# Linkage and Association Analysis of Radiation Damage Repair Genes XRCC3 and XRCC5 with Nevus Density in Adolescent Twins

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Previous studies have shown that a deficiency in DNA damage repair is associated with increased cancer risk, and exposure to UV radiation is a major risk factor for the development of malignant melanoma. High density of common nevi (moles) is a major risk factor for cutaneous melanoma. A nevus may result from a mutation in a single UV-exposed melanocyte which failed to repair DNA damage in one or more critical genes. XRCC3 and XRCC5 may have an effect on nevus count through their function as components of DNA repair processes that may be involved directly or indirectly in the repair of DNA damage due to UV radiation. This study aims to test the hypothesis that the frequency of flat or raised moles is associated with polymorphism at or near these DNA repair genes, and that certain alleles are associated with less efficient DNA repair, and greater nevus density. Twins were recruited from schools in south eastern Queensland and were examined close to their 12th birthday. Nurses examined each individual and counted all moles on the entire body surface. A 10cM genome scan of 274 families (642 individuals) was performed and microsatellite polymorphisms in XRCC3 and adjacent to XRCC5 were also typed. Linkage and association of nevus count to these loci were tested simultaneously using a structural-equation modeling approach implemented in MX. There is weak evidence for linkage of XRCC5 to a QTL influencing raised mole count, and also weak association. There is also weak evidence for association between flat mole count and XRCC3. No tests were significant after correction for testing multiple alleles, nor were any of the tests for total association significant. If variation in XRCC3 or XRCC5 influences UV sensitivity, and indirectly affects nevus density, then the effects are small.

The presence of melanocytic nevi is the strongest established risk factor for malignant melanoma (Swerdlow & Green, 1987). Common moles can be categorized according to their profile as either flat (junctional or compound) or raised (intradermal) (Green & Swerdlow, 1989). However, whether there is an etiological relationship between raised and flat moles is far from clear, and it is also unclear whether one type is more strongly related to melanoma risk (Schmoeckel, 1997; Worret & Burgdorf, 1998).

One of the major environmental risk factors for moles is solar ultraviolet radiation (Hofmann-Wellenhof et al., 1998; Husain et al., 1991; Longstreth, 1988). 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; Luther et al., 1996). It is also known that there is familial aggregation for moliness, and we have estimated heritabilities of 0.42 for flat mole count and 0.69 for raised mole count in a sample of 352 pairs of 12-year-old twins, on whom the current report is also based (Zhu et al., 1999).

In speculating on which particular genes might contribute to the high heritability of mole count, it seems reasonable to ask whether polymorphism in genes involved in radiation sensitivity and radiation damage repair might play a role. In particular, we consider the two X-ray damage repair genes, XRCC3 and XRCC5. XRCC3 (MIM 600675; X-ray repair, Complementing defective, in Chinese hamster, 3) was originally identified as a human gene able to complement DNA damage sensitivity, chromosomal instability and impaired growth of the mutant hamster cell line irs1SF. Recently, it has been cloned, sequenced and found to bear sequence homology to the highly conserved eukaryotic repair and recombination gene RAD51. The phenotype of irs1SF and the identification of XRCC3 as a member of the RAD51 gene family have suggested a role for XRCC3 in repair of DNA damage by homologous recombination. Homologous recombinational repair (HRR) of a specifically induced chromosomal double-strand break (DSB) was assayed in irs1SF cells with and without transient complementation by human XRCC3. Complementation with XRCC3 increased the frequencies of repair by 34 to 260-fold. A recent study confirmed a role for XRCC3 in HRR of DNA DSB, and the importance of this repair pathway for the maintenance of chromosomal integrity in mammalian cells (Brenneman et al., 2000).

