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BRAF Polymorphisms and Risk of Melanocytic Neoplasia

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Somatic mutations of the *BRAF* gene are common in melanomas and nevi but the contribution of polymorphisms in this gene to melanoma or nevus susceptibility remains unclear. An Australian melanoma case–control sample was typed for 16 single nucleotide polymorphisms (SNP) within the *BRAF* gene, and five SNP in three neighboring genes. The sample comprised 755 melanoma cases from 740 families stratified by family history of melanoma and controls from 635 unselected twin families (2239 individuals). Ancestry of the cases and controls was recorded, and the twins had undergone skin examination to assess total body nevus count, degree of freckling, and pigmentation phenotype. Genotyping was carried out via primer extension followed by matrix-assisted laser desorption ionization-time of flight mass spectrometry. SNP in the *BRAF* gene were found to be weakly associated with melanoma status but not with development of nevi or freckles. The estimated proportion of attributable risk of melanoma due to variants in *BRAF* is 1.6%. This study shows that *BRAF* polymorphisms predispose to melanoma but the causal variant has yet to be determined. The burden of disease associated with this variant is greater than that associated with the major melanoma susceptibility locus *CDKN2A*, which has an estimated attributable risk of 0.2%.

Key words: BRAF/genetic predisposition/melanoma/single nucleotide polymorphisms
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The field of cancer genetics has produced notable successes applying genetic linkage analysis to identify genes that confer significant inherited susceptibility to particular cancers that show specific familial clustering. So far, these have been variants in genes of relatively large effect but which are rare in the general population, e.g., *BRCA1* and *BRCA2* in breast cancer and *CDKN2A* in melanoma, and thus do not account for a major contribution to overall population risks. Unlike these prominent cases it seems likely that most common cancers with an inherited component involve more genes, each conferring a fraction of the risk, and perhaps particular variants in multiple genes acting epistatically. Clearly, these are not readily amenable to discovery using the linkage approach and instead an association design may be more successful (Barrett *et al*, 2005). Thus far, such association studies have had mixed results, arguably due to inadequate sample size and suboptimal study design (Risch, 2001). Here, we present a large association study of the role in genetic predisposition to melanoma susceptibility of the *BRAF* gene, in a systematic and comprehensive haplotyping approach across the entire locus and adjacent genes.

The Ras/Raf/MAPK (mitogen-activated protein kinase) pathway is a critical molecular signaling cascade through which extracellular signals can be transmitted into the nucleus to regulate cell proliferation or differentiation through altered gene expression. Constitutive activation of this pathway is a frequent event in melanoma development (Cohen *et al*, 2002; Dong *et al*, 2003; Satyamoorthy *et al*, 2003). Recently, *BRAF* gene somatic mutations have been shown to be associated with malignant melanoma (Davies *et al*, 2002), being present in 40%–88% of cutaneous melanomas (Brose *et al*, 2002; Davies *et al*, 2002; Dong *et al*, 2003; Gorden *et al*, 2003; Kumar *et al*, 2003; Pollock *et al*, 2003; Satyamoorthy *et al*, 2003), whereas being essentially absent in control tissues. Mutations are also extremely common (74%–82%) in benign melanocytic nevi (Pollock *et al*, 2003; Yazdi *et al*, 2003), an observation arguing for a critical role for B-Raf in initiating melanocytic neoplasia.

All somatic mutations documented to date in melanoma have been found in the kinase domain of B-Raf, encoded by exons 11 and 15 of the *BRAF* gene (Brose *et al*, 2002; Davies *et al*, 2002; Pollock *et al*, 2003; Satyamoorthy *et al*, 2003; Casula *et al*, 2004) (Fig 1). The vast majority (over 90%) of these mutations affect codon 599 and result in a valine to glutamic acid substitution, see note added in proof which is thought to lead to constitutive activation of Ras/Raf/MAPK signal transduction (Davies *et al*, 2002). Other less frequent *BRAF* coding somatic mutations in melanoma are G468A in exon 11 and L596V and Q608H in exon 15 (Davies *et al*, 2002; Pollock *et al*, 2003; Casula *et al*, 2004). In some melanomas without *BRAF* mutations, the MAPK pathway is constitutively activated through mutation of *NRAS* (van Elsas *et al*, 1996). BRAF and NRAS mutations appear to have similar effects in melanoma development

