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Polymorphisms of the *BRAF* gene predispose males to malignant melanoma

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Abstract

The incidence of malignant melanoma has rapidly increased in recent years. Evidence points to the role of inheritance in melanoma development, but specific genetic risk factors are not well understood. Recent reports indicate a high prevalence of somatic mutations of the *BRAF* gene in melanomas and melanocytic nevi. Here we report that germ-line single nucleotide polymorphisms (SNPs) in *BRAF* are significantly associated with melanoma in German males, but not females. At-risk haplotypes of *BRAF* are shown. Based upon their frequencies, we estimate that *BRAF* could account for a proportion attributable risk of developing melanoma of 4% in the German population. 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 less than 1%.

Introduction

Melanoma is an aggressive skin cancer which, once metastasized, is resistant to most current treatments. Malignant melanoma is the leading cause of death from skin diseases, and its mortality rate is increasing faster than for any other malignant disease except lung cancer [1]. In 2002, 53,600 new cases of melanoma were projected for the U.S.: 30,100 in males and 23,500 in females [2]. The lifetime probability of developing melanoma is 1.72% for males and 1.22% for females, and older males have double the prevalence compared with older females [2,3].

Familial melanoma represents 8–12% of all melanoma cases. Germ-line mutations in *CDKN2A* also called *p16*-gene [4,5] and *CDK4* [6] have been identified in different proportions ranging from 5% to approximately 90% in monogenic melanoma patients [7]. **Sporadic** malignant melanoma accounts for the vast majority, i.e. about 90% of cases, and genetic factors that mediate susceptibility to this form of melanoma are not well understood. Several candidate genes have been reported to predispose to sporadic melanoma, including vitamin D receptor (*VDR*; MIM# 601769) [8], the melanocyte-stimulating hormone receptor (*MC1R*; MIM# 155555) [9,10], glutathione-S-transferase M1 (*GSTM1*; MIM# 138350) [11], a gene of the cytochrome P450 family (*CYP2D6*; MIM# 124030) [12], as well as epidermal growth factor (*EGF*; MIM# 131530) [13]. The hitherto best known melanoma risk factor *CDKN2A* accounts for about 25% of familial melanoma cases [14], but less than 1% of all melanoma

Table 1: Allele frequencies of SNPs in the *BRAF* gene in the German cohort. Location of SNPs within *BRAF* and refSNP Ids are listed.

SNP Id.	dbSNP rs#	Exon/Intron*	M case	M control	p-value	F case	F control	p-value
BRAF-1	rs1639679	Intron 11	**C** = 0.888 A = 0.112	**C** = 0.927 A = 0.073	**0.045**	**C** = 0.943 A = 0.057	**C** = 0.934 A = 0.066	0.585
BRAF-2	rs1267646	Intron 9	**C** = 0.839 T = 0.161	**C** = 0.870 T = 0.130	0.240	**C** = 0.870 T = 0.130	**C** = 0.867 T = 0.133	0.914
BRAF-3	rs1267606	Intron 2	**T** = 0.890 G = 0.110	**T** = 0.925 G = 0.075	0.078	**T** = 0.942 G = 0.057	**T** = 0.930 G = 0.070	0.477
BRAF-4	rs1267621	Intron 1	**G** = 0.839 A = 0.171	**G** = 0.873 A = 0.127	0.147	**G** = 0.868 A = 0.132	**G** = 0.880 A = 0.120	0.584
BRAF-5	rs1267601	Intron 2	**A** = 0.894 G = 0.106	**A** = 0.932 G = 0.068	**0.041**	**A** = 0.944 G = 0.056	**A** = 0.935 G = 0.065	0.526
BRAF-6	rs1267609	Intron 3	**C** = 0.882 T = 0.118	**C** = 0.907 T = 0.093	**0.008**	**C** = 0.945 T = 0.055	**C** = 0.935 T = 0.065	0.539
BRAF-7	rs1267636	Intron 8	**A** = 0.891 G = 0.109	**A** = 0.939 G = 0.061	**0.010**	**A** = 0.945 G = 0.055	**A** = 0.935 G = 0.065	0.517
BRAF-8	rs1267649	Intron 5	**G** = 0.896 C = 0.104	**G** = 0.934 C = 0.066	**0.038**	**G** = 0.945 C = 0.055	**G** = 0.934 C = 0.066	0.474
BRAF-9	rs1639675	Intron 7	**A** = 0.891 G = 0.109	**A** = 0.930 G = 0.070	**0.041**	**A** = 0.945 G = 0.055	**A** = 0.939 G = 0.061	0.719
BRAF-10	rs1267610	Intron 3	**G** = 0.732 A = 0.268	**G** = 0.770 A = 0.230	0.187	**G** = 0.766 A = 0.234	**G** = 0.784 A = 0.216	0.507
BRAF-11	rs1267625	Intron 2	**T** = 0.833 G = 0.167	**T** = 0.870 G = 0.130	0.135	**T** = 0.872 G = 0.128	**T** = 0.864 G = 0.136	0.716
BRAF-12	rs1267618	Intron 1	**G** = 0.837 A = 0.163	**G** = 0.871 A = 0.129	0.146	**G** = 0.868 A = 0.132	**G** = 0.867 A = 0.133	0.961