XRCC5 (MIM 194364; X-ray repair, Complementing defective, in Chinese hamster, 5) is important for the fast

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repair of potentially lethal damage (PLD) in rat cells (Hayashi, 2001) XRCC5 is assigned to human chromosome 2q35 (Chen et al., 1994). A deficiency in DNA repair is associated with increased cancer risk, and exposure to UV radiation is a major risk factor for the development of malignant melanoma. DNA damage caused by UV radiation is thought to play a major role in carcinogenesis. Multiprotein pathways involved in repairing UV-DNA damage include base excision repair, nucleotide excision repair, and homologous double-strand break repair pathways. A recent study found that the presence of a T allele at position 18067 in exon 7 of the XRCC3 gene was significantly associated with melanoma development, suggesting a role for homologous recombination repair in UV damage (Winsey et al., 2000). Significant associations of XRCC3 with cancer status and clinical radio-sensitivity have been reported but there was no evidence of association for XRCC5 (Price et al., 1997). DNA repair defects are presumed to have a genetic origin and to be associated with polymorphic alleles in subgroups of the population (Berwick & Vineis, 2000; Mohrenweiser & Jones, 1998). The complementary nature of HRR and non-homologous end joining in dealing with double strand breaks may implicate variations in XRCC5 activity in UV associated repair also.

The present study aims to investigate the relationship between these genes and mole counts. We test the hypotheses that there are associations between Short Tandem Repeat (STR) polymorphisms in or near XRCC3 and XRCC5 genes and the number of moles in adolescent MZ and DZ twins. We were encouraged to do this by a recent observation (unpublished) of an inverse correlation between mitotic recombination frequency and the length of the longer STR polymorphism for both XRCC3 and XRCC5 in a population of healthy young adults. Mitotic recombination is a by-product of HRR. We also make use of the DZ twins to test simultaneously for linkage of mole counts with XRCC3 and XRCC5, in case there are other polymorphisms of major effect not in linkage disequilibrium with the typed STRs.

## Subjects and Methods

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, most parents and some sibs 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 (McGregor et al., 1999; Zhu et al., 1999). Details of mole counts and risk factors used as covariates in our analyses, including sun exposure and pigmentation, are given in Zhu et al. (1999). Zygosity was determined by typing nine highly polymorphic DNA microsatellite markers (ABI Profiler system) and three blood groups (ABO, MNS and Rh).

### Genotyping and IBD Estimation

DNA was extracted by use of standard methods (Miller et al., 1988). A genome scan of the first 274 families (642 individuals) from the Brisbane Twin Nevus study has recently been completed using the ABI-2 set of 400 microsatellite markers spaced at an average 10cM (Gyapay et al., 1994; Weissenbach et al., 1992). In addition we typed a (CA)n repeat polymorphism in intron 3 of XRCC3 (GenBank Accession number AF000735) and a further microsatellite polymorphism (tetranucleotide with compound repeats; GenBank Accession number: AF000736) about 120kb 5' of XRCC5 on 2q (Price et al., 1997). The polymorphism close to XRCC5 was analyzed using ALLEGRO (Gudbjartsson et al., 2000) with the other 30 markers from the genome scan on chromosome 2 to obtain the multipoint IBD sharing probabilities at the polymorphism close to XRCC5, and similarly for XRCC3 with 14 extra markers on chromosome 14. The estimated proportion of alleles shared IBD at the marker locus in a pair of sibs is then computed as $\hat{\pi} = p_2 + 0.5p_1$, where $p_2$ is the probability that the sib pair share both alleles identical by descent at the marker locus and $p_1$ is the probability that they share one allele IBD. For a given sib pair, $\hat{\pi}$ is used as the coefficient for the variance component due to the linked quantitative trait locus (QTL) effect in structural equation modeling of raw data (Almasy & Blangero, 1998; Amos, 1994; Eaves et al., 1996).

