



Estimating the relative risk of developing melanoma in INK4A carriers

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Estimation of the relative risk of cancer due to rare germline mutations using population-based epidemiological techniques is challenging, since studies with very large numbers of subjects are required. In this pilot study using a novel study design, we evaluated the role of INK4A mutations in melanoma by comparing patients with multiple primary melanomas to those with single primaries. Patients were ascertained from the Surgery and Dermatology Clinics at Memorial Sloan-Kettering Cancer Center and at the Yale University Pigmented Lesion Clinic. Subjects completed a questionnaire covering risk factors for melanoma and were tested for INK4A mutations. Five (8%) of 65 patients with multiple primaries had a mutation, compared with none of 88 patients with single primaries ($P=0.03$). Examination of other factors, such as number of nevi on the arms of the patients, fair skin, hair and eye colour, and other phenotypic characteristics associated with the risk of melanoma demonstrates that these factors

exhibit higher prevalence in the multiple primary cases than in the single primaries. These results provide evidence of the utility of the new study design in evaluating the impact of rare but highly penetrant cancer risk factors. *European Journal of Cancer Prevention* 13:000–000 © 2004 Lippincott Williams & Wilkins.

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Introduction

The inhibitor of cyclin dependent kinase 4, INK4A, was identified as a melanoma susceptibility gene in 1994 (Kamb *et al.*, 1994). With the exception of four families identified so far with germline mutations in cyclin-dependent kinase 4, CDK4 (Zuo *et al.*, 1996; Soufir *et al.*, 1998; Holland *et al.*, 1999), INK4A is the only inherited gene identified to date that is associated with susceptibility to melanoma. INK4A encodes a CDK inhibitor initially identified by its ability to bind CDK4, the kinase responsible for phosphorylation of the retinoblastoma protein pRB (Serrano *et al.*, 1993). INK4A codes for the protein p16, whose function is to induce cell cycle arrest at the G₁ checkpoint and is considered a tumour suppressor gene. Germline mutations have been detected in 20–30% of melanoma-prone families (Holland *et al.*, 1995; Borg *et al.*, 1996; FitzGerald *et al.*, 1996; Harland *et al.*, 1997; MacKie *et al.*, 1998; Soufir *et al.*, 1998).

However, mutations appear to be very rare in the population. In fact, even among patients with melanoma, only 4–12% report a family history of the disease and only a fraction of these familial cases are likely to be linked to INK4A. Thus, evaluation of the relative risk of INK4A mutations in the development of melanoma is challen-

ging. To address this issue we developed a novel study design. We hypothesized (Begg and Berwick, 1997) that second primary melanomas in individuals with a known risk factor will occur at a rate that is predictable based on knowledge of the relative risk. By comparing the prevalence of the risk factor in individuals with second primaries with the prevalence in an incident series of first primary melanomas, we can infer, by induction, the relative risk that could be obtained in a conventional case-control study. This approach is strategically advantageous when the risk factor is rare but the relative risk is high, in which case the patients with second primaries will be 'rich' in adverse genetic mutations.

This concept is borne out in a number of small, published series of individuals with multiple primary melanomas who were genotyped for INK4A mutations, presented in Table 1 (Fitzgerald *et al.*, 1996; Harland *et al.*, 1997; MacKie *et al.*, 1998; Monzon *et al.*, 1998; Burden *et al.*, 1999; Ruiz *et al.*, 1999; Hashemi *et al.*, 2001). These series are obtained from various countries in North America and Europe. The overall mutation frequency reported is 14%. The ascertainment of cases in these series was generally serendipitous, as is indicated in the table. The preponderance of the gene carriers reported a family history

Table 1 Previous studies of INK4A mutations in multiple primary melanomas

Case selection	Author	Country	Mutation/subjects (%)	Proportion of mutations
Population-based	MacKie <i>et al.</i> , 1998	Scotland	2 /17 (12%)	
Population-based	Burden <i>et al.</i> , 1999	Scotland	6 /23 (26%)	14%
Population-based	Hashemi, 2000	Sweden	9 /80 (11%)	
Mixed	Harland <i>et al.</i> , 1997	UK	0 /3	
Mixed	Monzon <i>et al.</i> , 1998	Canada/USA	5 /33	9.6%
	Ruiz <i>et al.</i> , 1999	Spain	0 /9	
Mixed	Auroy <i>et al.</i> , 2001	France	9 /100	
Family history	FitzGerald <i>et al.</i> , 1996	USA	4 /9	
Family history	MacKie <i>et al.</i> , 1998	Scotland	5 /5	64%

of melanoma, suggesting that this may have been an important factor in prompting ascertainment. Interestingly, Burden *et al.* (1999) compared their series of multiple primaries with a matched series of single primary cases, as we have proposed.

