ONLINE MUTATION REPORT

Search for genetic variants associated with cutaneous malignant melanoma in the Ashkenazi Jewish population

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Cutaneous malignant melanoma (CMM; MIM #155601) comprises about 1% of all cancer cases in European populations, but its mortality is over 2% because of the propensity of this tumour to metastasise. Surveillance of CMM-prone individuals and populations should decrease the morbidity and mortality associated with this disease.

In approximately 5–12% of all melanoma cases, there is a family history of the disease, defined as having one or more affected first degree relatives.¹ Germline mutations of *CDKN2A*, located on chromosome 9p21, cosegregate with affected cases in an estimated 20% of melanoma families worldwide (reviewed by Goldstein²). *CDKN2A* encodes two different tumour suppressor proteins: the CDK4/6 cell cycle inhibitor p16^{INK4A} from exons 1α, 2, and 3; and the MDM2 inhibitor p14^{ARF} from exons 1β, 2, and 3. The frequency of discovering a *CDKN2A* mutation within a family increases with the number of affected relatives, ranging from ~15% in families with two affected members, to 20–40% in those with three, and 70% in those with four or more (unpublished data from Hogg's laboratory; Kefford and Mann¹). In addition, mutations in the *CDK4* gene^{3,4} and polymorphisms in the *MC1R* gene⁵ have been implicated rarely in melanoma predisposition.

To date, mutations in the two major predisposition genes, *CDKN2A* and *CDK4*, account for melanoma susceptibility in less than half of all melanoma families,⁶ and the genetic bases of the disease in the remaining families have not been found. Gillanders *et al* recently provided strong evidence for another melanoma predisposition locus at chromosome 1p22.⁷ Current research in melanoma genetics is hindered by the allelic and locus heterogeneities, which render whole genome linkage analysis difficult. In addition, the sample sizes required to study the effects of weak or modifier alleles in a genetically heterogeneous population are large.⁸

To address the problem of both allelic and locus heterogeneities, we have focused on populations that have undergone genetic bottlenecks and are genetically isolated. We hypothesise that familial melanoma patients within such populations arose from a limited number of founders and thus display a restricted number of melanoma predisposition alleles. These alleles are in linkage disequilibrium with adjacent markers. By searching for melanoma associated alleles and genotypes, we can isolate the predisposition loci and subsequently determine the variant or variants responsible.

Ashkenazi Jews originated in the Near East, and subsequently migrated to eastern and central Europe. Throughout history, the Ashkenazi Jewish population has experienced numerous bottlenecks⁹ which led to highly altered allele frequencies relative to the diverse ancestral population. This genetic drift is evident from the high incidences of multiple genetic disorders, attributable to founder mutations,^{10,11} and the population bottlenecks and the practice of endogamy have also decreased genetic variability.^{9,12}

Key points

- Mutations in *CDKN2A* and to a lesser extent, *CDK4*, account for the genetic susceptibility to cutaneous malignant melanoma in only a subset of familial cases. The remaining families may have melanoma associated variants in the non-coding regions of *CDKN2A* (on chromosome 9p21), or in other regions of the genome, possibly in the newly suggested melanoma susceptibility locus on chromosome 1p22.
- The Ashkenazi Jewish population is genetically more isolated and less variable than the general population, so it was hypothesised that it would have a limited number of melanoma associated variants, and that they would exist in linkage disequilibrium with adjacent markers.
- The four exons of *CDKN2A* from the genomic DNA of 58 Ashkenazi Jewish melanoma patients were examined, but no mutations were uncovered. In addition, none of these patients bore the Arg24Cys and Arg24His mutations in *CDK4* that are associated with melanoma.
- Analysis of the frequencies of two *CDKN2A* polymorphisms that were previously shown to be different between patients and controls was also done, but no significant differences were found between Ashkenazi Jewish cases and ethnically matched controls. Genotype analyses using microsatellite markers at 9p21 and 1p22 did not reveal any allelic or genotypic associations with melanoma between these loci.
- Further investigation is required to determine the locus responsible for melanoma susceptibility in the Ashkenazi Jewish population.

The incidence of melanoma in Ashkenazi Jews is relatively high (table 1), but no *CDKN2A* mutations have been found in any Ashkenazi Jewish families.¹³ This population is therefore considered to be a promising setting for the identification of novel melanoma susceptibility genes.⁶ In this report we present the results of our search for *CDKN2A* mutations and polymorphisms, as well as 9p21 and 1p22 alleles and genotypes that are associated with melanoma, in our collection of Ashkenazi Jewish melanoma patients.