Abbreviations: BTNS, Brisbane Twin Nevus Study; CMM, cutaneous malignant melanoma; QFMP, Queensland Familial Melanoma Project; SNP, single nucleotide polymorphism

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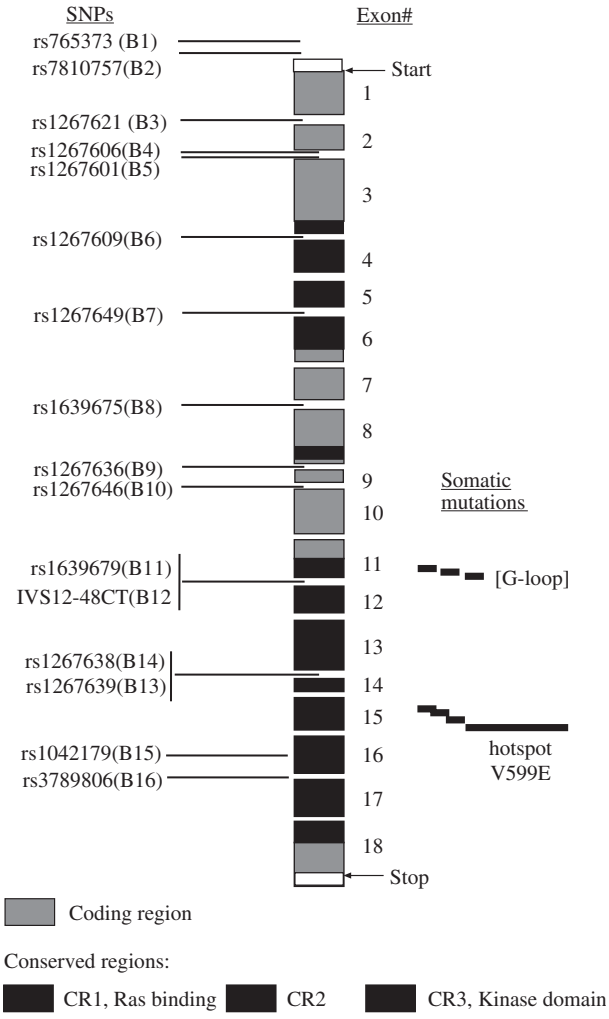

Figure 1
Positions of single nucleotide polymorphisms (SNP) within the *BRAF* gene. Each of the 18 exons is indicated by a box in the center of the figure, with the exon number adjacent to the right. Domains of the gene encoding the three conserved regions found among *Raf* family members are indicated by differential shading. SNP are shown by their unique database id (rs#) and the abbreviation used in this paper (B#). Other SNP in this study that flank *BRAF* are shown in Fig 2.

since their presence in any single tumor is mutually exclusive (Brose *et al*, 2002; Davies *et al*, 2002; Pollock *et al*, 2003; Satyamoorthy *et al*, 2003). This situation is similar in lung (Brose *et al*, 2002; Davies *et al*, 2002; Naoki *et al*, 2002), colon (Davies *et al*, 2002; Rajagopalan *et al*, 2002; Yuen *et al*, 2002), and thyroid cancers (Kimura *et al*, 2003), where *BRAF* and *RAS* mutations are rarely found together.

To date, only one common germline *BRAF* coding variant has been reported, a synonymous G642G change (rs1042179 (NCBI, 2003)). Although this particular variant was not tested, Meyer *et al* (2003b) recently described a case–control study (502 melanoma cases; 450 controls) assessing possible associations between intronic single nucleotide polymorphisms (SNP) in the *BRAF* gene and melanoma risk. Six of 12 SNP analyzed in this German case–control study were significantly ($p < 0.05$) more common in male cases than controls, but none of the SNP were significantly associated with melanoma risk in females, or when both sexes were combined. By contrast, Laud *et al* (2003) found no association between *BRAF* coding and intronic variants in a panel of 80 melanoma cases and 91 controls. More recently, a study of 569 Italian cases has revealed two types of germline variants (Casula *et al*, 2004), which were previously unreported codon changes (M116R, Q608H; note this was misreported as G608H (Casula *et al*, 2005)). The latter falls in exon 15 near the hotspot for somatic mutations that affect the kinase domain of the protein, whereas the former is of uncertain functional significance. One instance of the V599E substitution in germline DNA has subsequently been retracted (Casula *et al*, 2005).