In the following study we report a preliminary investigation of the concept that the comparison of the mutation frequency in incident cases with second primaries versus incident cases with first primaries is an efficient design for identifying rare cancer genetic risk factors, and to serve as the prototype for a much larger, definitive study that is now in progress in nine population-based centres around the world. The pilot study involves the comparison of 65 patients with multiple primary melanomas identified without regard to family history of the disease from the schedules of participating physicians at Memorial Sloan-Kettering Cancer Center and the Yale University Pigmented Lesion Clinic, and 88 patients with invasive first primary melanoma identified from MSKCC during the accrual period 1997–2000.

Materials and methods

The basic premise of the study is that the goals of a conventional case–control study, in which incident cases of melanoma are compared with age-matched ‘healthy’ controls from the general population with respect to the prevalence of any risk factor, can be replicated by instead comparing incident cases of second primaries as the ‘case’ group versus incident first primaries as the ‘control’ group. This type of design is especially advantageous for evaluating rare risk factors with a large relative risk, since in these circumstances few if any population controls will have the risk factor. The new design requires two key assumptions: second primaries are genuinely biologically independent occurrences of the disease; the risk factor does not affect case survival (this assumption can be tested). Further details of the theoretical justification are provided in Begg and Berwick (1997). The study reported here is a pilot version of the design, with the goal of establishing proof-of-concept.

Subjects

All subjects were interviewed in person between 1997 and 2000 after giving signed informed consent. The

Institutional Review Boards at Memorial Sloan Kettering Cancer Center (MSKCC) and Yale University reviewed this study. All subjects donated DNA samples as blood or buccal swabs, and interviewers counted nevi greater than 2 mm on both arms and the back. Both interviewers at MSKCC were trained by a dermatologist and received periodic re-training. Two interviewers enrolled all subjects at MSKCC; a dermatologist (JB) enrolled the 11 subjects from Yale University. Subjects completed a short, previously field-tested and validated questionnaire covering risk factors for melanoma (Berwick *et al.*, 1996). Items in this version included phenotypic variables (hair colour, eye colour, skin colour, freckling and skin type), age, race, education and family history of melanoma and non-melanoma skin cancer.

Single primary melanoma

Cases of invasive single primary cutaneous melanoma were identified from the schedules of two surgeons and the Dermatology Clinic at MSKCC. Cases newly diagnosed within one year of participation were eligible. One hundred and three subjects were eligible and 88 subjects had adequate DNA for mutation analyses. All melanomas were documented by pathology reports.

Multiple primary melanomas

It was not feasible to limit our pilot study solely to patients with an incident second primary melanoma, since we were able to identify only a few such cases in the 3-year accrual period at MSKCC. Thus, we expanded our accrual to any case of multiple primary cutaneous melanoma identified from the schedules of participating physicians at the Center with no restriction as to dates of diagnoses was eligible for this study. Additional cases were identified at Yale University Pigmented Lesion Clinic, and by referral. Seventy-four subjects were eligible and 65 had adequate DNA for mutation analyses. All melanomas were documented by pathology reports.

INK4A analyses

Genomic DNA was prepared from buccal brushes or blood. If DNA was obtained from buccal brushes, DNA was isolated following the method of Richards *et al.* (1993). If obtained from blood, the DNA was extracted using the Qiagen Qiap DNA kit (Qiagen Inc., Valencia, CA) following manufacturer’s recommendations.

Primers

Exons 1 α , 2 and 3 of the INK4A gene, and their splice junctions were analysed using primers described by Hussussian *et al.* (1994) with few modifications. Exon 2 was amplified using one set of primers (2A-forward and 2C-reverse), originating a 411 bp fragment.

