A Genome Scan for Epidermal Skin Pattern in Adolescent Twins Reveals Suggestive Linkage on 12p13.31

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Deterioration in stratum corneum reticular patterning (skin pattern or skin wrinkling) has been associated with increased rates of solar keratoses and skin cancer. A previous analysis of data from the twin sample used in this investigation has shown that 86% of the variation in skin pattern is genetic at age 12 and 62% in an adult sample (mean age = 47.5). Variation due to genetic influences is likely to be influenced by more than one locus. Here, we present results of a genome-wide linkage scan of skin pattern in adolescent twins and siblings from 428 nuclear twin families. Sib-pair linkage analysis was performed on skin pattern data collected from twins at age 12 (378 informative families) and 14 (316 families). Suggestive linkage was found at marker D12S397 (12p13.31, logarithm of the odds (lod) 1.94), when the effect of the trait locus was modelled to influence the skin pattern equally at both ages 12 and 14. In the same analysis, a peak was seen at 4q23 with a lod score of 1.55. A possible candidate for the peak at 12p13.31 is the protease inhibitor, α-2-macroglobulin.

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INTRODUCTION

Three studies have shown a moderate but significant association between rates of skin cancer and loss of evenness and clarity in epidermal reticular patterning (Beagley and Gibson, 1980; Holman *et al.*, 1984; Green, 1991). Deterioration of the fine reticular patterning of the stratum corneum layer of the epidermis occurs over time (Lavker *et al.*, 1980). To quantify this deterioration, Beagley and Gibson (1980) created a system for scoring silicone impressions of the stratum corneum layer of the epidermis. Using this scoring system, several studies have sought to determine the factors that influence skin pattern deterioration. Seddon *et al.* (1992) and Fritschi *et al.* (1995) found skin pattern deterioration to correlate approximately 0.2 with dermal elastosis, a form of cutaneous damage caused by ultraviolet radiation (Kligman, 1989). From this correlation, Seddon *et al.* (1992) concluded that skin pattern deteriorates as part of the natural ageing process. Conversely, Fritschi *et al.* (1995) believed that this correlation confirmed the Beagley–Gibson score's validity as a measure of photoageing. Seddon *et al.* (1992) showed that silicone mould scores correlate with age, but not sun exposure. Subsequently, Leung and Harvey (2002) confirmed that although sun exposure and age both explained deterioration in skin pattern over time, age was the better predictor of the two.

To estimate the extent to which changes to skin pattern are due to genetic or environmental influences, we studied 714 twin pairs between the ages of 12 and 14. Of the variation in skin patterning at age 12, 86% was found to be due to additive genetic influences, of which 12% was due to skin color. Reported sun exposure explained 0.9% of the variation in epidermal skin pattern at age 12, but increased to 3.4% by age 14. The percentage of variation in epidermal skin pattern at age 14 explained by additive genetic influences was 75%, with skin color explaining 6.9% of the total variation at this age (Shekar *et al.*, 2005). These results suggest that sun exposure may still influence skin pattern, but that its effect is modified by genetic factors. The sources of the majority of additive genetic influences are yet to be identified. Here we perform linkage analysis to determine the genomic regions that cause variation in skin pattern. In addition to analyzing the scores of silicone moulds gathered at ages 12 and 14 independently, an analysis combining the data collected at the two ages was performed.

RESULTS

In all, 50 silicone impressions were rescored by the same technician 9 months after the initial scoring. The interoccasion correlation of silicone mould scores was 0.87 (95% confidence interval: 0.67, 0.97), so the point estimate for intrarater measurement error is 0.13. The polychoric correlation between skin patterns at ages 12 and 14 for 768 twin

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Abbreviations: IBD, identical by descent; lod, logarithm of the odds QTL, quantitative trait loci

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individuals was 0.70 (95% confidence interval: 0.64, 0.74), and disattenuated for measurement error was 0.80 (95% confidence interval: 0.73, 0.85).

