fragment 3B, forward-GGC GAC TTC TCG TCG TCT C, reverse-CCG CCA TCT TAT AAC CGA GAG C.

DHPLC Screening. Temperatures for DHPLC analysis were selected on the basis of experimental melting curves and the theoretical prediction program http://insertion.stanford.edu/melt.html and were as follows: fragment 1, 53°C and 56°C; fragment 2, 55°C and 61°C; and fragment 3A, 64°C and 68°C. DHPLC was carried out using a Transgenomic WAVE nucleic acid fragment analysis system and DNASep column (Transgenomic, Crewe, United Kingdom; ref. 38). PCR products were prepared for DHPLC by denaturing at 95°C for 5 minutes and then cooling to 65°C to allow heteroduplex formation. Wild-type DNA for the region was used as a reference. Data analysis was by visual inspection of chromatograms, which were examined independently by two observers.

Sequencing. For the *BRAF* promoter fragments, samples that displayed aberrant DHPLC profiles were sequenced in both directions using Abi Prism BigDye Terminator Cycle Sequencing Kit version 1.1 (Applied Biosystems) according to the manufacturers instructions and analyzed on an ABI 3100. Fragment 3B was assessed by sequencing (in both directions). Single nucleotide polymorphisms rs1267621, rs1267606, and rs1267646 (in *BRAF* introns 1, 2, and 9, respectively) were also assessed by sequencing (in the reverse direction only). Primer pairs used for initial PCR amplification of introns 1, 2 and 9 are as follows: intron 1, forward-GGC TTG CTG TTC TTC CAG AG, reverse-AGG GAG GGA AGG GAG AGA C; intron 2, forward-TTT CTA TTC CCT TCA GCC ATT C, reverse-ACT GCT GCC TTC AGT GTG TG; and intron 9, forward-GCA AGT GGC ATT TCT TGT CC, reverse-TGG TTC AAA GGG GCT GTT AG.

Genotyping. DNA samples were screened for the intron 11 single nucleotide polymorphism (rs1639679) and for the insertion/deletion identified in promoter fragment 1 of

Table 2. Genotype and allele frequencies for the *BRAF* single nucleotide polymorphism rs1639679 in the case and control groups compared with the study by Meyer et al.

	Cohort cases			Late relapse cases		
	Total (n = 581)	Male (n = 247)	Female (n = 334)	Total (n = 258)	Male (n = 90)	Female (n = 168)
Allele frequency						
C allele (%)	94.7	94.7	94.9	92.8	92.6	93.3
A allele (%)	5.3	5.3	5.1	7.2	7.4	6.7
Genotype frequency						
CC (%)	523 (90.0)	221 (89.5)	302 (90.4)	222 (86.1)	79 (87.8)	143 (85.1)
CA (%)	56 (9.64)	26 (10.5)	30 (8.98)	35 (13.6)	10 (11.1)	25 (14.9)
AA (%)	2 (0.34)	0	2 (0.6)	1 (0.4)	1 (1.1)	0
Hardy-Weinberg (P)	0.70	0.38	0.2	0.76	0.31	0.30

BRAF by genotyping. The primers used are as follows: intron 11, forward (outer)-GTT ACA TAT ATA TAC ATG TGC CAT GCT G, forward (inner)-TAC TTT TAA GCA AAA TTC CAT AGG TCA, reverse (outer)-CCA TGG AAA TTT GGT AAA ATC AAT, reverse (inner)-TGC AAG TAA TGT TGA AAC TAC AAT TAC AAG; and fragment 1, forward-TGA AGA CAC CCT GTT TCA GAA G, reverse-GGA AGA GAG AAA CGG GAA TG. PCR samples were amplified using Amplitaq Gold as described above and analyzed on an ABI3100 automated sequencer with Genescan-500 Rox (Applied Biosystems) used as a size standard.

Statistical Analysis. Genotype/allele frequencies and tests for Hardy-Weinberg equilibrium were calculated both overall and for males and females separately. There was a small proportion of individuals with the rare (AA) genotype for the intron 11 polymorphism, therefore, the CA and AA genotypes were combined in all statistical analyses.

The association between genotype and melanoma risk among the cohort group was analyzed using logistic regression. Although there is no *a priori* reason to suspect that sex has an effect on these results, because the previous paper by Meyer et al. (32) suggested an effect in males only, this analysis was carried out in males and females separately as well as for both sexes combined. Comparisons of genotype frequencies between the male and female melanoma cases were examined using χ^2 tests. To replicate the findings of Meyer et al., allele frequencies were also compared.

Total nevus counts and atypical nevus counts were compared between genotype groups in both the cohort group and the general practitioner group using two-sided t tests. Before analyses, the natural logarithm of the nevus counts was taken to make the data more normally distributed.