### 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 (Martin et al., 1987). There are two parts to the model: (1) a model for the expected values of individual observations in terms of measured fixed effects such as age, sex, and other risk factors (the "model for the means"); (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. We test whether there is a gene of major influence on mole count linked to XRCC3 (or XRCC5), by fitting a path model to the raw data in which we condition the DZ/sib covariance on the estimated proportion of alleles shared IBD ($\hat{\pi}$) at the marker locus (Zhu et al., 1999). The likelihood for each family under the model is calculated and then summed over all twin families, MZ and DZ. Including older non-twin siblings in the analysis with the 12-year-old twins makes it vital to describe accurately the relationship between the phenotype (mole count) and age, so we include both linear and quadratic age regression terms in the model for the means. We also include in the model for the means, deviations for each allele at the XRCC3 repeat polymorphism, and for the repeat polymorphism close to XRCC5. Since we estimate allelic effects, we test only an additive model, but it is possible to elaborate this to models with various types of allelic interaction (dominance).



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### Other Linkage and Association Analysis Methods

Where there was any evidence for linkage or association detected by Mx analysis, we used other programs to seek confirmation. The program Sib-pair (Duffy, 1995) performs Haseman-Elston regressions for all marker loci versus the trait using full and half-sib pairs. The contribution of each pair can be weighted by the mean of their values for a quantitative trait. For association, the empirical *p*-value is estimated by comparing the observed test statistic for association with the empirical null distribution obtained by simulation using "gene dropping" (Monte-Carlo) of unassociated alleles.

The program Quantitative Transmission Disequilibrium Test (QTDT) (Abecasis et al., 2000) can use all the information in a pedigree to construct powerful tests of association that are robust in the presence of stratification and was also used for analyzing our data. It builds on recently developed methods for linkage disequilibrium mapping of quantitative traits to construct a general approach that can accommodate nuclear families of any size, with or without parental information. Variance components are used to construct a test that utilizes information from all available offspring but that is not biased in the presence of linkage or familial relatedness.

## Results

### Linkage Analysis

Complete genotype and phenotype data were available for 36 MZ twin pairs and 238 DZ twin pairs. To increase the number of sib pairs available for linkage analysis, 125 siblings (aged 9–23 years) from 101 twin families were also phenotyped and genotyped. In our twin sample, raised moles comprised 21.7% of the mean total mole count, and the correlation between raised and flat moles (both on log scales) was 0.35. Since we cannot assume that these two nevus types have the same etiology, we performed separate analyses.

We hypothesised that some of the genetic variance in mole count might be due to variation in the DNA repair genes XRCC3 and XRCC5. QTL linkage effects for raised mole count were estimated from Mx for XRCC3 and XRCC5 at 10.5% and 13.1% of total variance respectively, but both effects could be dropped from the model without significant deterioration in fit (Table 1). By contrast, there was absolutely no evidence for any QTL effect on flat mole count linked to either XRCC3 or XRCC5; in either case, dropping Q from the model made no change to the fit statistic. These negative results were corroborated using the Haseman-Elston estimation of linkage in Sib-pair. The genome scan using Allegro is shown in Figure 1 for both chromosomes.

### Association Analysis

It is well known that the power of sib-pair linkage analysis to detect QTL's of modest effect size is very low (Risch, 1990), so our failure to detect significant QTL linkage to XRCC3 and XRXCC5 does not mean that variation at or near these genes is having no effect on variation in mole count — merely that we do not have the power to detect it. It is quite possible that there are effects of modest size at these loci which might be detected by association analysis, specifically by linkage disequilibrium with polymorphic markers at or close to these X-ray damage repair genes. To guard against spurious associations due to population stratification, some of the association tests we want to employ are within-family tests so, for consistency, we used only DZ twin pairs with complete genotypic and phenotypic data — 211 pairs for XRCC3 and 205 pairs for XRCC5. We first tested for association in Mx by adding to the means model a deviation for each allele in turn, with effect size constrained equal for maternal and paternal

