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A Major Quantitative-Trait Locus for Mole Density Is Linked to the Familial Melanoma Gene *CDKN2A:* A Maximum-Likelihood Combined Linkage and Association Analysis in Twins and Their Sibs

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Summary

Important risk factors for melanoma are densely clustered melanocytic nevi (common moles) and mutations in the p16 (CDKN2A) gene. Nevi may be subclassified as raised or flat. In our sample, raised nevi were 27% of the total, and the two kinds had a correlation of .33. Correlations for total-nevus count (TNC) in 153 MZ and 199 DZ twin pairs were .94 and .60, respectively, which are compatible with a very-high degree of genetic determination. We hypothesized that some of the genetic variance might be due to variation in the p16 gene. Analysis of linkage to a highly polymorphic marker (D9S942), located close to p16, detected quantitative-trait–loci (QTL) effects accounting for 27% of variance in TNC, rising to 33% if flat but not raised moles were considered. Total heritability was higher for raised (.69) than for flat (.42) moles, but QTL linkage was 0 for raised moles, whereas it accounted for 80% of the heritability of flat moles; additionally, family environment accounted for only 15% of variance in raised versus 46% in flat moles. These findings suggest that raised and flat nevi have very different etiologies. Longer alleles at D9S942 were associated with higher flat-mole counts, and a novel modification to a within-sibship association test showed that this association is genuine and not due to population stratification, although it accounts for only 1% of total variance. Since germline mutations in the exons of CDKN2A are rare, it is likely that variants in the noncoding regions of this gene, or in another gene nearby, are responsible for this major determinant of moliness and, hence, of melanoma risk.

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Introduction

For the last two decades, the incidence of malignant melanoma (MIM 155600) has more than doubled in white populations throughout the world (MacLennan et al. 1992). The presence of melanocytic nevi is the strongest-known risk factor for this disease (Swerdlow and Green 1987). Common nevi can be categorized according to their profile as either flat (junctional or compound) or raised (intradermal) (Green and Swerdlow 1989). Since the incidence of melanocytic nevi peaks around the age of puberty (Green and Swerdlow 1989), knowledge of nevus development in adolescents may further our understanding of the causes of malignant melanoma. The etiological relationship between raised and flat nevi is far from clear, and it is also unclear whether one type is more strongly related to melanoma risk than the other (Davis 1991; Schmoekel 1997; Worret and Burgdorf 1998).

Another important risk factor for melanoma is germline mutation of the CDKN2A gene (MIM 155601, 600160), which encodes the cyclin-dependent kinase 4 (CDK4) inhibitor $p16^{ink4A}$ (Kamb et al. 1994*a*). Such mutations have been detected in multiplex melanoma families (Ohta et al. 1994; Hayward 1998*a*, 1998*b*), although it seems that they account for only a small fraction of cases of familial melanoma (Kamb et al. 1994*b*; Platz et al. 1997; Hayward 1998*b*). In Queensland, Australia, mutations were found in 10% of the most melanoma-dense families in a large population-based sample, but were estimated to occur in only 0.2% of all melanoma cases (Aitken et al. 1999). A second melanoma-predisposition gene, CDK4 (MIM 123829), has also been identified, but only three families with germline mutations have been documented (Zuo et al. 1996; Soufir et al. 1998). In some melanoma kindreds, individuals have been observed with a high prevalence of nevi classified as "dysplastic" or "atypical" (Gruis et al. 1995) but this phenotype has not been found to segregate with CDKN2A mutations in melanoma-prone

families (Gruis et al. 1995; Hayward 1996, 1998*a*). However, some evidence for linkage of *normal* nevus count with CDKN2A has been found in three large CDKN2A-linked melanoma pedigrees (Cannon-Albright et al. 1994).

Solar ultraviolet radiation is the major environmental risk factor for nevi. Nevus counts in young children are strongly related to episodes of sunburn, cumulative sun exposure, and markers of sun sensitivity, such as fair complexion and tendency to burn (Harrison et al. 1994). In a study of nevus counts in children in three Australian cities spanning 20° of latitude, significant differences in total-mole count were seen between cities, with numbers increasing toward the equator (Kelly et al. 1994). However, at age 12 years (the age of subjects in the present study), 95% of the variance still occurred within cities (MacLennan et al., in press). It is the source of these large differences among individuals living in a small geographic region with uniformly high sun exposure that we seek to explain, and our chosen design is the comparison of similarity in MZ and DZ twins (Martin et al. 1997). Strong familial correlations between total-nevus number and nevus density have been reported (Easton et al. 1991; Goldgar et al. 1991; Duffy et al. 1992; Briollais et al. 1996); in fact, the very first twin study to formally compare the similarity between MZ and DZ twins happened to be on melanocytic nevi (Siemens 1924). However, all twin studies of nevi to date have been small and heterogeneous in age, ethnicity, or region. Here we present data on moliness of 352 pairs of twins aged 12 years living in southeastern Queensland (McGregor et al. 1999).