PCR reaction

Between 10 and 100 ng of genomic DNA were amplified in a reaction mixture containing 0.4 μ mol/l for each forward and reverse primer, 200 μ mol/l dNTPs (PE, Roche Molecular Systems Inc., Branchburg, NJ, USA), 0.06 U/ μ l Taq Polymerase (PE), 10 nmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l MgCl₂ and 1 mol/l betaine (Sigma-Aldrich, St. Louis, MO, USA). PCR reaction mixtures were subjected to the following cycling conditions: 95°C for 2 min; 95°C for 25 s, 55°C for 25 s, 72°C for 35 s for 35 cycles, and a final extension at 72°C for 7 (exon 3) to 10 (exons 1 alpha and 2) min. All PCR products were tested on ethidium bromide stained agarose gels in order to verify the size of the amplified band.

Denaturing high-performance liquid chromatography (DHPLC) analysis

We first screened all samples on DHPLC (Orlow *et al.*, 2001). All PCR products were mixed in approximately equimolar proportions with an amplified sample known to contain a wild-type INK4A fragment. Mixed samples were heated at 95°C for 5 min and allowed to cool down to room temperature for approximately 20 min. Five to 10 μ l of each sample were run on a Wave DNA Fragment Analysis System (Transgenomic, Omaha, NE, USA) using a DNASep column, and the run was monitored by UV (260 nm). Each elution profile or chromatogram was compared with profiles associated with samples versus those obtained from a wild-type control and was assessed by comparing band intensities. Samples showing an altered chromatographic profile were repeated starting with a new PCR reaction. These samples were either mixed or unmixed with wild-type control. This allowed differentiation between heterozygote or homozygote variants.

Sequencing analysis

An independent PCR reaction was performed in all cases. Specific bands were gel purified with a gel purification kit according to the manufacturer's recommendations (Qiagen). The amount of purified DNA varied according to the fragment size and sequencing instrument used, but approximately 8–25 ng of each purified sample were mixed with 3.2 pmol of specific primer, 4 μ l of termination mix, and distilled H₂O to a final volume of 10 μ l. Samples were subjected to 25 cycles at 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. Samples were then purified by ethanol and sodium acetate precipitation, and run in an ABI310 instrument (PE-Applied Biosystems, Foster

City, CA, USA). A subset of samples was sequenced in the Sequencing Facility of MSKCC on an ABI377 instrument (PE-Applied Biosystems). Sequencing electropherograms were read at least twice.

Statistical analysis

The design of the study is based on the premise that the odds ratio for an epidemiologic risk factor, obtained from a comparison of incident first primary melanomas and incident second primary melanomas, will be a bona fide estimate of the rate ratio for the development of melanoma. Consequently, for the purpose of relative risk estimation, the study is equivalent to a conventional case-control comparison of incident cases of melanoma with population controls. The advantage of this design is that rare risk factors, such as mutations in INK4A, will occur too infrequently in the population for a conventional case-control study to be feasible. The assumptions required for the equivalence of the two designs are that: (a) a tumour designated as a second primary is a genuinely independent occurrence of the disease, as opposed to an unrecognized metastasis of the first primary; and (b) the risk factor is not associated with subsequent case survival. The second assumption can be formally tested by evaluating whether the risk factor is correlated with the time intervals between occurrences of the first and second primaries, measured solely in the series of second primaries. Furthermore, in this pilot study, our case series includes patients identified with multiple primaries, rather than being restricted to incident second primaries. This restriction was eased as a practical step to increase accrual, and might affect the validity of the relative risk estimates. Further details of the rationale for the novel study design are contained in a methodological article by two of the authors (Begg and Berwick, 1997).

In our analyses we have used Fisher's exact test to compare the frequencies of INK4A mutations. Other analyses involving relative risks were accomplished by calculating odds ratios directly and by using logistic regression for adjusted analyses. The logistic regression analyses involved adjustment for all risk factors: nevus number, skin colour, hair colour, eye colour, freckling, inability to tan, propensity to burn, and family history of melanoma. To address the possibility that a risk factor might be associated with patient survival, the risk factors were correlated with the times between diagnosis of the first and second primaries using a linear regression analysis on the logarithms of these time intervals, an analytic strategy that has been shown to be a valid approach for identifying bias induced by length-biased sampling (Begg and Gray, 1987).