**Table 1**

Variance Components (% variance) and 95% CI for Log Raised and Flat Mole Counts After Model Fitting to Raw Observations for Twins and Siblings

| Mole Type | QTL | Polygenic | Family Environment | Individual Environment |
|---|---|---|---|---|
| XRCC3 | | | | |
| Raised moles | 10.5 | 63.6 | 6.9 | 19.0 |
| 95% CI | (0.0–35.1) | (32.2–86.9) | (0.0–24.5) | (12.5–30.0) |
| Drop $Dc^2_1$= | 0.69[a] | 21.2[b] | 0.6[c] | — |
| XRCC5 | | | | |
| Raised moles | 13.1 | 60.6 | 7.3 | 19.1 |
| 95% CI | (0.0–33.9) | (31.3–86.1) | (0.0–24.9) | (12.5–30.1) |
| Drop $Dc^2_1$= | 1.63 | 21.9 | 0.6 | — |
| Both genes | | | | |
| Flat moles | 0.0 | 22.4 | 51.1 | 26.5 |
| 95% CI | — | (0.0–43.9) | (35.2–66.8) | (17.4–40.9) |
| Drop $Dc^2_1$= | — | 2.5 | 33.9 | — |

Note: All models include mean effects for sex, age, age$^2$ and six risk factors – body surface area, sun exposure, sunburn, skin colour, hair colour and freckling. The significant risk factors are freckling for raised ($\chi^2_1$ = 16.31) and flat ($\chi^2_1$ = 12.42) moles, skin colour for raised ($\chi^2_1$ = 46.43) and flat ($\chi^2_1$ = 19.74) moles, hair colour ($\chi^2_1$ = 31.17) for raised moles, and BSA ($\chi^2_1$ = 19.06) for flat moles; components of variance include that due to a linked QTL (Q), residual additive polygenetic variance (A), common family environment (C) and individual environment (E).

[a] ACE vs. ACEQ model.

[b] CE vs. ACE model.

[c] AE vs. ACE model.



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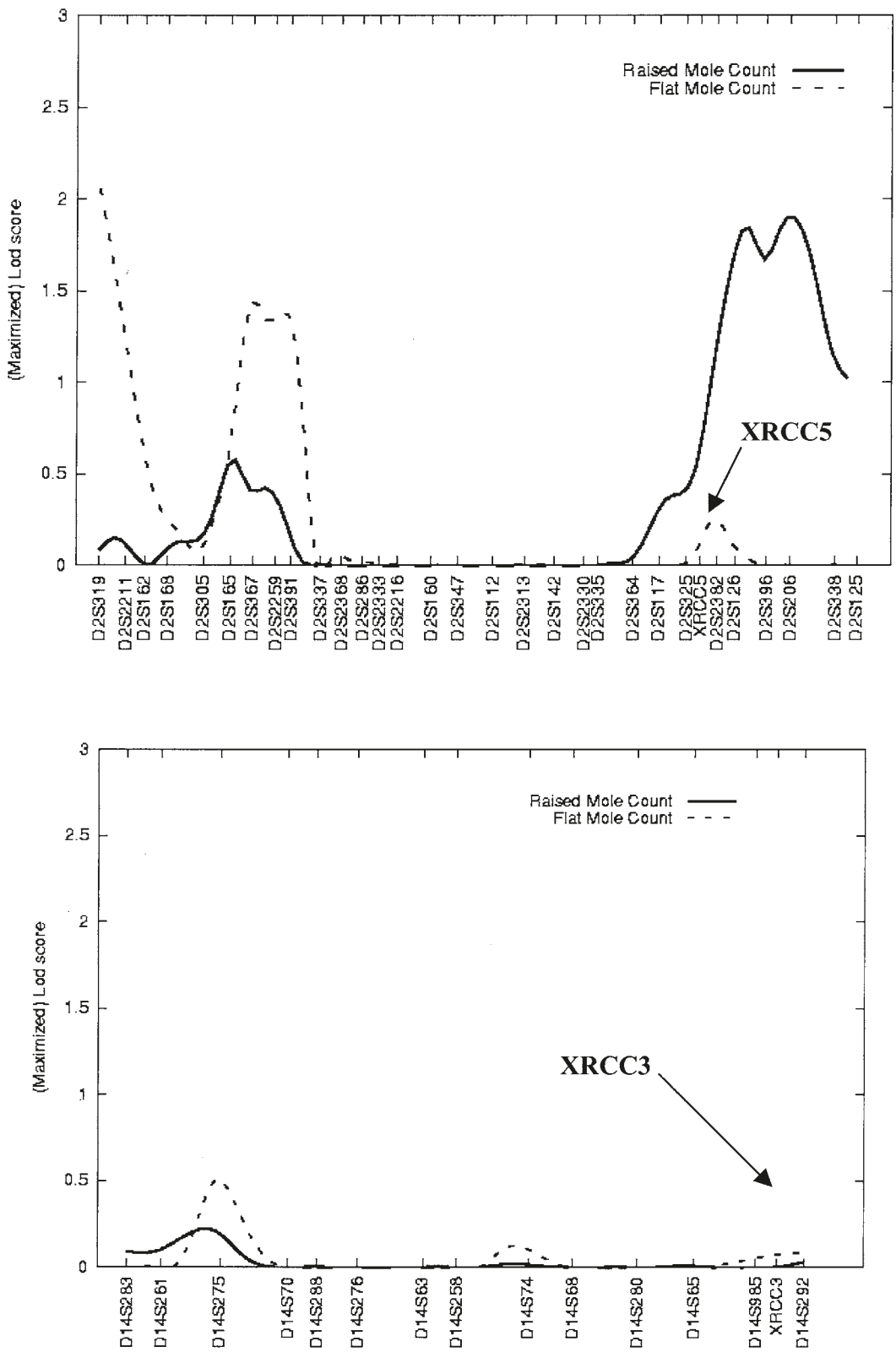


