Genome-wide search for nevus density shows linkage to two melanoma loci on chromosome 9 and identifies a new QTL on 5q31 in an adult twin cohort

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The density of acquired melanocytic nevi represents an important risk factor for malignant melanoma. Total body nevus counts were collected in a cross-sectional study of 1730 healthy females from the UK Adult twin registry comprising 709 dizygous and 156 monozygous pairs. Nevus density (ND) increased up to the age of 35 years and then gradually declined. Quantitative genetic analysis showed a smaller genetic influence (36%) on ND up to 35 years, compared with after 35 years where it rose to 59%. Using a sub-sample of 1238 genotyped individuals, we performed distinct genome-wide scans for individuals above and below 35 separately. In the younger sub-sample, we confirmed a quantitative trait locus (QTL) for ND on chromosomes 9p21 (LOD = 2.54), a region already linked to both familial melanoma and ND. We also observed a linkage signal on 9q21 (LOD = 2.55) overlapping a recently reported susceptibility locus for ocular and cutaneous melanoma in Danish families. The strongest evidence of linkage identified a novel QTL on chromosome 5q31–32 (LOD = 3.47). None of these linkages was observed in the group aged 35 years and over, which showed suggestive linkage on chromosome 2p24 (LOD = 2.75). To the best of our knowledge, this is the first genome-wide search for ND in a large sample of healthy adults. The results suggest that different sets of genes are likely to influence the processes leading to the appearance of nevi and their involution. They provide both novel and replicated QTLs for nevus development, some of which might overlap with those for melanoma and warrant detailed investigation.

INTRODUCTION

Cutaneous malignant melanoma incidence has risen markedly over the past 40 years. The number of nevi is the strongest predictor of melanoma occurrence (1). Nevi are benign tumours of melanocytes and their density per unit of skin surface has been shown to be heritable. Our group and others have found heritability for nevus density (ND) both in adults and in children ranging from 36 to 84% (2–4). ND is maximal in the teens and twenties and starts to decrease from ~30 to 35 years of age (5–7). What influences the appearance and involution of nevi with age is still not clear. It has been hypothesized that ultraviolet exposure increases the genesis of nevi in young people (8–10), but it is also likely to facilitate their disappearance in older subjects (10–12).

It is possible that different gene expressions and/or genetic–environmental interactions are involved in each phase of the induction and involution of nevi. Constitutional risk factors, such as skin type, might also play a role in nevus expression because they determine the propensity to burn. Indeed, it has been observed that the number of nevi in non-white populations is substantially lower when compared with Caucasian populations (13). Better understanding of the genetic and environmental factors involved in the induction and disappearance of benign melanocytic nevi is, therefore, important in the understanding of the development of melanoma.

More than 50% of families with multiple cases of melanoma have been linked to the chromosomal region 9p21 (14,15). This region contains two candidate tumour suppressor...
genes, which presumably arose by tandem duplication: CDKN2A, which encodes two different transcripts from different promoters for proteins p16 and p14, and CDKN2B, which encodes the protein p15. p15 and p16 are structurally similar proteins that arrest the cells in late G1 by inhibitions of CDK4/CDK6, involved in promoting the progression of the cell cycle from G1 to S through the phosphorylation of the retinoblastoma protein (Rb). The protein p14 can arrest cells in both G1 and G2/M by sequestering MDM2, a protein involved in both the Rb (16) and p53 (17) pathways. Most of the germline mutations in the 9p21 region have been identified in the CDKN2A exons encoding for p16 (18). Germline mutations have also been detected for p14 (19,20), whereas none has been yet identified in p15.

One of the most frequent genetic alterations in malignant melanoma tumours is loss of heterozygosity (LOH) that occurs non-randomly at certain chromosome loci, thus suggesting the involvement of putative tumour suppressor and regulatory genes. In addition to the CDKN genes, LOH studies have proposed that there are additional tumour suppressor genes in the chromosome 9p21 region that are implicated in melanoma (21,22).

Chromosomal regions showing linkage with melanoma have also been reported for chromosomes 1p22, 1p36 and 6p (23–25). Evidence of linkage on chromosome 9q21 has recently been reported in a sample of Danish families with multiple cases of both cutaneous and ocular malignant melanoma (26).

A quantitative trait locus (QTL) for ND has been identified by studying the candidate region 9p21 through combined linkage and association in a sample of 199 Australian dizygous (DZ) twin pairs aged 12 years (2) and partially replicated in 115 UK DZ twin pairs aged 11–18 years (27). However, to the best of our knowledge, no genome-wide search for ND has been performed in a healthy sample at this time. In this study, we present data on ND for 1730 twin females from the TwinsUK Adult Twin registry and the results of a linkage analysis genome-wide search on a sub-sample of 1238 genotyped individuals.