Multipoint variance components linkage analysis

The logarithm of the odds (lod) scores for each of the 761 autosomal markers under the three analyses are shown in Figure 1. The lod score indicates how well allelic similarity at any given marker location between related individuals explains their phenotypic similarity. Markers that produced lod scores greater than 1.2 are listed in Table 1. Under the univariate analysis of skin pattern at age 12, there are three locations with a lod score above 1.2. The highest of these was at 12p13.31 with a lod score of 1.34, followed by 6q24.3 (lod 1.27) and 4q21.21 (lod 1.23) (Table 1). In the age 14 analysis, there were only two peaks with a lod score above 1.2. In both these cases, the equivalent lod score for the age 12 univariate analysis was less than 0.05. It is expected that the addition of siblings in the analysis of skin pattern at age 12 should increase power to detect a quantitative trait locus (QTL) effect from the greater number of quasi-independent sib pairs. Linkage analysis with the QTL effect loading equally on skin pattern at age 12 and 14 (bivariate) resulted in three linkage peaks with a lod score greater than 1.3. The highest peak, which was suggestive of linkage, was for marker D12S397 (12p13.31), with a lod score of 1.94 (Figure 2). The empirically derived, pointwise probability value for this peak, as obtained from the distribution of log-likelihoods from fitting the simulated data, was 0.00167. This is consistent with the asymptotic *P*-value of 0.0014 associated with a lod score of 1.94. At this location, information from the marker is estimated to explain 38% of the variation in skin patterns at ages 12 and 14. However, this may be overestimated as a result of the association between the estimated effect size and statistical significance (Goring *et al.*, 2001). Comparing lod scores across analyses for the peak at 12p13.31, power appears to be gained from performing a bivariate analysis, which is important for future collection strategies for ordinal traits (see also Neale *et al.*, 1994). Marker D4S1647 (4q23) had a lod score of 1.55 under the bivariate model, below the threshold of suggestive linkage.

Table 1. Cytogenetic locations with lod scores above 1.2 (in bold) for univariate analyses at ages 12 and 14 and the bivariate analysis of epidermal skin pattern

Cytogenetic location		Marker	Age 12 lod	Age 14 lod	Bivariate lod
1p21.1	[	D1S1631	0.01	**1.37**	1.09
		D1S2726	0.02	**1.25**	0.95
4q21.21	[	D4S2964	**1.21**	0.03	0.68
		D4S3243	**1.23**	0.03	0.68
4q23	[	D4S1647	0.89	0.01	**1.55**
4q24		D4S1572	0.48	0.00	**1.31**
6p22.3		D6S289	**1.24**	0.08	0.97
6q24.3		GATA184A08	**1.27**	0.00	0.32
8p23.1		D8S550	0.00	**1.20**	0.48
9q34.3		D9S1838	0.31	0.81	**1.50**
12p13.31	[	D12S99	**1.23**	0.61	**1.47**
		D12S397	**1.34**	0.87	**1.94**
		D12S336	0.77	0.87	**1.60**

Adjacent markers within the same linkage peak are bracketed.