**Figure 1**

QTL-scan for linkage on Chromosome 2 and 14 with all available markers. Positions of XRCC 5 (chromosome 2) and XRCC 3 (chromosome 14) are indicated.



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**Table 2**

Testing of Association of Mole Count to XRCC3 and XRCC5 Alleles Using 3 Different Statistical Methods. (A Total of 211 Complete DZ Pairs Were Used in These Analyses.)

| | | | | Program Used for Association Test | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Flat Mole Count | | | | MX | | Sib-pair | | QTDT | |
| | | Alle_freq | Count | $\chi^2$ | $p$ | $\chi^2$ | $p$ | $\chi^2$ | $p$ |
| **XRCC3** | **[AC]n** | | | | | | | | |
| allele_1 | 14 | 0.3270 | 276 | 0.00 | 1.00 | 0.00 | 1.00 | 0.00 | 1.00 |
| allele_2 | 15 | 0.0415 | 35 | 2.12 | 0.15 | 1.89 | 0.17 | 2.25 | 0.13 |
| allele_3 | 16 | 0.4893 | 413 | 1.10 | 0.30 | 0.11 | 0.74 | 0.67 | 0.41 |
| allele_4 | 17 | 0.0391 | 33 | 0.16 | 0.69 | 0.01 | 0.91 | 0.14 | 0.71 |
| allele_5 | 18 | 0.0024 | 2 | 2.15 | 0.14 | 2.27 | 0.13 | 2.15 | 0.14 |
| allele_6 | 19 | 0.0344 | 29 | 0.48 | 0.49 | 0.11 | 0.74 | 0.13 | 0.72 |
| allele_7 | 20 | 0.0628 | 53 | 0.65 | 0.42 | 2.80 | 0.09 | 1.18 | 0.28 |
| allele_8 | 21 | 0.0036 | 3 | **3.31** | **0.07** | **6.17** | **0.01** | **4.20** | **0.04** |
| Total association[a] | | | | 2.33 | 0.94 | 16.62 | 0.02 | 4.69 | 0.20 |
| **XRCC5** | **[GAPyA]n** | | | | | | | | |
| allele_1 | 14 | 0.0062 | 5 | 1.05 | 0.31 | 0.00 | 1.00 | 0.53 | 0.47 |
| allele_2 | 14.5 | 0.0087 | 7 | **3.26** | **0.07** | **3.00** | **0.08** | **3.26** | **0.07** |
| allele_3 | 15 | 0.4054 | 328 | 2.02 | 0.16 | 0.27 | 0.61 | 1.35 | 0.25 |
| allele_4 | 15.5 | 0.0507 | 41 | 0.00 | 1.00 | 0.16 | 0.69 | 0.03 | 0.87 |
| allele_5 | 16 | 0.3684 | 298 | 0.27 | 0.60 | 0.19 | 0.67 | 0.06 | 0.81 |
| allele_6 | 16.5 | 0.0371 | 30 | 0.05 | 0.83 | 0.09 | 0.77 | 0.04 | 0.85 |
| allele_7 | 17 | 0.1162 | 94 | 0.69 | 0.41 | 0.21 | 0.65 | 0.57 | 0.45 |
