Cathryn Lewis
is senior lecturer in Genetic Epidemiology and Statistics at Guy's, King's and St. Thomas' School of Medicine. Her research interests are the localisation of genes for complex disorders through linkage and association.

Genetic association studies: Design, analysis and interpretation

Cathryn M. Lewis

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Abstract

This paper provides a review of the design and analysis of genetic association studies. In case control studies, the different contingency tables and their relationships to the underlying genetic model are defined. Population stratification is discussed, with suggested methods to identify and correct for the effect. The transmission disequilibrium test is provided as an alternative family-based test, which is robust to population stratification. The relative benefits of each analysis are summarised.

Keywords: *case control study, TDT, population stratification*

INTRODUCTION

The Human Genome Project has generated a wealth of data that will determine the genetic contribution to common human disorders. Genetic studies have already proved highly successful in cloning genes for simple Mendelian diseases, such as cystic fibrosis, Huntington's disease and many rare syndromes, but progress has been slower in complex diseases. This class of diseases covers a broad spectrum of human health, including inflammatory bowel disease, asthma and heart disease, where several genes are likely to control disease risk, and gene–gene or gene–environmental interactions may be important. Identifying the genetic contributions to complex diseases will lead to advances in diagnosis and therapy (especially pharmacogenomics) and with far-reaching implications for public health.

The major tools from the Human Genome Project for identifying disease susceptibility loci are the single nucleotide polymorphisms (SNPs). These single base-pair changes are common across the genome, and over 1.4 million such polymorphisms have been detected.[1] SNPs occur ubiquitously across the genome, in coding, non-coding and untranslated regions. Such variants are strong candidates for disease susceptibility mutations, and gene localisation studies screen large numbers of SNPs to test the co-occurrence of SNP alleles and disease. These genetic association studies are performed to determine whether a genetic variant is associated with disease: an individual carrying one or two copies of a high-risk variant is at increased risk of developing a disease.

This review paper will consider two different study designs for association studies: the case control study and the transmission disequilibrium test (TDT), focusing on study design, statistical analysis methods and interpretation of results. Web sites for downloading analysis software are given.

CASE CONTROL STUDIES

Case control studies compare the frequency of SNP alleles in two well-defined groups of individuals: cases who have been diagnosed with the disease under study, and controls, who are either known to be unaffected, or who have been randomly selected from the population. (Both choices of controls form a valid study.) An increased frequency of a SNP allele or genotype in cases compared with controls indicates that presence of the SNP allele may increase risk of disease. The major problem in case control studies is ensuring

Cathryn Lewis,
Division of Medical and Molecular Genetics,
8th Floor,
Guy's Tower,
Guy's Hospital,
London SE1 9RT, UK

Tel: +44 (0) 20 7955 8761
Fax: +44 (0) 20 7955 4644
E-mail: cathryn.lewis@kcl.ac.uk

Case control studies are a widely used and powerful study design

a good match between the genetic background of cases and controls, so that any genetic difference between them is related to the disease under study and not to biased sampling. Clearly, cases and controls should be from similar ethnic groups. More subtle genetic differences can be guarded against by collecting controls from the same geographical area as cases, or by collecting information such as the birth place of grandparents to check a similar distribution between cases and controls.

Analysis methods

For a single SNP with alleles A and B tested in a case control study, the data generated consist of six counts of the numbers of genotypes (AA, AB and BB) in cases and controls (Table 1(a)). We assume a total of n_{case} cases and n_{cont} controls have been tested, and the total number of AA genotypes observed is n_{AA}, etc. This 2×3 contingency table can be analysed directly using an observed-expected test statistic, which has a chi-squared distribution on two degrees of freedom (df). Contingency tables can be analysed using any standard statistical package (Stata, SAS, SPSS, Splus, etc.) or using Excel.