Results

We examined the known risk factors of subjects who had inadequate DNA for mutation analysis and found no evidence that the excluded patients differed from the patients with adequate DNA for any of the variables measured. We found 5 (8%) mutations among 65 multiple primary subjects and one silent mutation among 88 single primary subjects. The mutation frequencies were significantly different ($P = 0.03$). However, the frequencies are too small to reliably estimate the relative risk. The five mutations were Leu32Pro, Ile49Thr, Met 53Ile, and Gly101Trp (2 subjects) (Table 2). The one silent mutation was at codon 106.

The median age at diagnosis was 52.7 years for the first primary cases, while the median age at diagnosis of the second primary cases was 53.8. However, the five mutation carriers experienced their cancers at generally younger ages (see Table 2). The sex distribution for single primaries and multiple primaries was similar with approximately 60% males in each group.

Although we cannot reliably estimate the relative risk due to a germline *INK4A* mutation since there were no occurrences in the single primary group, we can estimate the relative risks for phenotypic characteristics that are known to be associated with melanoma. The most important of these is the number of nevi (counted on both arms and the back), and this is shown to be strongly associated with the occurrence of second primaries, with relative risks similar to those observed in conventional case-control studies in both the adjusted as well as the unadjusted analyses (Table 3). The other phenotypic characteristics in the table are mostly moderately associated with risk, again paralleling the results of conventional studies, though the sample sizes in this study are too small to reliably evaluate factors with modest relative risks.

The fact that the relative risks of individual risk factors are mostly unaffected by adjustments for other risk factors suggests that they possess 'independent' influences on cancer risk. Due to the limited sample size we

Table 2 Characteristics of the five mutation carriers

Risk factor	Case 1	Case 2	Case 3	Case 4	Case 5
Mutation	Leu32Pro	Met53Ile	Ile49Thr	Gly101Trp	Gly101Trp
Ages at diagnosis ^a	32, 36	24, 31	16, 18	56, 71	48, 48
Race	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian
Number of nevi ^b	3 (2%)	45 (90%)	6 (15%)	18 (59%)	22 (73%)
Skin colour	Fair	Fair	Fair	Fair	Fair
Hair colour	Light brown	Red	Black	Medium brown	Medium brown
Eye colour	Grey	Medium brown	Medium brown	Blue	Dark brown
Freckles	No	No	No	Yes	No
Inability to tan	No	No	Unknown	Yes	No
Propensity to burn	Yes	Yes	No	No	No
Family history of melanoma	No	Yes	No	No	Yes
Sex	Male	Female	Male	Male	Female

^aAge at diagnosis of first primary and second primary, respectively.

^bNumber of nevi counted on both arms: percentile of distribution among multiple primary cases in parentheses.

Table 3 Risk factors for melanoma, comparing subjects with single primaries and those with multiple primaries

Risk factor	SPM	MPM	Unadjusted relative risk	Adjusted relative risk	P-value	Test for survival bias
<5 nevi ^a	17 (21%)	6 (10%)	1.0	1.0		
5–9 nevi	31 (38%)	13 (22%)	1.2 (0.4, 3.7)	1.1 (0.3, 3.9)		
10–14 nevi	15 (18%)	10 (17%)	1.9 (0.6, 6.4)	3.1 (0.8, 12.6)	0.001 ^h	0.24
15–19 nevi	8 (10%)	11 (19%)	3.9 (1.1, 14.3)	4.8 (1.1, 21.8)		
≥ 20 nevi	11 (13%)	19 (32%)	4.9 (1.5, 16.1)	4.9 (1.3, 19.4)		
Fair skin colour	62 (76%)	47 (80%)	1.3 (0.6, 2.8)	1.3 (0.5, 3.6)	0.57	1.00
Light hair colour ^b	55 (64%)	48 (75%)	1.7 (0.8, 3.5)	2.6 (1.0, 7.1)	0.06	0.70
Light eye colour ^c	55 (64%)	47 (73%)	1.6 (0.8, 3.2)	1.0 (0.4, 2.6)	0.95	0.76
Freckles ^d	37 (45%)	28 (44%)	1.0 (0.5, 1.9)	0.7 (0.3, 1.6)	0.38	0.45
Family history ^e	6 (7%)	9 (15%)	2.3 (0.8, 6.7)	1.8 (0.4, 8.2)	0.44	0.43
Inability to tan ^f	13 (15%)	12 (19%)	1.3 (0.6, 3.1)	1.1 (0.4, 3.3)	0.81	0.47
Propensity to burn ^g	31 (36%)	27 (44%)	1.4 (0.7, 2.7)	1.6 (0.7, 3.9)	0.29	0.54

^aNumber of nevi on arms.