A nominal significance level of 5% was used in all analyses. All statistical analyses were carried out using Stata Statistical Software (version 8, Stata Corporation, College Station, TX).

Results

One hundred and eighty-four melanoma family samples were screened by DHPLC and sequencing for mutations in the putative promoter region of *BRAF*. Six variants were found across the region, two common and four rare with allele frequencies ranging from 0.3% to 7.1% (Table 1). One of these, a common insertion/deletion (g.−1207_−1204delGTTinsTGAATTC), proved to be in linkage disequilibrium with the *BRAF* intron 11 single nucleotide polymorphism, rs1639679 in these samples. The genotyping of a further 633 samples from the cohort and general practitioner control groups for the *BRAF* promoter insertion/deletion and the intron 11 polymorphism identified 66 samples which carried both variants. Three discordant samples were also identified which carried the *BRAF* intron 11 polymorphism but not the promoter insertion/deletion. One of these samples was from the general practitioner control group and the other two from the cohort group.

Haplotype analysis of 91 samples using additional single nucleotide polymorphisms in *BRAF* introns 1, 2, and 9 (rs1267621, rs1267606, and rs1267646) estimated that in every instance, the presence of the insertion/deletion in the *BRAF* promoter corresponded with the H2 haplotype reportedly associated with melanoma (32). Of the three samples identified with the intron 11 polymorphisms but not the promoter insertion/deletion, one was found to have the H2 haplotype. In the remaining two samples, the intron 11 polymorphism

Table 3. Comparison of *BRAF* intron 11 genotype distribution and allele frequency between male and female melanoma cases using χ^2 tests

	Female general practitioner controls n (%)	Cohort n (%)			Late relapse n (%)			Familial cases n (%)		
	Females	Females	Males	P	Females	Males	P	Females	Males	P
Genotype										
CC	581 (86.3)	302 (90.4)	221 (89.5)	0.71	143 (85.1)	79 (87.8)	0.56	102 (91.1)	60 (83.3)	0.11
CA + AA	92 (13.7)	32 (9.6)	26 (10.5)		25 (14.9)	11 (12.2)		10 (8.9)	12 (16.7)	
Total	673 (100)	334 (100)	247 (100)		168 (100)	90 (100)		112 (100)	72 (100)	
Allele										
C	1,249 (92.8)	634 (94.9)	468 (9.7)	0.90	311 (92.6)	168 (93.3)	0.75	214 (95.5)	130 (90.3)	0.05
A	97 (7.2)	34 (5.1)	26 (5.3)		25 (7.4)	12 (6.7)		10 (4.5)	14 (9.7)	
Total	1,346 (100)	668 (100)	494 (100)		336 (100)	180 (100)		224 (100)	144 (100)	

Table 2. Genotype and allele frequencies for the *BRAF* single nucleotide polymorphism rs1639679 in the case and control groups compared with the study by Meyer et al. (Cont'd)

Familial melanoma cases			Female general practitioner controls	Study by Meyer et al. (32)			
Total (n = 184)	Male (n = 72)	Female (n = 112)	Female (n = 673)	Male cases (n = 236)	Male controls (n = 217)	Female cases (n = 266)	Female controls (n = 233)
93.5	90.3	95.5	92.8	88.8	92.7	94.3	93.4
6.5	9.7	4.5	7.2	11.2	7.3	5.7	6.6
162 (88.0)	60 (83.3)	102 (91.1)	581 (86.3)				
20 (10.9)	10 (13.9)	10 (8.9)	87 (12.93)				
2 (1.1)	2 (2.8)	0	5 (0.74)				
0.14	0.08	0.62	0.39				

seemed to be present as part of a rarer haplotype (H6) that was not associated with the promoter insertion/deletion.

The prevalence of the intron 11 polymorphism in each study population of melanoma cases and controls is shown in Table 2. The genotypes observed in the various study populations were all found to be in Hardy-Weinberg equilibrium.

Due to the observation that the intron 11 polymorphism and the promoter insertion/deletion variant are not in complete linkage disequilibrium, separate statistical analyses were done to establish whether the insertion/deletion variant alone was associated with melanoma.

There was no statistically significant difference in the genotype or allele distribution for the intron 11 polymorphism between male and female cases either in the cohort, or in the late relapse study groups (Table 3). There was a marginally higher frequency of the ''*A*'' allele in the male familial cases compared with the female familial cases (P = 0.05, Table 3). There was a marginally higher proportion of general practitioner controls with the rare genotype group compared with the cohort cases (P = 0.05), but the genotype distribution of the general practitioner controls was consistent with the results of the late relapse and the familial cases (tests not shown).

Table 4 shows the comparison of the promoter insertion/deletion genotype distribution between male and female cases (the late relapse group was not assessed). There was no difference in genotype or allele distribution between males and females in either the cohort or the familial study group. There was also no difference in genotype/allele distribution between the general practitioner controls and either melanoma case study group (tests not shown).