Contingency table analysis methods allow for different genetic models

The chi-square statistic tests for departure from the expected values across cells in the table. Thus the observed value for AA genotype in cases ($O_1 = a$) is compared with its expected value given the total number of cases and the total number of AA genotypes, so $E_1 = n_{\text{AA}} n_{\text{case}}/n$. The full test statistic is

$$\mathrm{X} = \sum_{i=1}^{6} \frac{(O_i - E_i)^2}{E_i} \sim \chi_2$$

where the summation is over all six cells in the table, and O_i are the observed values a, b, c, d, e, f in each cell.

Notice that this test statistic compares the observed number of AA genotypes in cases with that expected assuming both cases and controls have the same frequency of AA genotypes. The analysis does not provide any sense of ordering across the genotypes AA, AB and BB. The test statistic approximation to the chi-square distribution is asymptotic, implying that the analysis becomes more accurate with larger data sets. A small count in any cell can violate the distributional assumptions, and an expected value of at least five observations in each cell is regarded as a minimum number.

The data may also be analysed assuming a prespecified genetic model. For example, with the hypothesis that carrying allele B increased risk of disease (dominant model), the AB and BB

Table 1: Contingency tables for case control analyses, by genetic model. Test 1 is a baseline analysis, and any further analysis should be driven by prior hypothesis. *a*, *b*, *c*, *d*, e, *f* are genotype counts observed in cases and controls

(a) Full genotype table for a general genetic model

	AA	AB	BB
Cases	*a*	*b*	*c*
Controls	*d*	e	*f*

(b) Dominant model: allele B increases risk

	AA	AB+BB
Cases	*a*	*b* + *c*
Controls	*d*	e + *f*

(c) Recessive model: two copies of allele B required for increased risk

	AA + AB	BB
Cases	*a* + *b*	*c*
Controls	*d* + e	*f*

(d) Multiplicative model: *r*-fold increased risk for AB, r^2 increased risk for BB. Analysed by allele, not by genotype

	A	B
Cases	2*a* + *b*	*b* + 2*c*
Controls	2*d* + e	e + 2*f*

(e) Additive model: *r*-fold increased risk for AB, 2*r* increased risk for BB. Genotypes analysed by Armitage's test for trend

	AA	AB	BB
Cases	*a*	*b*	*c*
Controls	*d*	e	*f*

genotypes are pooled giving a 2×2 table (Table 1(b)). This is particularly relevant when allele B is rare, with few BB observations in cases and controls. Alternatively, under a recessive model for allele B, cells AA and AB would be pooled (Table 1(c)).

Allele frequency methods assume a multiplicative genetic model

Analysing by alleles provides an alternative perspective for case control data. This breaks down genotypes to compare the total number of A and B alleles in cases and controls, regardless of the genotypes from which these alleles are constructed (Table 1(d)). This analysis is counter-intuitive, since alleles do not act independently, but it provides the most powerful method of testing under a multiplicative genetic model, where risk of developing a disease increases by a factor r for each B allele carried: risk r for genotype AB and r^2 for genotype BB. If a multiplicative genetic model is appropriate, both case and control genotypes will be in Hardy–Weinberg equilibrium,[2] and this can be tested for (see below).

An additive genetic model is tested using Armitage's test for trend

A fourth possible genetic model is additive, with an increased disease risk of r for AB genotypes, and $2r$ for BB genotypes (Table 1(e)). This model shows a clear trend of an increased number of AB and BB genotypes, with the risk for AB genotypes approximately half that for BB genotypes. The additive genetic model can be tested for using Armitage's test for trend.[3]

Testing for Hardy–Weinberg equilibrium can identify genotyping problems

The contingency table can also be analysed using a logistic regression model, where the outcome (0/1) is a case or control, and the explanatory variable of genotype has three levels (AA, AB, BB). Statistically, this method is equivalent to the contingency table, but the logistic regression modelling can be easily extended to further SNPs, epidemiological risk factors or clinical variables such as disease severity or age at onset. Standard statistical packages will fit this model. This method can also be used to analyse a codominant model, where the disease risk associated with AB individuals lies between that of AA and BB individuals, but not in the specific relationship of a multiplicative or additive model.