Subjects and Methods

Subjects

Twins were recruited from schools in Brisbane and surrounding areas of southeastern Queensland and were examined close to their 12th birthday. Blood was obtained from all twins and most parents for blood grouping and DNA extraction. Parents were asked the ancestry of all eight great-grandparents of the twins. More than 95% of great-grandparents were identified as being of northern European ancestry, mainly from Britain and Ireland. Further details are reported by McGregor et al. (1999).

Mole Counts

A nurse (A. Eldridge) examined each individual and counted all nevi on the entire body surface, which was divided into 17 sites on each side of the body excluding buttocks, chest, and abdomen. Melanocytic nevi were defined as pigmented macules or papules, which were reasonably well defined and were darker in color than the surrounding skin. The total number of nevi with a diameter <2 mm, 2–5 mm, and >5 mm, as measured by stencils, and the number of raised nevi in each size category were also recorded for each site. The total number of these nevi, at each site, classified as atypical was also recorded. To be classified as atypical, a lesion needed to show at least two of the following attributes: (1) a diameter $\geqslant$6mm, (2) variegate color, and (3) atypical morphology, defined as an irregular border, an ill-defined border, or the presence of a macular component at the periphery. Mole counts (x) have a distribution with a long tail to the right, so for most purposes they are transformed with use of *ln* to stabilize variances and improve normality. Previous authors (e.g., see Goldgar et al. 1991; Cannon-Albright et al. 1994) have analyzed nevus density by dividing total-mole count by body surface area (BSA; see below). In this paper, we begin by analyzing mole counts and then correct them for regression on BSA, which is tantamount to analyzing nevus density.

Other Measures

During the testing session, subjects completed a questionnaire about sun exposure. Nurses collected other risk-factor information, as follows.

Sun exposure.—Twins and sibs were asked to report the number of hours they spent in the sun during a normal school week in summertime and during a normal weekend in summertime. These two amounts were summed (x), and sun exposure was transformed as $\log(x + 1)$.

Sunburn.—Twins and sibs were asked how many painful sunburns they had gotten in the last six months. They were asked to number these sunburns by using a four-point scale for 0, 1, 2–6, or >6 sunburns.

Skin color and hair color.—Skin color on the inner upper-arm was given a rating by the nurse of 1 (fair/pale), 2 (medium), or 3 (olive/dark). Hair color was given a rating of 1 (fair/blonde), 2 (light brown), 3 (red/auburn), 4 (dark brown), or 5 (black).

Freckling.—The presence of freckling on the face was ascertained. The nurse gave a rating of 0 (absent), 1 (mild), 2 (moderate), or 3 (severe).

BSA.—Since the number of moles increases with body size, it is appropriate to correct for BSA, calculated as $\text{BSA}(m^2) = (\text{height [cm] x weight [kg]}/3{,}600)^{0.5}$. This correction is equivalent to measuring nevus density. For the purposes of regression analyses, all categorical variables were transformed to normal weights.

Zygosity

Zygosity of twins was diagnosed by typing eight highly polymorphic DNA microsatellite markers on different chromosomes and three blood groups (ABO,

MNS, and Rh) in the twins and (in most cases) in both parents. The probability of dizygosity, given concordance for all markers in our panel, was $<10^{-3}$.

Genotyping and IBD Estimation

DNA was extracted by use of standard methods (Miller et al. 1988). All families were typed for marker D9S942 (Genome Database 370738), and where this marker was not fully informative we typed other markers (shown in fig. 1) to resolve identity-by-descent (IBD) status. Samples were typed on an ABI 373 sequencer (Perkin-Elmer) with GENESCAN and GENOTYPER software after PCR amplification. IBD probabilities at D9S942, for each sib pair, were estimated with MAPMAKER/SIBS (Kruglyak and Lander 1995), and all available marker data for the family and allele frequencies were estimated from our data. If one of the three probabilities—p_0, p_1, or p_2 (i.e., that sibs share 0, 1, or 2 alleles IBD)—was not >.8, then further flanking markers of CDKN2A (fig. 2) were typed, and probabilities were recalculated until all DZ twins and sib pairs could be classified with a probability of >.8 (except for one recombinant). The estimated proportion of alleles shared IBD at the marker locus in a pair of sibs is then computed as $\hat{\pi} = p_2 + 0.5p_1$, and this is used as the coefficient for the quantitive-trait–loci (QTL) effect in structural-equation modeling of raw data (Amos 1994; Boomsma 1996; Eaves et al. 1996; Fulker and Cherny 1996; Martin et al. 1997; Almasy and Blangero 1998).