There was no difference in the mean total number of banal or atypical nevi between genotype groups, in either the general practitioner controls or the cohort cases (Table 5). This was also the case when the results were adjusted for age (tests not shown).

With regard to the other variants identified in the *BRAF* putative promoter, g.−599A > G was the most common (allele frequency of 7.1%) and remains a possible susceptibility variant. Preliminary work however suggests that allele frequencies are the same in case (n = 184) and control groups (n = 176, results not shown).

Discussion

The high prevalence of oncogenic somatic mutations in melanoma tumors and nevi (39) suggests that mutation in this gene is a key event early in the process of melanocyte proliferation which may lead to melanoma. Given that the presence of a large number of nevi is the most potent risk factor for melanoma yet identified, polymorphisms in the gene leading to changes in expression of the protein were hypothesized to be putative low-penetrance melanoma and/or nevus genes. A study published recently suggests that a polymorphism in the *BRAF* gene might predispose to melanoma. The first study to address this provided some supportive evidence for association of a haplotype (named the H2 haplotype) with melanoma but only in males (32). We hypothesized that promoter variants in *BRAF* would be the most likely determinants of risk and therefore screened the putative promoter, identified using promoter prediction software, for variants. Six variants were found by screening DNA samples from familial melanoma cases but the promoter insertion/deletion was of particular interest because it was common, and was shown to be in linkage disequilibrium with the *BRAF* intron 11 polymorphism rs1639679. Indeed, this insertion/deletion proved to correspond more accurately

Table 4. Comparison of insertion/deletion genotype distribution and allele frequency between male and female melanoma cases using χ^2 tests

	Female general practitioner controls (%)	Cohort n (%)			Families with two or more cases n (%)		
	Females	Females	Males	P	Females	Males	P
Genotype							
Wild-type	148 (89.2)	244 (90.0)	175 (89.3)	0.79	101 (90.9)	59 (83.1)	0.11
Insertion/deletion	18 (10.8)	27 (10.0)	21 (10.7)		10 (9.01)	12 (16.9)	
Total	166 (100)	271 (100)	196 (100)		111 (100)	71 (100)	
Allele							
Common	314 (94.6)	514 (94.8)	371 (94.6)	0.90	211 (95.1)	128 (90.1)	0.07
Rare	18 (5.4)	28 (5.2)	21 (5.4)		11 (4.9)	14 (9.9)	
Total	332 (100)	542 (100)	392 (100)		222 (100)	142 (100)	

NOTE: Samples from the late relapse study were not screened.

Table 5. Univariate analysis of nevus characteristics with genotype in the cohort and general practitioner control groups

	Cohort group			Female general practitioner controls		
	CC, mean (SD)	CA/AA, mean (SD)	*P* value from two-sided *t* test	CC, mean (SD)	CA/AA, mean (SD)	*P* value from two-sided *t* test
Atypical mole syndrome 0-5: (number)	327	38		540	88	
Log total nevus count	3.8 (1.0)	3.8 (1.0)	0.81	3.7 (0.9)	3.7 (0.9)	0.49
Log atypical nevus count	0.4 (0.7)	0.3 (0.5)	0.35	0.2 (0.4)	0.1 (0.4)	0.62

with the H2 haplotype previously reported in association with melanoma (32), than the *BRAF* intron 11 polymorphism rs1639679. This promoter variant therefore seemed a good candidate as a melanoma/nevus susceptibility gene.

In the study of German melanoma patients by Meyer et al. (32), male cases were significantly more likely to carry the H2 haplotype than male controls (whose haplotype frequency matched that of the female controls). There was no difference between female cases and female controls so the statistical evidence for an association came from the increased H2 haplotype frequency among the male cases as compared with the female cases, female controls, and male controls. In this much larger study, we found no difference in the prevalence of the intron 11 polymorphism associated with the H2 haplotype between cases and controls in three groups of cases (incident cases, familial cases, and cases taking part in a study of late relapse) and general practitioner controls. No difference was seen in genotype or allele distribution between male and female cases in the cohort and late relapse groups. The only difference was seen in the familial cases, where there was a higher allele frequency in males compared with females. This result is only just significant ($P = 0.05$) and may be due to chance, especially as the allele frequency estimated from the female familial cases is the lowest of the sample series that we studied (Table 3). In addition, we have found no evidence for an association between a common promoter insertion/deletion (present in the haplotype H2 described by Meyer) and melanoma risk. Furthermore, we found no association between this variant and the nevus phenotype.

With regard to the other variants identified in the *BRAF* putative promoter, g.−599A > G is the most common and remains a possible susceptibility variant, although preliminary data suggests that it is not. Work is ongoing to look at the other four rare promoter variants.

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