In this study, we sought to test the hypothesis that inherited polymorphisms of the *BRAF* gene predispose to the development of melanoma using a large case–control study. In addition, we attempted to detect association with nevus count and freckling phenotype in the general population.

Results

After data cleaning (Materials and Methods), there were 755 CMM (cutaneous malignant melanoma) cases with genotype information, and 46 unaffected genotyped relatives from the QFMP (Queensland Familial Melanoma Project). From the BTNS (Brisbane Twin Nevus Study), there were 2229 controls with genotype information after data cleaning and removal of one of each pair of the 159 monozygotic (MZ) twins. For the case–control analyses, there were 720 unrelated cases (mean age 52.9 y, 43.8% male) and 1170 unrelated controls (mean age 42.6 y, 44.8% male). We and others (Martin *et al*, 1997; Risch, 2001) have shown that unselected twin collections provide valid population controls since they represent a truly random proportion (1% of all births) of the general population. Previously, we reported no differences in mole counts between the twins and the general population from Queensland (Zhu *et al*, 1999, 2004). Over 95% of grandparents of both cases and controls are of Northern European ancestry, mainly from the British Isles. Mean Breslow thickness of melanomas in cases was 0.3 mm, and 21.4% were histologically graded as level I tumors.

All 16 SNP minor alleles were more common in cases than in controls, and the differences in frequency were statistically significant for 11 SNP (exceeding a critical threshold of 0.05, uncorrected for multiple testing; see Table I) After correction for multiple testing, the best individual SNP permutation in p-value was 0.065. No SNP was significantly associated with melanoma when either males or females were considered separately, and indeed, there was no heterogeneity of risk between sexes. The highest genotypic relative risk was for the B11 SNP (rs1639679) in intron 11 (1.30, 95% CI = 1.00–1.69, $p = 0.018$). Adjusting for ancestry (proportion of grandparents born in the British Isles) did not alter the strength of this association.

The B5 (rs1267621) SNP minor allele frequency was highest in the high familial risk cases (Table I), and a test for trend in family risk was significant ($p = 0.008$). In the 10 cases (from eight high-risk families) carrying a mutation in *CDKN2A*, the major familial melanoma predisposition gene, none carried the rare allele (Table II).

Table I. SNP genotyped around the *BRAF* gene

Gene	SNP ID	dbSNP_ID	Exon/intron[a]	Change (transcribed strand)	Frequency[b]	Association χ^2 with melanoma	p-value
MRPS33	M1	rs1533933		C>G	0.205	1.29	0.525
BRAF	B1	rs765373	Promoter	C>T	0.138	4.32	0.116
	B2	rs7810757	5′UTR	A>G	0.073	1.32	0.517
	B3	rs1267621	Intron-1	G>A	0.133	5.41	0.067
	B4	rs1267606	Intron-2	T>G	0.061	4.24	0.120
	B5	rs1267601	Intron-2	A>G	0.061	9.79	0.007
	B6	rs1267609	Intron-3	G>A	0.060	7.02	0.030
	B7	rs1267649	Intron-5	C>G	0.061	7.02	0.030
	B8	rs1639675	Intron-7	T>C	0.061	9.07	0.011
	B9	rs1267636	Intron-8	A>G	0.061	6.88	0.032
	B10	rs1267646	Intron-9	C>T	0.135	4.17	0.124
	B11	rs1639679	Intron-11	C>A	0.060	7.11	0.029
	B12	IVS12-48CT	Intron-12	C>T	0.062	2.59	0.274
	B13	rs1267639	Intron-13	C>T	0.062	9.04	0.011
	B14	rs1267638	Intron-13	T>C	0.062	6.97	0.031
	B15	rs1042179	Exon-16	A>G	0.140	6.6	0.037
	B16	rs3789806	Intron-16	C>G	0.141	6.71	0.035
ADCK2	A1	rs1046515		C>T	0.061	6.63	0.036
Q9ULE3	Q1	rs269238		T>G	0.290	3.39	0.184
	Q2	rs3748088		C>T	0.337	4.16	0.125
	Q3	rs4726882		A>C	0.500	1.58	0.453

[a]Within the 18 exon transcript ENST00000288602.
[b]Minor allele frequency in controls.
SNP, single nucleotide polymorphism.