Structural-Equation Modeling of Genetic Hypotheses

We used the MX software package (Neale 1997) to perform maximum-likelihood (ML) analyses of the individual observations, in which one simultaneously tests hypotheses about the means, variances, and covariances under the assumption of multivariate normality (Lange et al. 1976; Martin et al. 1987). There are two parts to the model, as follows: (1) a model for the expected values of individual observations in terms of measured fixed effects such as age, sex, and allele size, and risk factors such as sun exposure (the "model for the means"); and (2) a model for the covariance matrix of the residuals after removal of the fixed effects. The variances and covariances (or correlations) may be estimated directly or parameterized in terms of genetic and environmental variance components.

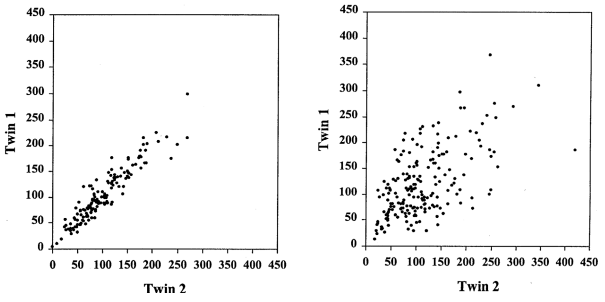

Figure 1 Plot of total-mole counts of twin 1 versus twin 2 for 153 MZ (*left*) and 199 DZ (*right*) twin pairs, age 12 years, living in southeastern Queensland.

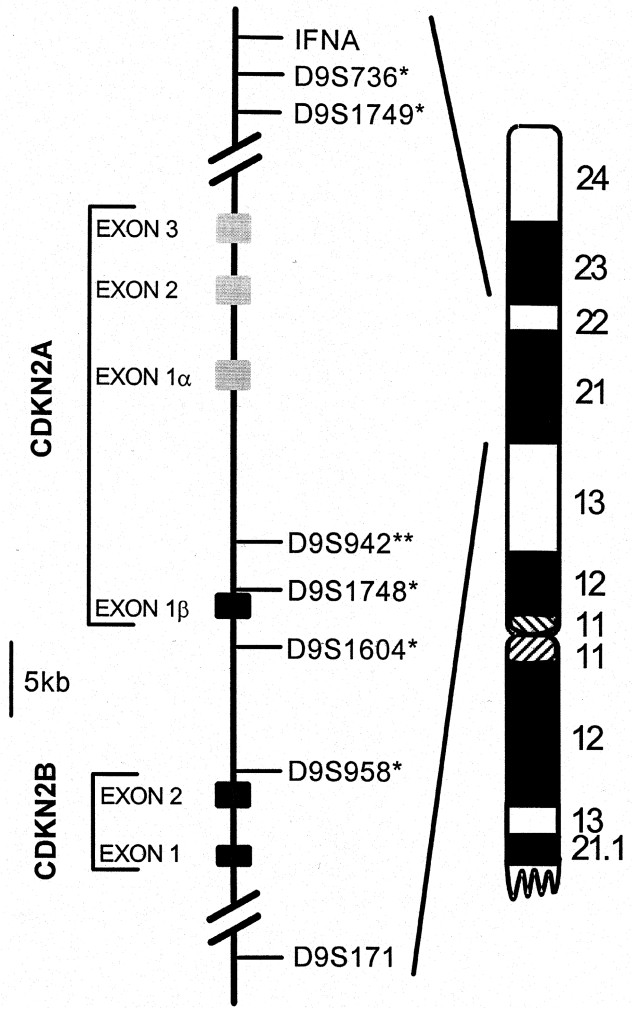

Figure 2 Genetic map of the CDKN2A region on chromosome 9p. All families were typed for D9S942 (denoted by a double asterisk [**]) to determine IBD status but where this was not clear, further markers (denoted by a single asterisk [*]) were typed. Genome Database accession numbers for these markers are D9S736, 270736; D9S1749, 595876; D9S942, 370738; D9S1748, 595589; D9S1604, 567048; and D9S958, 373624. Note that marker D9S958 is identical to D9S1605 and D9S1752, which have been independently isolated and mapped to this chromosome segment. The region between the breaks in the vertical line has been drawn to scale using information derived primarily from GenBank entries AC000048 and AC000049, which contain the complete sequence of cosmids from this region.

Significant twin correlations establish that there is familial aggregation for the measure of interest. Our task

is to distinguish between the possible mechanisms by which this familial likeness may arise. One can conceive of four broad causes of variation, three of which (additive genetic influences [A], genetic dominance [D], and common environment [C]) make family members more alike than random pairs of individuals and one of which (unique environmental experiences, including error, [E]) makes MZ twins and siblings different. A component of variance (Q), due to variation at a QTL linked to a marker locus for which IBD information is available on DZ twins and sib pairs, can also be added to the model if the covariance of each sib pair is conditioned on the proportion of shared parental alleles, $\hat{\pi}$. However, it must be recognized that there is a substantial negative correlation between estimates of Q and A and this results in a large standard error for the estimate Q.