METHODS

Recruitment of patients and controls

Fifty eight Ashkenazi Jewish patients with malignant melanoma (table 2; fig 1) were recruited to this study as

Abbreviations: CMM, cutaneous malignant melanoma

Table 1 Age standardised rates of melanoma incidence in the year 2000

Population/region	Male	Female
Israel: Jewish*	16.87	12.38
Israel: non-Jewish*	1.39	2.63
Israel	9.44	9.81
Western Asia	0.60	1.21
Eastern Europe	5.22	5.01
Western Europe	7.01	8.13
Australia/New Zealand	39.88	32.32
North America	12.74	9.26

Sources: Globocan 2000: <http://www-dep.iarc.fr/globocan/globocan.html> and *personal communication: M Barchana, Head, Israel National Cancer Registry.

described by Yakobson *et al.*¹³ Twenty of these reported a family history of the disease, defined as having at least one first degree relative affected by melanoma. All tumours were confirmed to be malignant melanoma by hospital pathologists. Genomic DNA was extracted from peripheral blood of all 58 patients.

The 86 control DNA samples were a kind gift from Dr Steve Narod (Toronto). They were obtained from individuals of Ashkenazi Jewish background who had no personal history of cancer, but may have had a family history of breast or ovarian cancer.

Germline *CDKN2A* and *CDK4* sequencing

For all samples, we sequenced exons 1α and 2 of *CDKN2A* as described by Liu *et al.*,¹⁴ and exon 3 as described in Loo *et al.*¹⁵ We also amplified exon 1β using primers p14E1b-f and p14E1b-r (table 3), and sequenced using the same primers. We amplified exon 3 of *CDKN2A* using primers p16E3TMF and p16E3TMR (table 3) and the following amplification profile: 95°C for three minutes; 38 cycles of 95°C for 21 seconds, 66°C for 16 seconds, 72°C for 51 seconds; and 72°C for 22 minutes.

To screen for the two known melanoma associated mutations in *CDK4*³⁻⁴ in patient samples, we amplified exon 2 of the gene using primers CDK4E2f and CDK4E2r (table 3), and sequenced the polymerase chain reaction (PCR) products using the same primers.

Genotype analysis at the 9p21 and the 1p22 chromosomal regions

To determine whether the chromosomal locus 9p21 and 1p22 are associated with melanoma, we carried out genotype analysis to search for alleles shared as a result of founder effect. The microsatellite markers used were *D9S1749*, 974, 942, 1748, 1604, and 171 for 9p21; and *D1S435*, 2779, 236, 2664, and 2726 for 1p22 (fig 2). We obtained primer sequences for all markers from the Genome Database (<http://www.gdb.org/>). We added the sequence GTTCT to the 5' of the forward primer of *D9S1749* according to the principles of the "PIGtailing" technique.¹⁶ This increased the clarity of the genotypes by ensuring that non-templated adenylation by Taq polymerase occurs to 100% of labelled PCR products. We carried out PCR (annealing at 58°C) and genotyping as described by Loo *et al.*¹⁵

Statistical analyses

For each marker, alleles were analysed if their frequencies were more than 10% in either patients or controls, and all other alleles were grouped together. We then compared the allele frequencies in the patients *v* the controls for each marker. In examinations of genotypic associations, genotypes made up of alleles with frequencies of more than 10% in either patients or controls were analysed, and all other

Table 2 Descriptive statistics for patients and controls in the present study

	Patients (n = 58)	Controls (n = 86)
Recruitment	Dr E Yakobson, Israel	Dr S Narod, Toronto
Sex*	26 male, 32 female	11 male, 75 female
Age (years) (mean (SD))	Diagnosis: 43.2 (16.2)	Recruitment: 54.4 (8.1)
Ethnic background	Ashkenazi Jewish	Ashkenazi Jewish
Personal history of melanoma	58 patients†(100%)	None
Multiple primary melanomas	4 patients (7%)	None
Family history of melanoma	20 patients‡ (34%)	Unknown

*Sex distribution significantly different: $\chi^2 = 16.98$, $p = 0.0000378$ (df = 1).

†Including 10 patients whose samples have been analysed for *CDKN2A* mutations using single strand conformation polymorphism (SSCP).¹³

‡Including four individuals from two families.

genotypes were grouped together. We then compared the genotype frequencies in the patients *v* the controls for each marker.