^bLight/medium brown, blond or red.

^cBlue, green or hazel.

^dTendency to develop freckles prior to age 25.

^eKnowledge of family history of melanoma.

^fNo tan after prolonged exposure to the sun.

^gPainful/severe burn after exposure to strong sunlight for one hour.

^hTrend test

cannot formally evaluate the independence of the influence of these factors with that of INK4A mutational status. However, the five cases with mutations have an unremarkable profile with respect to the phenotypic risk factors (Table 2), suggesting that the influences may indeed be independent.

The statistical tests for survival bias were all non-significant (shown in the last column of Table 3). Thus there is no evidence that the risk factors studied are associated with patient survival after the first primary diagnosis. However, again, the small sample sizes limit the statistical power of these analyses.

Discussion

The reported study is a pilot study to estimate the relative risk of genetic determinants of melanoma, in particular INK4A mutations, using subjects identified with an incident second primary melanoma as cases and subjects diagnosed with an incident first primary melanoma as controls. Though far from definitive, our results support the view that the design is a useful technique for evaluating relative risks of rare, highly penetrant risk factors, based on the observation that, collectively, the known melanoma risk factors, such as nevi count, hair and skin colour, etc., had relative risk estimates that are broadly consistent with the results from the large body of evidence from conventional case-control studies.

Although we cannot accurately estimate the relative risk of developing melanoma due to an INK4A mutation in this relatively small dataset, our analysis does demonstrate a statistically significant association with risk. We found that 8% of our subjects with multiple primaries had mutations in INK4A, compared with none of 88 subjects with single primaries ($P = 0.03$). This is very similar to the proportion, 9%, of mutations found in the only other moderately large study reported (Auroy *et al.*, 2001). In our study, only one case of first primary melanoma had a mutation, but it was one that did not change the amino acid or was 'silent'. Of the mutations identified Leu32Pro has been described among Australian melanoma kindreds (Walker *et al.*, 1995). This mutation results in an alteration of the conserved amino acid sequence within the ankyrin domain. Ile49Thr was first considered to be a polymorphism (Hussussian *et al.*, 1994; Ranade *et al.*, 1995). However it has since been shown to exhibit reduced binding to CDK4 (Reymond and Brent, 1995; Yang *et al.*, 1996). Met53Ile has been described in numerous populations, particularly though not exclusively Scottish (Soufir *et al.*, 1998; FitzGerald *et al.*, 1996; MacKie *et al.*, 1998; Burden *et al.*, 1999; Monzon *et al.*, 1998; Harland *et al.*, 1997). The one subject in our study with this mutation had a Scottish great-grandmother.

Mutations in Gly101Trp have been reported (Hussussian *et al.*, 1994; Kamb *et al.*, 1994; Whelan *et al.*, 1995; Soufir *et al.*, 1998; Jakobson *et al.*, 2000) from families in France, Australia, the US and Israel. Ciotti *et al.* (2000) have studied this mutation among 20 families (10 from Italy, four from the US and six from France), and the authors estimate that all families derived from a single ancestral haplotype that arose 97 generations ago. Polymorphisms at nucleotide (nt) 500 have been reported by Aitken *et al.* (1999) to be slightly higher among cutaneous malignant melanoma (CMM) cases than controls (14% versus 11%) and among CMM cases the prevalence of this polymorphism increased linearly with increasing familial risk and was highest in families with INK4A mutations. We did not observe an association in this study, although the prevalence is observed to be high for both multiple primary subjects (26%) and single primary subjects (22%). We found no evidence of melanoma risk associated with the common polymorphism at codon 148.

In summary, our results are consistent with the speculation that INK4A mutations are quite rare in patients presenting with single primary melanoma (possibly around 1%), very rare in the population at large, but much more common in patients with two or more primary melanomas.

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