| allele_8 | 17.5 | 0.0074 | 6 | 0.00 | 1.00 | 0.35 | 0.56 | 0.03 | 0.86 |
| allele_9 | 18 | 0.0136 | 11 | 0.18 | 0.67 | 0.19 | 0.67 | 0.13 | 0.72 |
| Total association[b] | | | | 4.74 | 0.79 | 6.68 | 0.57 | 1.70 | 0.64 |
| Raised Mole Count | | | | | | | | | |
| **XRCC3** | **[AC]n** | | | | | | | | |
| allele_1 | 14 | 0.3270 | 276 | 0.62 | 0.43 | 0.09 | 0.77 | 0.09 | 0.76 |
| allele_2 | 15 | 0.0415 | 35 | 0.79 | 0.37 | 0.09 | 0.77 | 0.73 | 0.39 |
| allele_3 | 16 | 0.4893 | 413 | 0.90 | 0.34 | 0.16 | 0.69 | 0.84 | 0.36 |
| allele_4 | 17 | 0.0391 | 33 | **3.57** | **0.06** | **1.20** | **0.27** | **3.47** | **0.06** |
| allele_5 | 18 | 0.0024 | 2 | 0.28 | 0.60 | 2.30 | 0.13 | 0.28 | 0.60 |
| allele_6 | 19 | 0.0344 | 29 | 1.74 | 0.19 | 0.00 | 1.00 | 1.72 | 0.19 |
| allele_7 | 20 | 0.0628 | 53 | 0.35 | 0.55 | 0.35 | 0.56 | 0.39 | 0.53 |
| allele_8 | 21 | 0.0036 | 3 | 0.00 | 1.00 | 0.06 | 0.80 | 0.00 | 1.00 |
| Total association[a] | | | | 5.20 | 0.64 | 5.99 | 0.54 | 2.51 | 0.47 |
| **XRCC5** | **[GAPyA]n** | | | | | | | | |
| allele_1 | 14 | 0.0062 | 5 | 0.17 | 0.68 | 0.01 | 0.91 | 0.15 | 0.70 |
| allele_2 | 14.5 | 0.0087 | 7 | 0.17 | 0.68 | 0.16 | 0.69 | 0.15 | 0.70 |
| allele_3 | 15 | 0.4054 | 328 | 0.24 | 0.63 | 0.89 | 0.34 | 0.21 | 0.65 |
| allele_4 | 15.5 | 0.0507 | 41 | **2.32** | **0.13** | **3.59** | **0.06** | **2.72** | **0.10** |
| allele_5 | 16 | 0.3684 | 298 | 0.05 | 0.83 | 0.00 | 1.00 | 0.04 | 0.84 |
| allele_6 | 16.5 | 0.0371 | 30 | 0.85 | 0.36 | 0.35 | 0.56 | 0.83 | 0.36 |
| allele_7 | 17 | 0.1162 | 94 | **3.81** | **0.05** | **5.28** | **0.02** | **3.70** | **0.05** |
| allele_8 | 17.5 | 0.0074 | 6 | 0.07 | 0.79 | 0.32 | 0.57 | 0.05 | 0.82 |
| allele_9 | 18 | 0.0136 | 11 | 0.62 | 0.43 | 0.11 | 0.74 | 0.56 | 0.45 |
| Total association[b] | | | | 13.44 | 0.10 | 6.83 | 0.56 | 4.20 | 0.24 |

Note:[a] For MX & Sib-pair, $df = 7$ and for QTDT $df = 3$.