* within the 18 exon transcript ENST00000288602

cases [15,16]. The etiology of sporadic melanoma is complex and likely involves interactions of multiple low-penetrance susceptibility genes, the influences of environmental exposures such as ultraviolet (UV) light, and the interaction of genotype and environment. Recently, single nucleotide polymorphisms (SNPs) have emerged as markers of choice for complex disease gene mapping because of their high density and even distribution across the human genome [17]. Feasibility studies to establish the strategies and methodologies of SNP use now allow direct comparison of allele frequencies in case-control populations using DNA pools [18,19].

Analysis and Results

Genome-Wide Association Study

To screen for major genetic factors contributing to the development of melanoma, we conducted a genome-wide SNP association study to systematically compare allele frequencies of approximately 25,133 SNPs in a melanoma case-control cohort. The SNP set covers 15,275 human genes that account for approximately 46% of the entire human genome. Frequencies of the minor allele of the SNPs analysed were at least 10%, with a median spacing of 38 kb and a mean spacing of 120 kb [20]. Initial results of this genome scan indicated a significant association of a SNP located in the BRAF gene with the disease in male melanoma patients. To further evaluate this, we proceeded with direct analysis of the BRAF gene.

BRAF Gene Analysis

Four non-coding SNPs (BRAF-1, BRAF-2, BRAF-3, BRAF-4) in the BRAF gene region were tested (Table 1). The allele frequency of BRAF-1, a SNP located in intron 11 of BRAF, was significantly different between male cases and control groups (p = 0.045). This corresponded to a male genotypic relative risk of 1.67 (multiplicative penetrance model; 95% confidence interval, 1.03–2.78). The frequency of BRAF-3, a further non-coding SNP, showed borderline significance (p = 0.078) in the male study population (Table 1), while BRAF-2 and BRAF-4 frequencies were not associated with melanoma risk.

Haplotype analysis of these four SNPs by the D' and r^2 tests indicated that they were in strong linkage disequilibrium (LD). Eight haplotypes were identified for these four SNPs, three of which accounted for approximately 98% of all haplotype combinations (Table 2). Comparison of haplotype frequencies in affected and control groups showed significant differences between male case and control groups, as well as between the combined case and control groups (Table 3). Haplotype-based genotype data (Table 3) were analysed using a standard chi-square test of

Table 2: Haplotype frequencies of *BRAF* in our German cohort.