In assessing the fit of different submodels, we use the χ^2 likelihood ratio to balance the joint criteria of fit and parsimony (Neale and Cardon 1992). The fit of each submodel is tested against the preceding, more-complex model within which it is nested. A particular point concerns the likelihood-ratio test for QTL linkage when Q is dropped from the model. Since the test for linkage is essentially a test of whether the IBD2 correlation is greater than the IBD0 correlation, it is a one-sided test and the likelihood-ratio statistic is a mixture of a χ^2 on 1 df and a point mass of 0. This is indicated by $\chi^2_{0.1}$ in the text; the practical consequence is that the probability associated with this χ^2 statistic may be halved.

Test for Linkage Disequilibrium

For each individual we calculate the mean length of their two D9S942 alleles. Then for each family we calculate the average of these means across all twins and sibs tested, and for each of these individuals we express his or her own mean allelic length as a deviation from the family average. The means model is then rewritten with two terms for the regression of the phenotype on mean allelic length for which we previously had just one; now we have one term for regression on the family average (b_f) and another for regression on each individual's deviation from the family average (b_i). If the allelic association we have detected is merely a result of population stratification, then we should expect all of it to be detected as b_f and none as b_i. However, if the association is genuine—if it is a functional effect of the polymorphism or of another polymorphism in linkage disequilibrium with it—then we should expect to see an appreciable b_i term detecting within-sibship differences arising from the allelic effect, as well as a b_f term detecting differences between families. Analysis of simulated data confirms the validity of this procedure for distinguishing and correctly estimating genuine associations from those due to stratification. Using methods described by Fulker et al. (1999), we simulated 10,000 sib pairs drawn from a population with a genuine allelic association and the formal within-sibship association test (a_w; the "Fulker test") was performed, with $\chi^2_1 = 415.29$. When this test was reparameterized as described previously (b_i), analysis of the same data yielded $\chi^2_1 = 415.06$. Another sample of 10,000 sib pairs was simulated from a second population, in which there was an allelic association due to population admixture but no genuine association. Here, the Fulker test yielded $\chi^2_1 = 3.115$ and the b_i test yielded $\chi^2_1 = 3.112$.

Results

Quantitative Genetics of Nevus Counts

Twins were recruited through schools in Brisbane and surrounding areas, and 98% of their great-grandparents were of European (95% northern European) origin. We endeavored to test all twins close to their 12th birthday, but a few pairs were a year older. To increase the number of sib pairs available for linkage analysis, 80 siblings (aged 9–22 years) from 67 twin families were also phenotyped and genotyped. For the purpose of estimating test-retest reliability, a subsample composed of 33 twin pairs was retested, early in the study, 2–11 wk (median 4 wk) after their first visit; the repeatability of TNC was .96 (Aitken 1994). Mean TNC in the twins (110) did not differ from that of an age-matched epidemiological sample of Brisbane school children (108), although the variance is slightly lower in the twins ($\chi^2_1 = 4.84$; $P = .03$) (Green et al. 1995), thus allaying our concern that twins with a higher-than-average mole count were being "volunteered" by their parents for our study.

Complete data were available for 77 MZ female-twin pairs, 76 MZ male-twin pairs, 49 DZ female-twin pairs, 54 DZ male-twin pairs, and 96 DZ opposite-sex–twin pairs. Plots of TNC in sex-pooled MZ and DZ twins are shown in figure 1. Note the very-wide range of moles (2–422) in this sample of 12-year-old children, all living in the small geographic region of southeastern Queensland. Mean counts for boys are slightly higher than for girls (table 1), but the MZ correlation remains the same when corrected for this sex difference because of differences in body size, as measured in BSA or when log transformed for correction. This extraordinarily high MZ correlation (.94 with 95% confidence interval [CI]; range .92–.96) is as high as the test-retest correlation on the same individuals (.96; Aitken et al. 1994) and between mole counts on the left and right sides of the body in these subjects (.93; range .92–.94; subjects [N] = 492).

The DZ correlation (.60; range .50–.68) is much lower than that of MZ twins, suggesting major genetic influences on TNC in this population. Furthermore, after the

correction was made for differences in age, the correlation between nontwin siblings in the families of our twins (.65; range .57–.71) is not significantly different from that between DZ twins ($\chi^2_1 = 0.60$; $P = .44$), denying any role for special twin environmental effects. ML estimation suggests that 68% of the variance in TNC is due to additive genetic factors, 26% to family environment, and 6% to unique environmental variance, including errors of measurement.