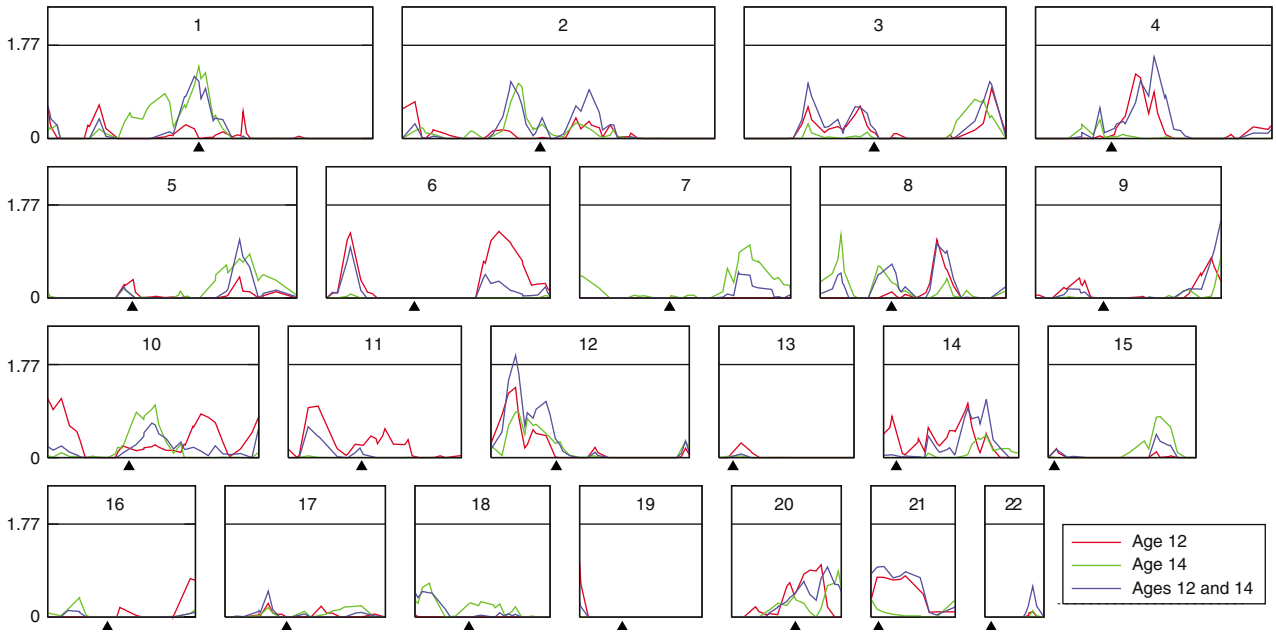

Figure 1. Linkage analysis of skin pattern data collected at ages 12 and 14, and for a bivariate analysis of twins at ages 12 and 14. The *x*-axis is the chromosomal position (▲ marks centromere). The *y*-axis is the lod score with the suggestive threshold score, 1.77, indicated.

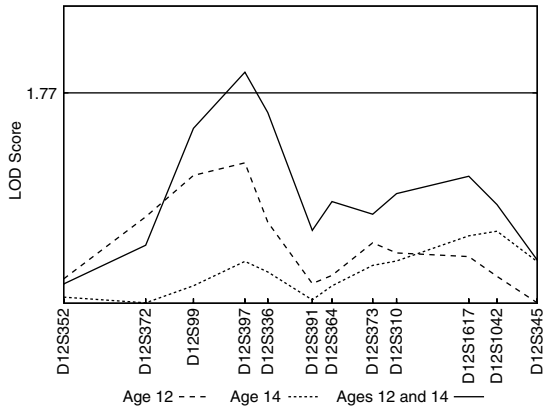

Figure 2. Linkage analysis of skin pattern for the p arm of chromosome 12.

DISCUSSION

This is the first linkage study of epidermal reticular patterning. Although no linkage peak exceeds the level indicating significance, the result for marker D12S397 at 12p13.31 (lod 1.94) shows suggestive linkage when a QTL is modelled to influence the skin patterns at both ages 12 and 14 equally. The suggestive lod score, according to simulations conducted by Wiltshire *et al.* (2002), is 1.77. The next highest peak under the bivariate model, at marker D4S1647 at 4q23 (lod 1.55), did not reach this suggestive threshold. These results are consistent with variation being due to more than one locus.

We expect the genes causing variation in epidermal reticular patterning to be those related to the structure, or regulation, of the epidermis, particularly those that also relate to sun exposure. We kept this in mind when performing bioinformatic searches under the peaks on chromosomes 4 and 12. The transcription factor activator protein (Ap-1), NF-κB, and macrophage inhibitory factor are all induced by sunlight and increase the transcription of matrix metalloproteinases in both keratinocytes and fibroblasts (Fisher *et al.*, 2001; Kang *et al.*, 2001; Watanabe *et al.*, 2004). Matrix metalloproteinases, which are often overexpressed in malignant tumours (Hidalgo and Eckhardt, 2001), break down collagen, which is the major structural component of the dermis. Genes for matrix metalloproteinases are found throughout the genome.