The 16 *BRAF* SNP were in extremely tight linkage disequilibrium (LD), such that D′ values for pairs of adjacent SNP ranged from 0.72 to 1 (Fig 2). This degree of LD results in only three haplotypes defining 98% of chromosomes (Table III). The strength of association of the common haplotypes combining the *BRAF* SNP was not greater than that evidenced by the individual SNP with melanoma (Table IV). For example, SNP B5, or equivalently, the "C" haplotype, accounted for a proportion attributable risk estimated as 1.6% (95% CI = 0.1%–3.1%). The histological level and Breslow thickness of tumors were not correlated with genotype, either for individual SNP or overall haplotype (Table IV).

There was no association between any of the *BRAF* SNP and counts of total, macular, papular, or atypical nevi in the Australian controls (best p-value = 0.16; see Table V). Similarly, no association was seen with freckling (Table V). Numbers of genotypes containing the "C" haplotype were not large, however.

Table II. Association analysis. Increased gradient of *BRAF* association with familial risk of melanoma

Population group	C/C	C/A	A/A	Minor allele frequency	p-value
Controls (n = 1170)	0.874	0.123	0.003	0.064	
Low familial risk (n = 448)	0.859	0.127	0.013	0.077	0.041
Intermediate familial risk (n = 150)	0.900	0.073	0.027	0.063	0.002
High familial risk (n = 59)	0.780	0.220	0.000	0.110	0.099
CDKN2A mutation families (n = 8)	1.000	0.000	0.000	0.000	0.614

Genotypic frequencies of the B2 SNP in the Australian case–control sample stratified by familial risk class and *CDKN2A* mutation status.
SNP, single nucleotide polymorphism.

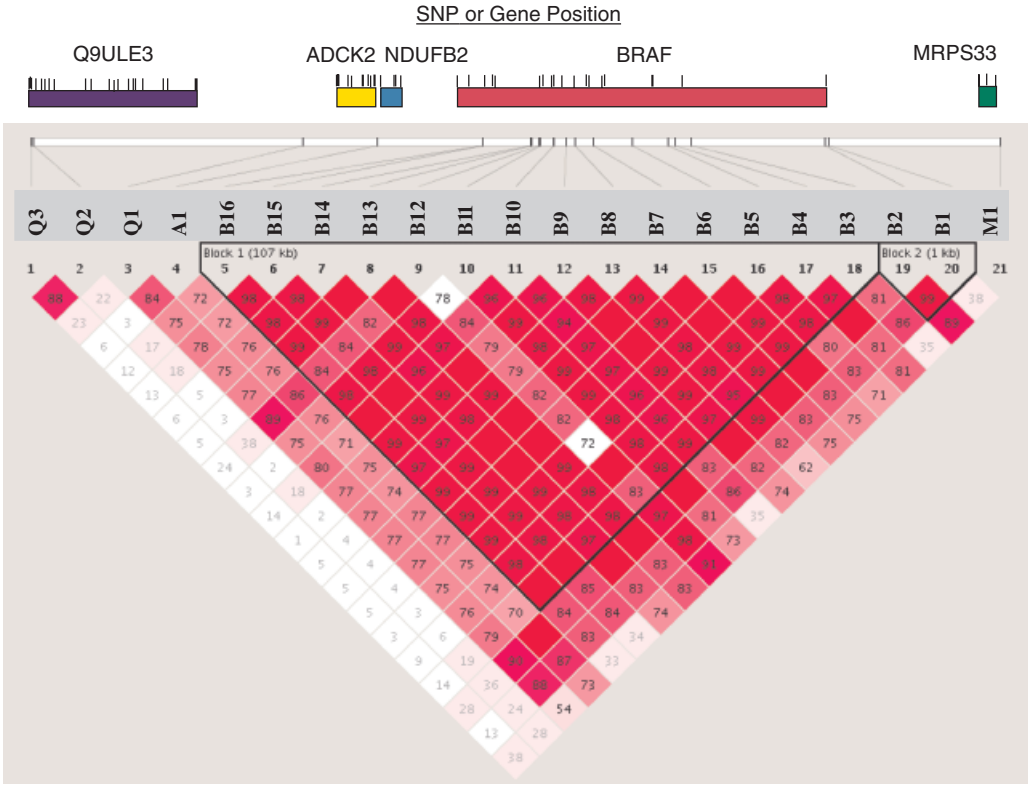

Figure 2
Linkage disequilibrium across 484 kb. Plot of inter-single nucleotide polymorphism (SNP) linkage disequilibrium, measured as Lewontin's D′, using Haploview (Barrett *et al*, 2005). Gene intron (boxes) and exon (tick marks) positions are represented along the X-axis, whereas SNP positions are shown as vertical dotted lines. SNP abbreviations are given in Table I.