Sib-Pair Linkage Analysis

We hypothesized that some of the genetic variance might be a result of variation in the CDKN2A gene and sought to detect it by typing all twins and available sibs and parents for a highly polymorphic $(CA)_n$ repeat marker (D9S942), located ~9 kb upstream of the p16 initiation codon located in exon 1α of the CDKN2A locus (fig. 2). We detected 26 alleles at D9S942, with a range in length of 91–145 bp and with 94% heterozygosity, in our sample, making D9S942 highly informative for linkage analysis (fig. 3). Flanking markers were also typed if the marker was insufficiently informative (fig. 2).

If there is a gene of major influence on mole count close to D9S942, then we should expect sib pairs carrying the same two parental alleles to be more-highly correlated for the trait than pairs sharing neither parental allele; pairs sharing one allele should have intermediate correlation. This is indeed what we observed (fig. 4); correlations for logTNC in DZ twins and sibs classified by IBD class 0, 1, and 2 are in the order expected (.44, .64, .74) for a marker close to a QTL having a major influence on the trait, and are heterogeneous ($\chi^2_2 = 12.58$; $P = .002$). The difference between the MZ correlation and that for DZ twins and sibs whose IBD class is 2 ($\chi^2_1 = 59.45$, $P < .001$) indicates the importance of polygenic variation elsewhere in the genome. Note

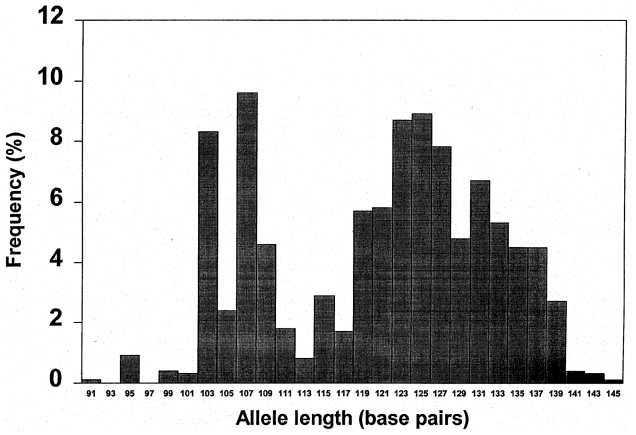

Figure 3 Frequency of the 26 alleles of D9S942, found in our sample of 352 twin pairs.

also that the number of DZ twins (51, 99, 49) in each IBD class is close to the expected 1:2:1 ratio.

We quantify the influence of the QTL and other sources of variation by fitting a path model (fig. 5) to the raw data by conditioning the DZ twin/sib covariance on the estimated proportion of alleles shared IBD at the marker-locus $\hat{\pi}$. Likelihood is calculated for each family under the model and then summed over all twin families, both MZ and DZ. The inclusion of older nontwin siblings in the analysis with twins aged 12 years makes it vital to describe accurately the relationship between the phenotype (logTNC) and age, so we included both linear- and quadratic-age–regression terms in the model for the means.

Fitting the full model, a QTL-linkage effect accounting for 27% of the total variance in logTNC is estimated, and the removal of this effect from the model results in significant worsening of fit ($\chi^2_{0.1} = 11.86$; $P < .001$). The

Table 1

Mean Mole Counts and Correlations in MZ and DZ Twins and Siblings for Raised, Flat, Atypical, and Total Moles

Subject Category (*N*)	Raised Moles	Flat Moles[a]	Atypical Moles	Total Moles
	Mean ± SD (Range)			
Boys (356)	29.6 ± 21.0 (1–123)	84.4 ± 52.2 (1–362)	.6 ± 1.2 (0.–7)	113.9. ± 62.3 (2–422)
Girls (348)	26.8 ± 19.7 (1–139)	80.1 ± 49.0 (4–334)	.6 ± 1.1 (0–9)	106.9 ± 56.4 (12–366)
	Correlation (95% CI)[b]			
MZ twins (153 pairs)	.88 (.84–.91)	.90 (.87–.93)	.38 (.24–.51)	.94 (.92–.96)
DZ twins (199 pairs)	.51 (.41–.61)	.65 (.56–.72)	.21 (.07–.34)	.60 (.50–.68)
Siblings (80 sibs)[c]	.50 (.33–.63)	.71 (.58–.80)	.17 (.00–.33)	.65 (.57–.71)

NOTE.—Independent counts were made of total, raised, and atypical moles.

[a] Flat mole count = no. of total moles − no. of raised moles.

[b] Correlations of mole count [$ln(x + 1)$] determined after allowance was made for sex and age differences among siblings.

[c] Correlation = the ML estimate from all possible pairings with twins and other sibs.