Genotype data were assessed for deviations from the Hardy–Weinberg Equilibrium separately for patient and control groups using the HWE program from the URL: <http://linkage.rockefeller.edu/ott/linkutil.htm#HWE>. To test whether specific alleles or genotypes were associated with the disease, the genotype data were analysed using the χ^2 test (EpiInfo6 from <http://www.cdc.gov>).

RESULTS

Table 2 presents the descriptive statistics for the patient and control groups in the present study. The statistically significant difference seen between the sex distributions in the patient and control groups is probably because the control group consisted primarily of individuals seeking genetic

Figure 1 Examples of Ashkenazi Jewish melanoma pedigrees. Black symbols represent melanoma patients, and grey symbols represent individuals who suffer from cancers but not melanoma. Ages at diagnosis of the cancers are given under the symbols. Individuals in this study are designated by M numbers. Additional information is available in table 2.

Table 3 New primers used in this study

Primer	Sequence (5' to 3')	Location of 5' end of primer	Direction	Size of PCR product (bp)
p14E1b-f	CGTGTCAAGTGACGGATGTAGCTA	c.-359	Forward	848
p14E1b-r	GGTACTGCGAGAACCACATGTCTAAG	IVS1+173	Reverse	
p16E3TMF	GATGCGGGAAGGGAAGGCCACATC	IVS2-380	Forward	581
p16E3TMR	GACATTACGGTAGTGGGGGAAGGC	c.658	Reverse	
cdk4E2f	GGAGTCCCTTTGTGCTGC	IVS1-78	Forward	287
cdk4E2r	CCAGTCGCCTCAGTAAAGC	c.190	Reverse	

bp, base pair; PCR, polymerase chain reaction.

Figure 2 Schematic representations of the genetic markers used in the present study. Upper panel: microsatellite markers on chromosome 9p21 and the four exons of the *CDKN2A* gene. Lower panel: microsatellite markers on chromosome 1p22. Measurements are not to scale. Source: UCSC Genome Browser (May 2004): <http://genome.cse.ucsc.edu/index.html>

testing for breast cancer susceptibility, a disease that predominantly affects women. As melanoma predisposition is not inherited in a sex restricted manner, the difference in sex distribution is unlikely to affect subsequent analyses. Twenty of the 58 patients (34%, including four patients from two families) reported a family history, with clustering patterns consistent with an autosomal dominant mode of inheritance (fig 1). The remaining 66% of the patients, though reported as sporadic cases, may still possess a genetic predisposition. Their “family history” of the disease may be unknown owing to dispersion of relatives, or is simply not observed if the penetrance is low and the family size is small.

We searched for melanoma associated mutations in the *CDKN2A* and the *CDK4* genes, but did not find any such

variants in our patient population. We did not find a statistically significant difference (data not shown) between the frequencies of the C500G (*rs11515*) and C540T (*rs3088440*) polymorphisms, previously shown to be associated with melanoma.^{17 18}

As the frequency of *CDKN2A* coding mutations does not account for all 9p21 linked families in other populations studied, we sought for a difference in allele and genotype frequencies between patients and controls, which may be indicative of candidate non-coding variants present in linkage disequilibrium with genetic markers in 9p21. The allele and genotype distributions for all six markers (fig 2) did not deviate from the HWE in either the patient or the control groups. Table 4 shows 9p21 allelic and genotypic frequency comparisons between patient and control groups, along with the calculated heterozygosities. Of the markers where the analyses were available, none showed allelic or genotypic associations with melanoma.

Allelic and genotypic associations at the 1p22 locus
Gillanders *et al*⁷ provided strong evidence that a novel melanoma susceptibility locus is present at 1p22. We therefore searched for associations between 1p22 and melanoma cases in the Ashkenazi Jewish population. We undertook the same types of analyses for 1p22 as we did for 9p21, but found no significant allelic or genotypic associations with melanoma (table 5).