[b] For MX & Sib-pair, $df = 8$ and for QTDT $df = 3$.

Allelic associations significant at the nominal level of 0.1 are shown in bold.



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alleles. The change in fit tests the significance of the allelic effect, and this is shown in Table 2 for each allele at both loci for both flat and raised moles. The strongest association between XRCC3 and raised mole count was seen at the 4th allele ($p$ = .06). For XRCC5 and raised mole count the 7th allele displayed the strongest association with ($p$ =.05). For flat mole count the 8th allele of XRCC3 and the second allele of XRCC5 displayed weaker evidence of association (both $p$ = .07) and these occurred in rarer alleles.

We also tested for quantitative trait association using the Sib-pair program using an additive model to predict an individual's trait value from their genotype at the marker locus. The residual sum-of-squares is compared to that obtained via a gene-dropping simulation of the pedigrees, giving an empiric $p$-value. By this method, association between XRCC3 and raised mole count at the 4th allele was not significant ($p$ = .27), but for XRCC5 the 7th allele was again significantly associated ($p$ = .02), and the 4th allele was marginally associated ($p$ = .06). For flat mole count the uncommon 8th allele of XRCC3 again displayed a significant association with ($p$ = .01) and the uncommon second allele of XRCC5 was marginally significant with ($p$ = .08). Allelic associations were broadly consistent between the MX and Sib-pair analyses. Seeking further confirmation we used QTDT which uses all the information in a pedigree to construct powerful tests of association and to test for the presence of stratification. The results from QTDT analysis were in close agreement with both the MX and Sib-pair findings (Table 2). Despite the consistency of association results between different programs, the nominally marginal significance of allelic effects disappears when Bonferroni correction for testing multiple alleles is made.

We can also test the total association effects of all alleles, by estimating all allelic deviations simultaneously. If there are $n$ alleles, $n$–1 deviations are estimated and so for MX and Sib-pair, the test of total association has 7 degrees of freedom. QTDT combines alleles with frequencies < .05, so the test of total association has only 3 degrees of freedom. These tests are also shown in Table 2 and there is no consistent evidence of association of either marker with either phenotype.

## Discussion

XRCC3 and XRCC5 are potential candidate genes for an effect on mole count through their function of repairing DNA damage. We used sib-pair linkage and association analysis to seek evidence for effects of variation in these genes on raised and flat mole counts. With only 274 twin families available, we have little power to detect any linked QTL accounting for less than 40% of total variance, so not surprisingly the point estimates for variance due to QTLs linked to XRCC3 and XRCC5 — 10% and 13% respectively — fall well short of significance.

That we find no significant evidence for linkage with either locus does not preclude the existence of more modest effect sizes that might be detected by association analysis. Polymorphisms found to be associated with mole count may be in disequilibrium with a causal variant nearby or, at least for XRCC3, could be the variant itself. Our association studies with the highly polymorphic microsatellites in XRCC3 and XRCC5 provide weak evidence of effects on nevus density, although their already marginal significance disappears when adjusted for testing of multiple alleles. That different alleles of the same locus are associated with raised and flat moles does not increase confidence in the robustness of the finding if there is any commonality in their etiology. To establish whether the associations we have observed are genuine will require their confirmation and we intend to extend our observations reported by collecting more samples to increase statistical power. Typing further polymorphisms in and close to the genes may also be warranted, especially since the maximized lod score for linkage to XRCC5 rises to around 2 just distal of the marker we typed (see Figure 1).

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