Although this paper focuses on biallelic SNPs, association studies may also be performed using multi-allele systems, such as microsatellite markers. Indeed, several disease genes have been identified through association studies with microsatellite markers in regions delineated by linkage studies.[4,5] The analysis methods remain similar, but problems arise when rare alleles lead to sparse contingency tables that cannot be analysed by chi-square statistics. Sham and Curtis[6] provide a solution to this in their program CLUMP, which analyses case control data from microsatellite markers using Monte Carlo simulation methods.

Testing for Hardy–Weinberg equilibrium

Control genotypes should be in Hardy–Weinberg equilibrium, provided the population they are selected from is random mating and is large in size. Suppose the population frequency of allele A is p and allele B is $q = 1 - p$, then the genotypes AA, AB and BB should have frequency p^2, $2pq$ and q^2. This may be tested in controls, comparing observed control genotype counts against those expected under Hardy–Weinberg equilibrium, using $p = (2d + e)/(2n_{\text{cont}})$ as an estimate of the frequency for allele A. The test statistic has a chi-square distribution with 1 df (reduced from 2 since the data have been used to estimate the parameter p).

A significant result showing that controls are not in Hardy–Weinberg equilibrium (HWE) could arise because of:

- random chance: one of every 20 markers tested will give a p-value of less than 0.05 by chance;
- genotyping problems, where genotypes are consistently mis-called, or specific genotypes give missing values;
- heterogeneous population: the controls

may be a mix of different populations with different allele frequencies (although the test has low power to identify this scenario).

Provided the controls are in HWE, the cases may then be tested. If the SNP has a true genetic effect that is not controlled by a multiplicative model, the cases will not be in HWE[2] (although again, the test has little power to detect small departures from HWE). If the cases are in HWE, the data may be analysed by allele counting, as any genetic effect is consistent with a multiplicative model.

Population stratification

Population stratification leads to false positive results

Case control association studies assume that any difference in SNP genotypes between cases and controls is due solely to their difference in disease status, and not to any difference in genetic background. This assumption is crucial to a successful study, but is difficult either to ensure at the design stage of a study, or to test for at the analysis stage. The problem arises if our underlying population is actually a mix of ancestrally distinct populations with different values of disease prevalence and SNP allele frequency. For example, with two populations where population 1 has high disease prevalence and (independently) a higher allele frequency at the SNP than population 2, cases will be preferentially drawn from population 1. This will give a higher frequency of the SNP allele than controls, and a spurious association between the SNP and disease. Notice that the frequencies of both the disease and the SNP allele must differ in the two populations in order for the population stratification to give a false positive result in the association study.

Odds ratios illustrate the risks conferred by genetic mutations

The lack of replication across many disease association studies may be due to population stratification, but there is little evidence that the genetic differences between populations are sufficient to lead to these results. Diverse genetic populations such as Africans and Caucasians have different disease prevalences (eg hypertension, prostate cancer), and large differences in SNP allele frequencies, but would not be analysed together in a genetic study. Within ethnic groups, more subtle genetic variation could lead to population stratification, but (aside from human leukocyte antigen, HLA, studies) little is known regarding genetic differences within broad population groups. Given the current level of concern over possible population stratification, stringently designed case control studies are essential for scientific credibility.