Discussion

The question of whether germline coding region mutations of the *BRAF* gene are responsible for predisposition in large multiple-case melanoma families has been investigated by several groups. The answer appears to be that such large impact mutations are not responsible for any significant proportion of familial or sporadic melanoma since no germline exon 15 *BRAF* mutations have been found after screening of 42 familial and two sporadic melanoma cases (Lang *et al*, 2003); 46 familial melanoma cases, 21 multiple melanoma cases, and 106 sporadic melanoma cases (Meyer *et al*, 2003a); 35 familial melanoma cases, 16 multiple melanoma cases, 18 uveal melanoma cases, and 11 probands from familes with melanoma nervous system tumors (Laud *et al*, 2003), respectively. Most recently, however, a germline variant, Q608H, in exon 15 has been found (Casula *et al*, 2004; Casula *et al*, 2005) though its functional significance is unknown. Nevertheless, as 569 CMM cases were screened, the low incidence (0.18%) of these mutations cannot play a major role in melanoma susceptibility.

In a similar vein, a recent study by Laud *et al* (2003) suggested that *BRAF* is also not a low-risk susceptibility gene for melanoma. This group screened the entire coding region of *BRAF* for germline mutations in melanoma-prone families and sporadic cases (n = 80). They found 13 variants (four silent exonic and nine intronic), but none segregated in melanoma-prone families, and there were no significant differences in heterozygote frequencies between cases and controls for any of the 13 SNP. In stark contrast, the results of our study and that of Meyer *et al* (2003b) suggest that *BRAF* is indeed a low-risk susceptibility gene for melanoma. There are several plausible reasons for the discrepant results found between these studies. Laud *et al* analyzed a much smaller number of samples (n = 80) compared to our study, in which 753 cases were utilized. The larger data set provides more power to detect SNP frequency differences, particularly if allele frequency differences are small

Table III. Frequency of *BRAF* haplotypes and association with CMM risk, adjusted for mole count, hair, and skin color

	Haplotype[a]																Frequency	Odds ratio (95% CI)	p-value
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16			
A	C	A	G	T	A	G	C	T	A	C	A	C	C	T	A	C	0.854	1.00	
B	T	G	A	T	A	G	C	T	A	T	A	T	C	T	G	G	0.072	1.07 (0.78–1.46)	0.68
C	T	A	A	G	G	A	G	C	G	T	C	C	T	C	G	G	0.059	1.24 (0.89–1.73)	0.21
Other	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.016	1.13 (0.461–2.75)	0.79

[a]SNP numbering according to Table I (1 = B1, etc.).
CMM, cutaneous malignant melanoma; SNP, single nucleotide polymorphism.

Table IV. Association of *BRAF* haplotypes with melanoma, and with tumor thickness (mm) among cases

BRAF genotype[a]	Odds ratio (95% CI)	Breslow thickness (95% CI)
A/A	1.00 (reference)	0.61 (0.54–0.69)
A/B	1.07 (0.80–1.42)	0.69 (0.52–0.86)
A/C	0.94 (0.69–1.29)	0.61 (0.43–0.78)
B/B	0.71 (0.15–2.57)	0.74 (0.00–1.85)
B/C	1.24 (0.41–3.60)	0.24 (0.00–0.94)
C/C	5.80 (1.40–39.07)	0.62 (0.03–1.21)

[a]Based on haplotypes given in Table III.

yet significant. In addition, for each SNP, Laud *et al* calculated heterozygote frequencies and determined that there were no significant differences in frequencies between cases and controls for any SNP analyzed. In our study, in addition to genotype frequencies, allele frequencies were determined for cases and controls, and for three SNP located in *BRAF*, significant differences in frequencies between cases and controls were found, suggesting *BRAF* is a melanoma predisposition gene. A similar conclusion was reached by Meyer *et al* (2003b), who found six SNP in *BRAF* were associated with melanoma risk in male cases ($n = 470$), although not female cases ($n = 530$), or males and females combined. The SNP B11 (rs1639679) and B5 (rs1267601) were associated with melanoma susceptibility in both the Australian and German case–control populations (see Table I).