NF-κB is involved in regulating keratinocyte differentiation and proliferation as well as epidermal carcinogenesis (Takao *et al.*, 2003; Ridky and Khavari, 2004). The gene for NF-κB subunit 1 resides at 4q23. Both NF-κB and the Beagley–Gibson scale of skin pattern deterioration have been associated with nonmelanotic skin cancers (Holman *et al.*, 1984; Green, 1991; Ridky and Khavari, 2004). Further along the q arm of chromosome 4, at 4q25, is the gene for epidermal growth factor, a mitogenic factor that also promotes epithelial cell proliferation. A polymorphism in the epidermal growth factor gene that increases epidermal growth factor production has been associated with an increased risk of malignant melanoma (Shahbazi *et al.*, 2002). Also at 4q25 is the gene for lymphoid enhancer factor 1, which is present in the ectoderm during skin development (Zhou *et al.*, 1995). lymphoid enhancer factor 1, which has been linked to the Wnt pathway, forms complexes with β-catenin, a protein that accumulates in melanoma (Rubinfeld *et al.*, 1997; de Lau and Clevers, 2001).

Alpha-2-macroglobulin, a major matrix metalloproteinase (Woessner, 1999) and protease inhibitor (Bergqvist and Nilsson, 1979), has been mapped to 12p13.31 (Fukushima *et al.*, 1988). It has the ability to mediate the degradation of A-β, a component of amyloid β deposits that are deposited just below the dermal, epidermal junction (Joachim *et al.*, 1989; Blacker *et al.*, 1998). Both α-2-macroglobulin and its receptor are localized to dermal fibroblasts and dendritic cells (Feldman and Sangha, 1992). Further research is required to determine the genetic variations at 12p13.31 and 4q23 that influence the rate of skin pattern deterioration. We are currently genotyping a further 170 families with a 100k single-nucleotide polymorphism chip which will enable simultaneous, genomewide, linkage and association analysis.

MATERIALS AND METHODS

Samples

The data for the adolescent cohort used in this study were collected as part of a longitudinal study investigating the development of melanocytic naevi (mol). Twins were enlisted through contacting principals of primary schools in the greater Brisbane area, through word of mouth and a range of media. The twins who registered their interest were contacted and participation was conditional upon the informed consent of the twins and their parents. Details of the clinical protocol are described in McGregor *et al.* (1999) and Zhu *et al.* (1999). Approval to undertake this study was granted by the Human Research Ethics Committee of the Queensland Institute of Medical Research. The study was conducted according to the Declaration of Helsinki Principles. Of the twin pairs used for the genetic analysis in Shekar *et al.* (2005), a subsample of twins and their siblings was genotyped for linkage analysis (Table 2). The non-twin siblings were, on average, older than the twins by approximately 2 years.

Silicone mold impression grades

Silicone mold impressions of the back of the hand were collected as close to the twins' 12th and 14th birthdays as possible. Silicone mould impressions for the non-twin siblings were collected at the same time as the twins' age 12 visit. Affinis light-body silicone elastomer (manufactured by Coltène AG, Altstätten, Switzerland) was used to take an impression of the dorsum of the left hand, which was held in a relaxed position by loosely gripping a cardboard cylinder (for more details, see Sarkany, 1962; Sarkany and Caron, 1965; Barnes, 1973; Battistutta, 1998). The silicone impressions of the skin were scored according to the Beagley–Gibson rating using a low-power dissecting microscope. The Beagley–Gibson method classifies skin pattern impressions into six categories, depending upon the evenness, clarity and depth of primary and secondary lines, with higher scores indicating greater epidermal skin pattern deterioration (Holman *et al.*, 1984).

Families of monozygotic twins with one or more siblings (Evans and Medland, 2003) and additional siblings in families of dizygotic twins (Dolan *et al.*, 1999) add significant power to detect QTL.