Haplotype	SNP				Frequency
	BRAF-1	BRAF-2	BRAF-3	BRAF-4	
H1	C	C	T	G	0.843
H2	A	T	G	A	0.074
H3	C	T	T	A	0.064
H4	C	T	T	G	0.012
H5	C	C	G	G	0.003
H6	A	C	T	G	0.002
H7	C	C	T	A	0.002
H8	A	C	G	A	0.001

Table 3: Haplotype analysis of the *BRAF* gene in our German case-control cohort.

	Total			Male			Female		
Haplotype	**Case (N = 1000)**	**Control (N = 898)**		**Case (N = 470)**	**Control (N = 434)**		**Case (N = 530)**	**Control (N = 464)**	
CCTG (H1)	84%(829)	86%(771)		81%(380)	86%(374)		85%(449)	86%(397)	
ATGA (H2)	8%(82)	7%(59)	p = 0.206	11%(52)	7%(30)	**p = 0.040**	6%(30)	6%(29)	p = 0.796
CTTA (H3)	6%(64)	6%(58)		5%(24)	6%(26)		8%(40)	7%(32)	
CTTG (H4)	2%(20)	0%(3)	χ^2 = 16.75 **p = 0.019**	2%(11)	0%(2)	χ^2 = 15.85 **p = 0.015**	2%(9)	0%(1)	χ^2 = 7.82 p = 0.25
CCGG (H5)	0%(1)	0%(4)		0%(0)	0%(2)		0%(1)	0%(2)	
ACTG (H6)	0%(1)	0%(2)		0%(0)	0%(0)		0%(1)	0%(2)	
CCTA (H7)	0%(2)	0%(1)		0%(2)	0%(0)		0%(0)	0%(1)	
ACGA (H8)	0%(1)	0%(0)		0%(1)	0%(0)		0%(0)	0%(0)	

independence. This analysis demonstrated that the haplotype CTTG (H4) was significantly associated with melonoma in both males and the total melanoma case cohort, but not for female case patients when analysed separately. ATGA (H2) was significantly associated with melanoma in males only. The frequency of the most common haplotype, CCTG (H1), did not differ significantly between cases and controls in any group.

We subsequently genotyped eight additional non-coding SNPs (BRAF-5 to BRAF-12) in this region. Five additional SNPs (BRAF-5 to BRAF-9) showed significant frequency differences between cases and controls in the male study populations. None of the 12 SNPs genotyped showed any significant association in female melanoma patients (Table 1).

Discussion

BRAF (MIM# 164757) encodes a serine/threonine kinase participating in the Ras/Raf/MAPK (mitogen-activated protein kinase) signal transduction pathway [21]. The *BRAF* gene is located on chromosome 7q34, and covers approximately 190 kb. It contains at least 19 exons and encodes a full-length transcript of 2,510 bp (NM_00433). At least seven variant transcripts have been identified, which are products of alternative splicing. From these various transcripts, several proteins are translated, including the full-length, 94–95 kD, 783 amino acid product [22].

Recently, *BRAF* was found to be mutated in six of nine (66%) primary malignant melanomas, with lower frequencies of mutation ranging from 0.5% to 14% in other primary tumours. Furthermore, 12 of 15 (80%) melanoma short-term cultures and 20 of 34 (59%) melanoma cell lines showed *BRAF* mutations. A single-base substitution, which alters codon 599 (V599E) in exon 15 of this gene, accounted for 35 (92%) of these 38 mutations observed. BRAF proteins with the V599E substitution showed elevated kinase and transforming activities compared to wildtype BRAF proteins [23]. Germline mutations of *BRAF* have not yet been detected in familial or sporadic melanoma patients [24-26]. Analysis of 13 non-coding or silent *BRAF* polymorphisms did not reveal significant frequency differences in 80 familial or multiple melanoma cases compared to 91 cancer-free controls [25].

Here, a genome-wide association study independently pointed to *BRAF* as a gene associated with melanomas, reinforcing the associations found in the previous studies.