The number of haplotypes spanning the 175 kb BRAF locus is surprisingly limited. In passing, the observed haplotypes exhibited what has been described as a "yin–yang" phenomenon (Zhang *et al*, 2003), in that the "C" haplotype differs at every site from the most common "A" haplotype. The causal variant(s) associated with melanoma risk is as yet unknown. As noted earlier, given the substantial sequencing of all exons and exon–intron junctions (Laud *et al*, 2003; Casula *et al*, 2004) it seems unlikely that there are any undiscovered coding changes common enough to account for our results. Newly described SNP in the promoter region are also unlikely to be relevant (Jackson *et al*, 2005).

The hitherto best known melanoma genetic risk factor, *CDKN2A*, accounts for about 25% of familial melanoma cases (Pollock and Trent, 2000), but less than 0.2% of the total melanoma burden (Aitken *et al*, 1999; Tsao *et al*, 2000). Based on the observed genotype frequencies, we estimate that *BRAF* could account for a proportion attributable risk to develop melanoma of 1.6% in the Australian population. Our results suggest that, in addition to the high somatic mutation rate of *BRAF* in melanomas and nevi, germline polymorphisms in this gene also predispose to melanoma, although not to the development of nevi or freckles.

One would expect that if germline *BRAF* mutations are associated with increased risk of melanoma, then a similar strength of relationship with cutaneous nevus count would also be observed. We did not observe such a relationship with heterozygotes carrying the "C" haplotype, and there were only eight C/C homozygotes in the entire dataset, yielding little power, suggesting that there may be multiple independent pathways to the different phenotypes. We were not surprised by the lack of association between *BRAF* genotype and freckling, since an overlap in the mechanisms giving rise to freckling and to melanoma is less likely.

Materials and Methods

Participants We studied an Australian case-control sample made up of 755 melanoma cases from 740 families participating in the QFMP (Aitken *et al*, 1994; Aitken *et al*, 1996; Palmer *et al*, 2000), and genotyped controls were 2239 individuals without melanoma from 635 twin families (476 DZ, 159 MZ) enrolled in the BTNS (Zhu *et al*, 1999, 2004). For purposes of a case–control analysis of melanoma, we used a subsample comprising 720 genotyped melanoma cases (one per family) and 1170 unrelated genotyped controls (parents of the twins individuals) used in calculations as indicated below. Ancestry of the cases and controls was recorded (grandparental country of birth and ethnicity), along with phenotypic risk factors such as hair and eye color, and tanning type.

Tumor thickness and level were recorded for the cases that included both *in situ* and invasive melanomas. A familial risk index was generated for the cases using a permutation-based procedure as described elsewhere (Aitken *et al*, 1994). From this, three familial risk levels ("low" (or "sporadic"), "intermediate," and "high") were classified that correspond approximately to having zero, one, or two or more first degree relatives affected with melanoma. These were used to further stratify the cases in terms of genetic risk. Only clinically verified cases among relatives were included in the analysis.

Table V. Association of *BRAF* haplotypes with nevus counts and freckling scores in adolescent twin controls[a]

BRAF genotype[b]	Number genotyped	Median total nevus count (95% CI)	Median papular nevus count (95% CI)	Mean freckling score (95%CI)
A/A	822	106 (101.9–110.1)	17 (15.8–18.1)	2.6 (2.4–2.8)
A/B	144	102 (92.0–111.9)	17 (14.6–19.4)	2.6 (2.1–3.1)
A/C	110	104 (94.0–113.9)	19.5 (16.6–22.4)	2.4 (1.9–3.0)
B/B	76	82 (63.2–100.8)	7 (1.6–12.4)	0.5 (−3.0 to 4.0)
B/C	12	68 (28.1–107.9)	10.5 (5.0–16.0)	4.1 (2.4–5.9)
C/C	1	242	41	2 (−3.0 to 7.0)

[a]No differences between genotypes are significant at the 5% level.
[b]Based on haplotypes given in Table III.