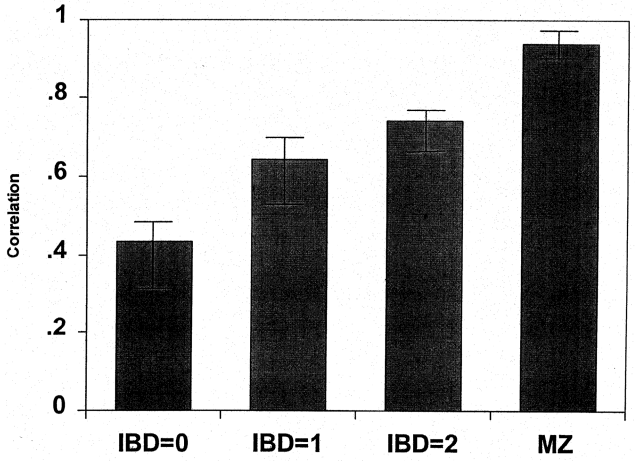

Figure 4 ML estimates of correlations for total-mole count by IBD status of DZ twins and sibs. The MZ correlation is included for comparison, and 95% CI are shown.

correlation between estimates of A and Q in this model is −.64. Allowance can also be made, in the model, for the mean of other risk factors, including sun exposure and sunburns, skin and hair color, and freckling—although the effects of these are modest in this sample, and it could be argued that this method is not the optimum treatment for covariates, some of which are themselves strongly genetically influenced (see table 2) (Martin and Eaves 1977). When these risk factors are included in the model for the means, the QTL linkage effect increases (31% of the total variance) as does its significance ($\chi^2_{0.1}$ = 15.70; $P < .001$). At the same time, the total variance decreases by 4.7%, which represents the proportion of variance in logTNC in our sample, accounted for by the six risk factors relating to sun exposure and complexion. It is interesting to note the composition of this variance change, since it comprises a 9% decrease in polygenic background variance and a 2% decrease in variance due to family environmental effects, reflecting the fact that the major epidemiologic risk factors in this sample are skin color and freckling—factors that are largely genetically determined (table 2). After removal of the risk factors, the estimated contribution of variation at the linked QTL to the residual variance actually increases by 4.3%.

Genetic Heterogeneity of Raised and Flat Moles

Our analysis so far has been on total-mole count, which is the sum of raised and flat moles. In our twin sample, the mean number of raised moles comprised 27% of the mean total-mole count, and the correlation between raised and flat moles (both on log scales) was .33 (.25 in 348 girls, and .40 in 356 boys). Since we cannot assume that these two nevus types have the same etiology, we performed separate analyses. Although the total heritability of raised moles (.69) was much higher than for flat moles (.44), the three IBD correlations were not heterogeneous (χ^2_2 = 3.63; n.s.), and the estimate for a QTL effect linked to CDKN2A was 0 for raised moles, since it was in a separate analysis of atypical moles (table 3). In contrast, the QTL linkage effect for flat moles was even more significant ($\chi^2_{0.1}$ = 26.98; $P < .001$ [equivalent to a LOD of 5.87]) and accounted for 32.9% of total variance in log flat-mole count, leaving only ~20% of the genetic variance unexplained, although this residual is still significant (χ^2_1 = 4.90; P = .027) (table 3). Further dividing flat moles into small (diameter <2 mm [56% of total-flat moles]), medium (diameter 2–5 mm [43%]), and large (diameter >5 mm [1%]), we find consistently large estimates of QTL variance (32%, 25%, and 22%), although not significantly so for large moles for which the small numbers provide little power (not shown).

Association with D9S942

Since there is clearly a strong effect of linkage with D9S942, we may ask whether the alleles at this polymorphism have any effect on the phenotype. We can test this by adding a regression term of the phenotype (total-flat moles) on length of the D9S942 allele(s) in base pairs. If we add to our existing model for the means a term for regression on the length of the longer allele, we find that the fit of the model improves giving χ^2_1 = 5.82 (P = .016), whereas regression on the smaller allele gives χ^2_1 = 5.64 (P = .018). Regression on the mean length of both alleles causes a greater improvement than either allele separately (χ^2_1 = 8.15; P = .004) suggesting that

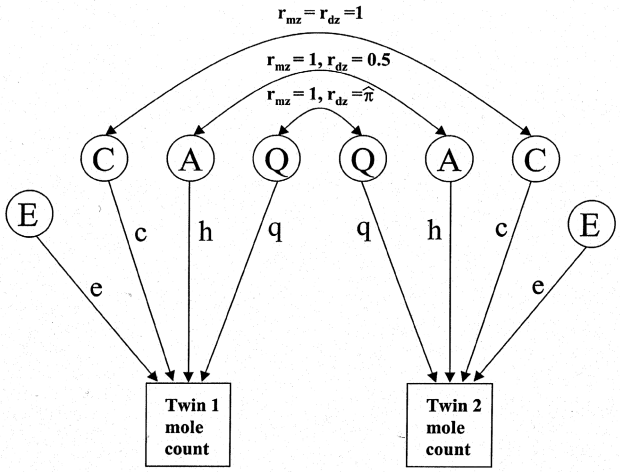

Figure 5 Path diagram showing model of twin/sibling covariation, with variation because of a linked QTL (Q), which is correlated 1 in MZ twins (r_{mz}) and $\hat{\pi}$ in DZ twins (r_{dz}); residual polygenic variation (A), which is correlated 1 in MZ and 0.5 in DZ twins; common family environment (C), which is equally correlated in MZ and DZ twins; and individual environmental influences (E), which are uncorrelated between twins.