Table 2. Categorization of families in the analysis of skin pattern at age 12 according to the number of siblings and parental genotype information

	Number of parents genotyped			
	0	1	2	Total families
Monozygotic twins, no siblings[1]	13	5	21	39
Monozygotic twins with one sibling	1	4	24	29
Monozygotic twins with two siblings	0	1	5	6
Dizygotic twins[2]	24	52	204	280
Dizygotic twins with one sibling	1	8	40	49
Dizygotic twins with two siblings	1	5	8	14
	40	75	302	417

At age 14, there were 399 families (including families of MZ twins) with 316 quasi-independent sib pairs.
[1]These twins are not informative for linkage, but are included in the analysis to assist in the resolution of additive genetic (A) and common environmental (C) influences.
[2]Four of these families contain one twin individual with a sibling.

Although monozygotic twins and families with one individual do not contribute linkage information, they increase the information used to estimate threshold parameters, and so enhance the stability of the model. Inclusion of monozygotic twins also enables residual familial variance to be dichotomized into additive genetic and common environmental sources, and provides an upper bound to the estimate of the linked QTL variance (Q) (Evans and Medland, 2003). While there were three families with three nontwin siblings, the univariate linkage analysis at age 12 only included up to two siblings per family to avoid numerical problems. The univariate analysis of skin pattern data collected at age 12 included 961 individuals, both twins and siblings, with phenotype and genotype information. These 417 families yielded 558 informative, sibling–sibling relationships, or quasi-independent sibling pairs (Table 2).

Zygosity testing

Venous blood samples were collected from twins, siblings and parents into ethylene diamine tetraacetic acid tubes. Deoxyribonucleic acid was extracted from buffy coats subsequent to salting out cellular proteins (Miller *et al.*, 1988). Zygosity of the twin pairs was established by typing them using the ABI Profiler Plus™ marker set consisting of nine highly polymorphic deoxyribonucleic acid microsatellite markers and the amelogenin sex marker. The probability of dizygosity given concordance at all markers was less than 10^{-4}. Zygosity was further confirmed in the genome scans, as below.

Genotyping

Two major genome scans of parents, twins, and siblings were carried out, at the Australian Genome Research Facility, Melbourne (Ewen *et al.*, 2000), and the Center for Inherited Disease Research, Baltimore (Weeks *et al.*, 2002). The intercalated genome scans resulted in a 796 marker map with an average intermarker spacing of 4.8 cM in Kosambi units (Zhu *et al.*, 2004). The data were cleaned for incorrect relationships between individuals, Mendelian inconsistencies, and improbable double recombinants as detailed elsewhere (Zhu *et al.*, 2004). Approximately 36% of individuals were genotyped in both the Center for Inherited Disease Research and Australian Genome Research Facility scans, these being mainly from families without genotyped parents; between 211 and 791 markers were genotyped per individual (Zhu *et al.*, 2004).

As very few of our subjects were scored in the lowest or highest categories, the data for skin pattern were recoded from six to four categories for twins and siblings (the first two categories were collapsed and the last two categories were collapsed). The data were analyzed using the multifactorial threshold model, which assumes that skin patterning is a continuous, normally distributed variable that has been divided into ordered categories by the rater. We estimate thresholds that cut into the underlying normal distribution according to the proportion of individuals in each category. Correlations under the threshold model take this into consideration such that the joint distribution of the liabilities for a pair of relatives is bivariate normal (Reich *et al.*, 1979; Martin *et al.*, 1988).

The correct specification of the threshold model is important in linkage analyses, where phenotypes between related individuals are compared to their genetic similarity. The threshold parameters for males were displaced by a constant from their female counterparts to account for the greater deterioration in epidermal reticular patterning in males (Shekar *et al.*, 2005). Although there was no influence of age when performing genetic analysis within age 12 twins, the increased age range with the addition of siblings necessitated the inclusion of regression on age in the threshold model. A quadratic regression on age did not significantly contribute to the threshold model ($\chi^2_1 = 0.03$).

Identity by descent estimation

When two offspring in a family receive the same allele from one parent, then those siblings share the parent's allele at that location identical by descent (IBD). Sibling pairs receive haplotypes from both parents and so can share 0, 1 or 2 alleles IBD at each locus. If variation at a genomic location is causing variation in skin pattern, then IBD similarity between siblings for that locus, and hence the nearby marker(s), will be related to skin pattern similarity between those siblings. Multipoint IBD was estimated at each of the 761 autosomal markers for all genotyped siblings using Merlin 0.10.1 (Abecasis *et al.*, 2002), which estimates marker allele frequencies from the observed sample. To more accurately estimate IBD between siblings, 81% of parents were genotyped (but not phenotyped). The genetic map was based on that of Kong *et al.* (2004).