RESULTS

The median (25–75% quartiles) age of twins was 43 years (34–51). The median (25–75% quartiles) of nevus count was 21 (9–43). Seventy percent of the individuals had a very wide range (0–67) and was correlated with skin type (34–51). The median (25–75% quartiles) age of twins was 43 years whereas the average contribution of additive genetic factors was 59% at age 35 and above. Genotypic data were available for 619 of the 709 DZ pairs existing in the whole sample—corresponding to the 82 and 89% of DZs belonging to the younger and older age groups, respectively.

Age-stratified QTL analyses were performed on the 194 (141 DZ and 53 MZ) and 579 (478 DZ and 101 MZ) twin pairs in the younger and older age groups separately (Fig. 2). For the twins aged <35 years, we found evidence of linkage for a QTL influencing ND on chromosome 5q31–32. The 1-LOD drop support interval (SI) identifies a 20 cM region flanked by microsatellites D5S2115 and D5S410 (peak LOD score 3.47 at marker D5S2115) and D5S410 (peak LOD score 3.47 at marker D5S638). On chromosome 9, we observed two distinct peaks 45 cM apart with LOD scores of 2.54 at marker D9S157 (SI: WIAF-3206–WIAF-1899) and 2.55 at marker D9S157 (SI: D9S157–WIAF-3325), respectively (Fig. 3). For twins aged 35 years and over, there was no evidence of linkage at the locations seen in the younger group, whereas suggestive evidence of linkage was seen on chromosome 2p25 with a peak LOD score of 2.75 at marker WIAF-933 (SI: D2S2268–WIAF-3348), 33 cM from the p-ter. When the two age groups were pooled together, we observed decreased LOD scores on chromosome 2p25 (LOD = 2.27) and on
expression is altered in melanoma cells: the melanoma recently identified by Jonsson signal for CDKN2A-unlinked ocular and cutaneous malignant marker D9S167, coincident with the parametric linkage cannot be excluded. The linkage peak on 9q21 was at tumours (21,22); therefore, other potential candidate genes 9p21 spans /C24 in this region has been suggested (2,27). The SI region on chromosome 9p21 was close to the well-known tumour sup-
pressor gene CDKN2A, and the presence of a QTL for ND already implicated for melanoma. The linkage peak on 5q31–32 and observed two linkage signals on chromosome 5q31–32 and observed two linkage signals on chromosome A

**DISCUSSION**

In this sample of randomly ascertained twins from the UK, the ND stabilized at ~30–35 years and afterwards gradually decreased with age. This observation was in agreement with previous epidemiological surveys (28). As ND was significantly influenced by age through a non-linear relationship, we examined the relative contributions of environmental and additive genetic effects in more detail by stratifying above and below 35 years of age separately. Quantitative genetic analysis showed that additive genetic effects accounted for 36% of total variability for those individuals aged under 35 years and for 59% in the older sub-sample. The significantly different distributional trends and amount of genetic influence observed in the two age sub-groups suggested heterogeneity between the nevus appearance and involution processes. In the light of these results, variance component linkage analysis was evaluated in the younger and older twins separately.

This approach has led to the replication of known and identification of novel regions linked to ND. For the twins aged <35 years, we identified a novel QTL on chromosome 5q31–32 and observed two linkage signals on chromosome 9 already implicated for melanoma. The linkage peak on chromosome 9p21 was close to the well-known tumour suppressor gene CDKN2A, and the presence of a QTL for ND in this region has been suggested (2,27). The SI region on 9p21 spans ~23 cM and encompasses also the CDNK2B gene and regions showing LOH in malignant melanoma tumours (21,22); therefore, other potential candidate genes cannot be excluded. The linkage peak on 9q21 was at marker D9S167, coincident with the parametric linkage signal for CDKN2A-unlinked ocular and cutaneous malignant melanoma recently identified by Jonsson et al. (26).

The identified region 5q31–32 harbours two genes whose expression is altered in melanoma cells: the α-catenin gene CTNNA1 (29) and the protocadherin gene PCDHB11 (30). Although these are obvious candidates, the chromosome 5 region spans 26 cM and ideally needs to be narrowed before initiating positional cloning studies.

Those linkage signals were absent in the sub-sample of twins aged 35 and over. Suggestive evidence of linkage was detected at the p-ter of chromosome 2. The chromosomal region, which spans ~40 cM and needs further fine mapping, contains the melanoma-associated gene 50 (MG50) involved in p53-dependent apoptosis (31).

The fact that at least two loci previously identified in relation to melanoma susceptibility are also linked to ND support the hypothesis that some melanoma genes are likely to be involved in nevogenesis. The analysis of the data by age groups also allowed us to investigate the potential genetic influence on nevi induction and involution separately, as different gene expressions and/or genotype–environment interactions are likely to be involved in these processes. The distribution of nevus counts has been suggested to differ between males and females (e.g. 4 and 10), and data in males need to be collected in order to confirm similar linkages in both genders. The next step of the research will be to refine the genome scanning for the two age groups with a denser marker map and to explore potential candidate genes in the linked regions.