Table 2

Correlations of Mole Counts with Epidemiological Risk Factors, and Twin Correlations for Risk Factors

Risk Factor	Raised Moles	Flat Moles	Atypical Moles	Total Moles	MZ Twins (153 Pairs)	DZ Twins (199 Pairs)
BSA	.05	.06	.01	.11	.94	.46
Sun-exposure hours	.10	.03	.04	.06	.50	.42
Sunburns	.05	−.03	−.02	.01	.24	.15
Skin color	−.21	−.06	−.05	−.14	.61	−.08
Hair color	−.24	−.03	−.06	−.11	.75	.42
Freckling	.24	.09	.04	.19	.91	.49

NOTE.—All mole counts and sun-exposure hours are $ln(x + 1)$ transformed; categorical variables are transformed to normal weights.

both are important, but no further improvement is obtained by joint regression on the two alleles ($\chi^2_2 = 8.21$; $P = .016$) suggesting that the allelic effects are additive. The total expected variance for log flat-mole count, under the base model, is 0.45388, whereas after adding average allele length to the model for the means it is 0.44962 suggesting that <1% of total variance is due to this polymorphism. However, for the same two models, the QTL variance is, respectively, 0.14938 and 0.14047, so the polymorphism, or another one in linkage disequilibrium with it, could account for ⩽6% of QTL linkage variance.

Linkage Disequilibrium or Population Stratification?

Given the large amount of QTL variance linked to D9S942, it is unlikely that the effect of the marker polymorphism on the phenotype is wholly an artifact of population stratification. We can use a modification of the within-sibship association test proposed by Fulker et al. (1999) to check this (see Methods). When we fit the between-b_f and within-b_i family-association–effects model to the observed data and drop both terms, the fit worsens ($\chi^2_2 = 8.20$; $P = .017$) close to the effect of the original parameterization of the mean-allele lengths (8.15). If we drop only b_i, the fit worsens to $\chi^2_1 = 4.98$ ($P = .026$), whereas if we drop only b_f it worsens to $\chi^2_1 = 3.24$ ($P = .072$). Since the association with D9S942 is largely a within-family effect, it cannot be because of population stratification.

Discussion

The strength of genetic influence on nevus density in twins aged 12 years and their siblings living in this high–sun-exposure environment is surprising: ~64% of the total variance is genetic and an additional 30% is due to family environment. Sun exposure, shared by cotwins in the course of family life (as 12-year-old children, most of their leisure and nonleisure activities will be together) is the most obvious explanation for this family component; the extent to which this remains after inclusion in the model of self-reported sun exposure and sunburns indicates how imperfect those measures are, since they only attempt to measure recent exposure and ignore exposures in earlier childhood, which are thought to be important but are very difficult to measure retrospectively. Our results complement and contrast with those from a recent study of basal-cell carcinoma of the skin in 13,000 adult twin pairs from Finland; no difference in MZ and DZ concordance was observed, indicating zero genetic influence on this type of skin cancer, but shared environmental factors (presumably sun exposure) accounted for about one-third of variance in liability (Milan et al. 1998).

The extent of a QTL effect linked to D9S942, a marker adjacent to the CDKN2A gene (33% of variance in flat moles, after adjustment for risk factors), was unexpected. The D9S942 polymorphism itself seems to have some small effect that is not due to population stratification but whether it is a functional effect of the $(CA)_n$ repeat length or is in disequilibrium with a functional polymorphism nearby is unknown. Since germline mutations in the exons of CDKN2A are rare (Hayward 1996, 1998*b*; Aitken et al. 1999), it is likely that variants in the noncoding regions of CDKN2A or in another gene nearby are responsible for this major determinant of moliness and hence of melanoma. Our result adds resonance to an earlier report of large CDKN2A-linked melanoma-dense pedigrees (Cannon-Albright et al. 1994) in which nevus counts were higher in presumed CDKN2A-mutation carriers than in noncarriers (although only one of three families carried an exonic mutation) suggesting that other variants in the region, outside of the exons encoding p16, were affecting nevus density. We intend to extend the observations reported here by typing a dense panel of markers through this region in the hope that linkage disequilibrium mapping will further localize the responsible variants.