DISCUSSION
Multiple lines of evidence support a genetic basis for melanoma in Ashkenazi Jews. First, the incidence of cutaneous malignant melanoma in the Jewish population differs from that found in non-Jewish populations (table 1). Although differences in incidence rates among ethnic groups may reflect different environments and habits, they can also suggest that genetic backgrounds play a significant role in melanoma predisposition. Second, familial clustering suggests the segregation of mutant predisposition alleles. In our patient group, 20 of 58 patients (34%; including four patients

Table 4 Association analyses for six microsatellite markers in 9p21

Marker	Heterozygosities		Allelic association			Genotypic association		
	Patients	Controls	χ^2	df	p value	χ^2	Df	p Value
D9S1749	0.95	0.99	0.07	1	0.78	Not analysed*		
D9S974	0.95	0.74	0.51	3	0.51	14.9	6	0.021†
D9S942	0.93	0.93	0.27	3	0.26	1.41	4	0.84
D9S1748	0.90	0.87	0.92	6	0.92	Not analysed‡		
D9S1604	0.55	0.36	2.67	1	0.10	4.94	2	0.084
D9S171	0.75	0.75	3.26	3	0.35	7.10	5	0.21

*The analysis for D9S1749 could not be carried out because there were no genotypes that were comprised of alleles that both had frequencies of >10%.
†With the Bonferroni correction, the critical p value becomes 0.05/6 = 0.0083. As 0.021 is >0.0083, the result is not statistically significant.
‡D9S1748 had too many genotypes that were comprised of alleles that both had frequencies of >10%; they therefore could not be analysed using the EpiInfo6 χ^2 function.

Table 5 Association analyses for six microsatellite markers in *1p22*

Marker	Heterozygosities		Allelic association			Genotypic association		
	Patients	Controls	χ^2	df	p value	χ^2	Df	p Value
D1S435	0.75	0.70	5.78	3	0.123	3.60	6	0.730
D1S2779	0.70	0.76	1.65	2	0.437	1.76	3	0.625
D1S236	0.82	0.85	2.38	3	0.497	2.20	6	0.901
D1S2664	0.74	0.70	1.55	3	0.670	4.31	6	0.635
D1S2726	0.68	0.76	4.57	3	0.205	5.42	5	0.366

from two families) reported a family history of the disease, with some having multiple affected relatives (fig 1). Third, some individuals—such as the four (7%) in our series—are afflicted with multiple primary melanomas, or develop melanoma at an early age, observations that are also consistent with an underlying genetic predisposition.¹⁹ The early age of diagnosis of melanoma for some of the patients (for example, two patients diagnosed at age 22 in fig 1) possibly reflects accelerated tumour formation kinetics.

Despite the evidence pointing to the cosegregation of genetic variants with melanoma predisposition in the Ashkenazi Jewish population, no such variants have so far been uncovered. In an earlier study, Yakobson *et al*¹³ found two *CDKN2A* mutations, Gly101Trp and Gly122Val, in two Israeli families, but neither is of Ashkenazi Jewish origin. Later, Yakobson *et al*²⁰ uncovered a Val59Gly mutation in four melanoma pedigrees. The concordance of the microsatellites at this chromosomal region shows that the mutation in these four families is probably derived from one Mediterranean non-Ashkenazi founder. The genetic basis for melanoma susceptibility in the Ashkenazi Jewish population thus remains unknown and is the focus of the present study.

Analyses of mtDNA haplogroups¹⁸ and the non-recombining regions of the Y chromosome¹² have shown that a major genetic drift has occurred in the history of the Ashkenazi Jewish population. Our strategy of taking advantage of the linkage disequilibrium between markers that resulted from recent founder effects should have enabled us to define genetic associations with melanoma at the *9p21* and *1p22* candidate loci. However, we were unable to find any allelic or genotypic associations between either of the two single nucleotide polymorphisms (exon 3 of *CDKN2A*) or the 11 microsatellite markers (*9p21* and *1p22*) and melanoma. There are several possible reasons for our findings; for example, it is possible that even within the Ashkenazi Jewish population, numerous melanoma predisposition variants exist, and our sample size did not allow us to find a definitive association that is statistically significant; furthermore, the melanoma predisposition alleles for melanoma are not located in the *9p21* or *1p22* chromosomal regions for the Ashkenazi population. These two possibilities are not mutually exclusive.

Investigation by Catalin *et al* (Catalin M, personal communication) on 24 Ashkenazi Jewish families with malignant melanoma and neural system tumour syndrome (MMNST) has also found no predisposing mutations in *CDKN2A*. These results, together with ours (82 families in all) show that *CDKN2A* is very unlikely to be the melanoma susceptibility locus for the Ashkenazi Jewish population. Further investigation should uncover a novel locus (or multiple loci) or environmental causes that underlie melanoma susceptibility of Ashkenazi Jews. The knowledge will allow the identification and surveillance of at-risk families, and thus decrease the morbidity and mortality associated with this disease.

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