**MATERIALS AND METHODS**

**Subjects**

We recruited 1730 healthy females from the UK Adult Twin Registry (32) at St Thomas Hospital in London between January 1997 and December 2003 on whom nevus counts were collected. The study was approved by the St Thomas’ Hospital Research Ethics Committee. As well as answering a comprehensive questionnaire on many common diseases and traits, the subjects were given a validated questionnaire on sun and sunbed exposure. A skin examination was performed by trained research nurses, which included a record of skin type, freckling, hair and eye colour and a total body nevus count, divided into 17 body sites (excluding the genital area, breasts and posterior scalp). The nevus count was performed by nurses trained by VB for 4 weeks before the start of the study. The nevus count protocol has previously

**Figure 2.** Chromosomal LOD score results for the total nevus count for individuals aged <35 years (continuous line) and >34 years (dotted line), including BSA, age and number of sunburns as covariates.

**Figure 3.** LOD score results on chromosome 9 for individuals aged <35 years. The two peaks coincide with a region identified for melanoma susceptibility and ND near the CDKN2A locus (A) and with a susceptibility locus for ocular and cutaneous melanoma in a Danish familial sample (B).
been validated and found to be reproducible (3,12). Nevi were recorded by size in three categories (>2 and <5 mm, >5 and <10 mm, >10 mm). Total body nevus count was defined as the sum of all nevi >2 mm in diameter. Skin type was assessed according to the Fitzpatrick classification (type I: always burn and never tan; type II: often burn and tan gradually; type IV: burn minimally and tan easily and type V: never burn and tan deeply). Sunburns were defined as a sunburn severe enough to cause redness and/or peeling for several days. ND was evaluated as the proportion of nevi per unit of skin surface, calculated using the Mosteller formula (33) for the BSA:

$$\text{BSA} \left( \text{m}^2 \right) = \left[ \left( \text{Height} \, (\text{cm}) \times \text{Weight} \, (\text{kg}) \right) / 3600 \right]^{1/2}$$

Genotyping

Genome scans were performed using DNA extracted from venous blood samples of 1238 subjects. Scans involved the use of standard fluorescence-based genotyping methodologies (34) for the analysis of up to 737 genetic markers from the ABI Prism linkage mapping set (Applied Biosystems) and Genethon Genetic Linkage Map (35). The average spacing was of approximately one marker at least every 10 cM for all sib pairs, as many markers were not informative for linkage. Allele frequencies were estimated from the whole sample of genotyped subjects. The map positions for each marker were taken from Rutgers combined linkage physical map (MAP-O-MAT web site) (36–38).

Statistical analysis

The distribution of ND by age in the cross-sectional sample was examined using lowess (locally weighted scatterplot smoother), a robust smoothing technique that calculates a locally weighted least-squares estimate for each point in the scatter plot to determine the shape of the relation. The loess function (39) is available in R (R project web site).

Age-stratified ND heritability was estimated using structural-equation modelling implemented in the MX software package (40). MX allows simultaneous modelling of the variance–covariance of ND observations according to the expected proportion of alleles shared identical-by-descent (IBD) in the genome and of the expected values of individual observations in terms of measured fixed effects, i.e. age, number of sunburns and BSA. Parameters are estimated by maximum likelihood under the assumption of multivariate normality (41). Additive genetic and environmental components of variance were evaluated by pooling one hundred random samples from each of the two age sub-groups.

Genome-wide screen through variance-components analysis was performed using Merlin (42) and incorporating a simultaneous correction for age, number of sunburns and BSA. A linear mixed model is fit to the data so that the phenotypic variance about the trait mean is partitioned into a monogenic component representing the contribution of a QTL, a polygenic component attributable to residual additive genetic variance and a residual component attributable to environmental effects. The phenotypic variance–covariance in sibs is modelled using the expected proportion of alleles shared IBD over the genome to estimate the polygenic component and the proportion of alleles shared IBD estimated from the genotypic data at a point in the genome to estimate the QTL effect. LOD scores are calculated as the difference between the maximum of the log$_{10}$ likelihood of the model estimating the QTL effects and the maximum of the log$_{10}$ likelihood of the model in which the QTL effect is constrained to equal 0. As in MX, parameters are estimated by maximum likelihood under the assumption of multivariate normality (41). Approximate SIs were generated using a 1-LOD drop approach.

**ELECTRONIC DATABASE INFORMATION**

MAP-O-MAT, http://compgen.rutgers.edu/mapomat
R project, http://www.r-project.org

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**Conflict of Interest statement.** None declared.

**REFERENCES**