On further evaluation, six non-coding SNPs and two combined haplotypes were shown to confer a significantly increased risk for developing melanoma in male patients of German origin. Thus, aberrations of *BRAF* would seem to cause disease predominantly in carrier males, which correlates with the overall increased incidence of melanoma in males as compared to females. A mechanism to explain a possible sex-linked effect of *BRAF* is not yet clear, and might be related to either genetic or environmental factors.

Summary and Conclusion

In this report, we describe six SNPs and two haplotypes of *BRAF* significantly associated with melanoma in male patients. One haplotype (H4) was also associated with an increased melanoma risk for combined female and male populations. Based on the observed genotype frequencies, we estimate that *BRAF* could account for an attributable risk of developing melanoma of approximately 4% in the German population. This risk estimate is much higher than that attributed to *CDKN2A*, whose contribution to the population burden of melanoma is less than 1%. Our results suggest that, in addition to the high somatic mutation rate of *BRAF* in melanomas documented in previous studies, germline polymorphisms in this gene predispose males to melanoma. Thus, *BRAF* may be one explanation of why males have an increased life-time incidence of melanoma compared to females. Further studies, both in German and other ethnic patient populations, will be necessary to confirm a correlation between *BRAF* polymorphisms or haplotypes and disease. Additional investigation is also needed to search for causal genetic *BRAF* variants, possibly in 5' or 3' regions of this gene. Furthermore, somatic mutations of *BRAF* have been observed at high frequency (39%–69%) in papillary thyroid cancers [27-31], and to a lesser extent in lung [23,32,33] and colorectal cancers [23,34-36]. It remains to be investigated whether germline polymorphisms of *BRAF* also contribute to the risk of these common malignancies.

Methods

Clinical sample and phenotype collection

Blood samples were collected from caucasian Germans at the Dermatology Department of the University Hospital in Tuebingen, Germany. The study protocol was approved by the local ethics committee and informed consent was obtained from all individuals recruited. Case patients were those diagnosed histologically with melanoma, while controls were unrelated age-matched individuals free of any cancer at the time of enrollment. A total of 236 male and 266 female patients diagnosed with cutaneous malignant melanomas were enrolled with mean ages of 51 and 49, respectively. The control population consisted of 217 males and 233 females with mean ages of 48 and 47, respectively.

DNA extraction and genotyping

DNA from 6–9 ml blood was extracted using a desalting method (Gentra Systems, Minneapolis, MN, USA), and quantitated using Pico green reagents and a Fluorometer (Fluoroskan Ascent CF, Labsystems, Franklin, MA, USA). All PCR and MassEXTEND reactions were conducted using standard conditions as described previously [37]. Each reaction product was dispensed onto four silicon chips and analyzed on a SEQUENOM-Bruker mass spectrometer (Sequenom Inc., San Diego, CA, USA). Spectra were then analysed using SpectroTYPER™ software that includes quantitative peak analysis functions for peak area calculation and baseline correction. The corrected peak areas for both alleles were used to calculate relative allele frequencies.

Statistical Analysis

Haplotypes were reconstructed from SNP genotypes using the statistical method developed by Stephens *et al*. [38], and implemented in the PHASE computer program (version 1.0). This method reconstructed a haplotype for each genotyped individual. The extent of linkage disequilibrium (LD) between each pair of SNPs was estimated as the difference between the observed two-locus haplotype frequency using the major alleles at each SNP and the product of the observed major allele frequencies. The LD between SNPs was also expressed by two other common standardized metrics, D' ($D/\min(p_1q_2, p_2q_1)$) and r^2 ($D^2/p_1p_2q_1q_2$), where p_1 and q_1 were the minor allele frequencies at two SNPs, and p_2 and q_2 were the corresponding major allele frequencies. Significant deviation of this disequilibrium from zero was tested by the use of a chi-square goodness-of-fit test.

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