We used variance components and maximum likelihood estimation to decompose variation in silicone mould scores into environmental and genetic sources, with the latter including variance due to a hypothetical QTL located at each of the autosomal markers in turn. Covariance between siblings for a linked QTL, Q, is conditioned by $\hat{\pi}$,

which is the proportion of alleles shared IBD at the trait locus between siblings (Sham, 1998): $\hat{\pi}=\frac{1}{2}\Pr[\text{IBD1}]+\Pr[\text{IBD2}]$. The path coefficient for the additive genetic QTL effect (q) is a function of the recombination fraction between the marker and the trait locus, as well as the magnitude of the genetic influence at that trait locus. Although monozygotic twins were included to partition familial aggregation into additive genetic and common environmental influences, the latter could be removed as a source of variation in skin pattern at ages 12 and 14, without a significant drop in the fit of the model. Residual additive genetic influence (a) is modelled such that the expected phenotypic covariance between siblings and dizygotic twins is $\hat{\pi}q^2+\frac{1}{2}a^2$, where $\hat{\pi}$ for each pair is calculated from the IBD values estimated in Merlin. The expected covariance between monozygotic twins is q^2+a^2. The expected phenotypic covariance matrix, $\sum$, is partitioned into: $\hat{\Pi}\sigma_q^2+2\Phi\sigma_a^2+\mathrm{I}\sigma_e^2$, such that $\hat{\Pi}$ is a matrix of estimated $\hat{\pi}$ between individuals i and j, Φ is a matrix of kinship coefficients between individuals i and j, I is the identity matrix, and the three $\sigma^{2\prime}$s indicate the additive genetic variance caused by the linked QTL (q), the residual additive genetic variance (a), and the unique environmental variance (e), respectively (Posthuma *et al.*, 2003). In the bivariate model, a single common QTL factor was modelled such that its influence on skin pattern at age 12 was the same as its influence on skin pattern at age 14. The additive genetic influence on skin pattern at age 14 was decomposed into that influencing skin pattern at age 12 and that unique to itself.

The computer program Mx (version 1.54; Neale *et al.*, 1994) was used to estimate the parameters for thresholds and variance components. The test for linkage at a particular marker is the statistical significance of σ_{q}^2. The difference in log likelihoods between the model where σ_{q}^2 is free and the model where σ_{q}^2 is zero is distributed as a $\frac{1}{2}:\frac{1}{2}$ mixture of χ_1^2 and a point mass at zero and designated $\chi_{0,1}^2$ (Self and Liang, 1987). Dividing this difference in log likelihoods by $2\ln 10$ (≈ 4.6) produces a lod score equivalent to that for parametric linkage analysis (Williams and Blangero, 1999). Since the effect of the QTL factor on skin pattern was constrained equal at ages 12 and 14 in the bivariate analysis, all analyses had the same test for linkage, dropping a single estimated parameter. Although marker information was available for the X chromosome, linkage analyses were performed on the autosomal markers due to current limitations of the computer program Mx.

To determine whether the link between genetic information at a particular marker and skin pattern is significant, the threshold P-value of 0.05 needs to be adjusted for the 761 tests performed in the linkage analysis, that is, a level above which a linkage peak is expected once in every 20 genome scans. Wiltshire *et al.* (2002), using locus-counting methods on simulated data, have suggested that for a 5 cM scan, a lod score of 1.77 is expected once every scan and a lod score of 3.17 would be expected once every 20 genome scans. For the highest linkage peak, 3000 simulations of the chromosome under a hypothesis of no linkage using the marker informativeness, spacing, and missing data patterns observed in the data sample were used to obtain an empiric, pointwise P-value. The simulations were generated using the –simulate option in Merlin 0.10.1.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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