Standard melanoma risk factors, including propensity to burn in the sun, pigmentation (skin color, hair color at 21 y, eye color, total freckling in summer, and density of melanocytic nevi) were obtained by mailed questionnaire with intensive telephone follow-up. Hair color was recorded as either black, dark brown, light brown, fair, or red/auburn and skin color was recorded as fair, medium, or olive/dark. A three-point scale was also used for eye color, including the categories blue/gray, green/hazel, and brown. Total freckling in summer was self-reported as either nil, 1–100, or 101 +, and density of melanocytic nevi was estimated using a scale of nil, few, moderate, or many.

Skin color (on a three-point scale), hair color (on a five-point scale), and eye color (on a three-point scale) is therefore available for most of the cases and controls. Although nevus counts were only carried out in the adolescents, self-assessed nevus number on a four-point scale ("none," "few," "moderate," "many") is available for parents of the adolescents, as well as the melanoma cases.

Additional analyses of mole count has been performed using the BTNS twins and their siblings closest in age who have all undergone total body skin examination at age 12 y by a trained nurse who assessed nevus count, degree of freckling, and pigmentary phenotypes (Zhu *et al*, 1999). Parents of the twins were genotyped, but only self-reported pigmentary characteristics are available, employing identical measures to those used to record these characteristics in the melanoma individuals. A four-point scale, however, was used to record freckle density, including no freckling, a few, some, and many freckles. No members of the BTNS had been diagnosed with melanoma at the time of this study. Approval to undertake this study was granted by the Human Research Ethics Committee of the Queensland Institute of Medical Research. All participants gave their signed informed consent. Australian National Health and Medical Research Council encompassing the Declaration of Helsinki Guidelines for human research were adhered to.

Genotyping Fifteen intronic/promoter and 1 exonic *BRAF* SNP were typed chosen on the basis of polymorphism in our collection and distribution across the gene (Table I, Fig 1). In order to define flanking areas where linkage disequilibrium decayed around the 175 kb BRAF gene region we also typed five SNP in three flanking genes (*MRPS33*, *ADCK2*, and *Q9ULE3*), which extended an additional 232 and 77 kb on either side and spanning 484 kb in total. SNP identity and type is given in Table I; full sequence and other linked information may be found in the public databases by using the unique "rs" accession number (NCBI, 2003). Genotyping was performed via a primer extension reaction and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MassARRAY, Sequenom, San Diego, California) as previously described (Bansal *et al*, 2002; James *et al*, 2004). All SNP had dropout rates of $<0.5\%$. Data cleaning involved examining Mendelian inconsistencies, departures from Hardy–Weinberg Equilibrium, and discordances between MZ pairs. After examination, where the Mendelian errors were encountered the entire family was dropped from analysis. Error rates due to genotyping technical causes, estimated by replicate typing and use of 159 MZ twin pairs, were found to be 0.11%–0.2%, respectively.

Statistical analysis The use of the BTNS families as controls greatly increases our ability to detect genotyping problems, but makes the analysis more complex. We have extracted unrelated cases and controls, and performed multiple logistic regression analysis *versus* individual SNP, and Fisher–Irwin Exact tests comparing genotype counts in cases and controls. Correction for multiple testing *versus* individual SNP was done by repeatedly permuting cases and controls, retaining the best text statistic out of 21 tests from each of 100,000 replicates. Given that this is a replication of a previously reported association it is not necessary to apply a genome-wide level correction. Haplotypic association analysis was performed using the *haplo.stat* package (Lake *et al*, 2002) running in R (RDCG, 2004), cross-checking with the COCAPHASE program (Lang *et al*, 2003). These programs use an EM algorithm to enumerate all legal haplotypes that could give rise to an observed genotype and estimate the posterior probabilities for these haplotypes for each individual. Association is then assessed via binomial (or ordinal logistic or linear) regression using the haplotype probabilities as weights. This approach allows covariates to be easily included. Proportion attributable risk was calculated using the control genotype frequencies and the estimated genotypic odds ratios ($PAR = 1-1/(p^2g_2 + 2p(1-p)g_1 + (1-p)^2)$, where p is the risk allele frequency, and g_2 and g_1 the genotypic relative risks for risk allele homozygotes and heterozygotes).

Note added in proof

NCBI-Genbank has renumbered Braf codons such that the previous 599 is now 600.

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