Interestingly, within CDKN2A there are several sequence polymorphisms (Holland et al. 1995; Pollock et al. 1996), the two most common of which occur in the 3′ untranslated region (3′ UTR) and lead to a cytosine to guanine substitution at nucleotide position (nt) 500 (nt500C→G, numbering from the initiation codon) and

Table 3

Variance Components and 95% CI for Log Mole Counts After Model Fitting to Raw Observations for Twins and Siblings

Mole Type and Variance	QTL	Polygenic	Family Environment	Individual Environment
Raised:	.0	69.0	15.4	15.6
95% CI	…	(50.8–84.9)	(1.9–31.8)	(12.1–20.2)
Drop $\Delta\chi_1^2$	…	60.75[a]	2.55[b]	…
Flat:	32.9	10.9	45.7	10.5
95% CI	(20.1–48.4)	(2.1–23.1)	(30.6–55.8)	(8.2–13.7)
Drop $\Delta\chi_1^2$	26.89[c]	70.82	32.76	…
Atypical:	.0	42.8	.0	57.2
95% CI	…	(3.9–54.8)	(.0–32.6)	(46.2–70.8)
Drop $\Delta\chi_1^2$	…	8.65	.00	…
Total:	31.1	32.6	30.0	6.3
95% CI	(15.6–48.5)	(20.6–49.3)	(13.4–43.8)	(4.9–8.3)
Drop $\Delta\chi_1^2$	15.70	124.13	13.22	…

NOTE.—All models include mean effects for sex, age, age^2, and six risk factors—BSA, sun exposure, sunburn, skin color, hair color, and freckling. The only risk factors determined to be significant are freckling, for raised ($\chi_1^2 = 22.42$) and flat ($\chi_1^2 = 12.18$) moles, and hair color ($\chi_1^2 = 15.19$) and BSA ($\chi_1^2 = 8.05$), for raised moles; components of variance include those that are due to a linked QTL (Q), residual additive polygenetic variance (A), common family environment (C), and individual environment (E).

[a] CE versus ACE model.

[b] AE versus ACE model.

[c] ACE versus ACEQ model.

a cytosine to thymidine change at nt 540 (nt540C→T). Since neither of these polymorphisms causes an amino acid change in p16, it is not known what, if any, effect they have on the expression or function of this protein. The third most common polymorphism within CDKN2A is a guanine to adenine substitution at nt 442 (nt442G→A), which results in an alanine to threonine change at nt 148 in CDKN2A (A148T). Functional analysis indicates that this amino acid substitution does not adversely affect the capacity of this variant protein to inhibit CDK4 enzymatic activity (Ranade et al. 1995; Reymond and Brent 1995). Elsewhere we show that the first of these polymorphisms (nt500C→G) is significantly associated with melanoma risk (Aitken et al. 1999). If it is assumed that this association is genuine and not a result of ethnic stratification, then the polymorphism simply might be in disequilibrium with a causal variant nearby. Alternatively, the polymorphism might be the causal variant itself and its effect on melanoma risk might be mediated through increased nevus density. We are typing all our twin families for the polymorphism to investigate this possibility.

A third finding is that the QTL effect observed in our sample appears to be confined to flat moles, with a 0 estimate for raised moles although raised moles have a considerably higher heritability (.69 with 95% CI; range .51–.85) than flat moles (.42; range .32–.56). In fact, the QTL linked to D9S942 appears to account for ~80% of the genetic variance in flat moles, and bivariate analysis shows that the remaining polygenic variance overlaps completely with that affecting raised moles. The converse is true for shared environmental effects, which account for 47% of variance in flat moles but only a nonsignificant 15% in raised moles, with ~0 correlation between these effects in the two types of mole. This is surprising, since it is thought that raised moles represent a later stage of nevus development than flat moles, in which case one would expect to see some carryover of the predominant influences on flat-mole etiology—namely, the D9S942-linked QTL effect and the large family environmental effects. The fact that the predominant etiological factor for raised moles is polygenic variance, which accounts for very little flat-mole variation, suggests quite distinct etiologies for these two mole types. It is also interesting that we find no evidence for a D9S942-linked QTL effect on atypical moles, although we do detect significant polygenic variation. However, since ours is an unselected sample and the numbers of atypical moles are mainly low, we have much less power to dissect their etiology.

Finally, using linkage mapping with a highly heritable quantitative trait we have located a region containing an important tumor-suppressor gene, albeit one that we already knew about and that is not necessarily the causal agent. Extension to a genomewide scan (now underway) may lead to the detection of novel genes of major effect on nevus density or of novel effects of genes known in other contexts.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank/index.html (for cosmid sequences [AC000048 and AC000049]
Genome Database, http://www.gdb.org/ (for markers used)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for malignant melanoma [MIM 155600], CDKN2A [MIM 155601, 600160], and CDK4 [MIM 123829])

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