Several methods have been developed to test and correct for potential population stratification in association studies, using genotypes from genetic markers unlinked to the SNP(s) involved in the association study to obtain information on population diversity among the cases and controls. Pritchard *et al.*[7] estimate the ancestry of study members, and then incorporate this information into the association study, essentially testing for association within subpopulations. The genomic control method treats the population ancestry as a nuisance parameter within the modelling, and removes the effect from the association test statistic.[8]

Odds ratios for disease from case control studies

The contingency table methods above provide an assessment of departure from equal SNP allele frequencies in cases and controls (a *p*-value). These studies can also be used to estimate the disease risk conferred by the SNP allele. Suppose we have a dominantly acting SNP allele, which other studies have confirmed as a functional mutation. The odds ratio (OR) for disease is the ratio of allele carriers to non-carriers in cases compared with that in controls (Table 1):

$$\mathrm{OR} = [(b + c)d]/[a(e + f)]$$

which gives the increase in disease risk for carriers compared to non-carriers. The relative risk can also be estimated, but the OR has the advantage of easily calculated confidence intervals based on cell entries.[9] Further evidence for the underlying

genetic model is provided by calculating odds ratios separately for each genotype (AB, BB) compared with the wild-type AA homozygotes. For example, in inflammatory bowel disease, the pooled effect of mutations in the *NOD2* gene gives an odds ratio of 3.0 for heterozygote carriers and 23.4 for homozygotes.[10] Odds ratios should only estimated once strong evidence has accumulated that the tested SNP is indeed the true disease mutation.

TRANSMISSION DISEQUILIBRIUM TEST

TDT is robust against population stratification

The TDT tests for both linkage and association in families with observed transmissions from parents to affected offspring.[11] It was originally developed to test for linkage in the presence of association, but its most common usage is now to test for association in the presence of linkage, since it is robust against population stratification.

The TDT tests for distortion in transmission of alleles from a heterozygous parent to an affected offspring (Figure 1). Under no association with the disease, alleles A and B have an equal chance of being transmitted from a heterozygous parent. If, however, allele B increases risk of disease, this allele will be preferentially transmitted to the affected offspring. The sampling scheme for the TDT is the family trio, with DNA available from both parents and a single affected offspring (although further affected offspring can be included in a test of linkage). The test statistic T considers all heterozygote AB parents, and compares the number of transmissions of allele A and allele B, in a McNemar's test (Figure 1). T has a chi-square distribution with 1 degree of freedom, provided the sample size (of heterozygous parents) is sufficiently large. For a smaller number of parents, an exact binomial test can be used. The TDT treats parental contributions as independent, and therefore assumes a multiplicative model. In case control studies, different tests can be used for specific genetic models (recessive, dominant, etc.), and similar methods are available for the TDT,[12] although these are not widely used.

TDT counts transmissions of an allele from heterozygous parents to an affected offspring

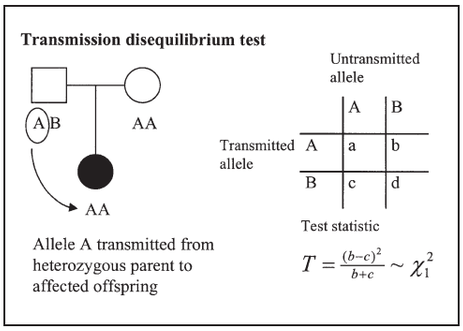

Figure 1: TDT diagram and test statistic

The sampling scheme of the TDT is convenient where families have previously been collected (for example in a linkage study), and in young-onset traits. However for older onset traits, collecting complete family trios may be difficult. An alternative is the sibTDT[13] and other similar tests, where unaffected siblings replace the parents. In the sibTDT, the number of A alleles carried by affected siblings is compared with those carried by unaffected siblings. The sample size required for the sibTDT is substantially larger than that for the TDT: for families where two unaffected siblings replace the parents, 50 per cent more families are required to obtain the same power to detect association.[14] Results for the TDT and sibTDT may be combined into a single test, giving a method that is flexible to different family structures. Including families where only a single parent is genotyped is difficult: reconstructing parental transmission can bias the result.[15]

Many programs can analyse TDT data and are available through the WWW, with a complete listing given on the Rockefeller linkage site (Table 2). The TDT/Sib-TDT program by Spielman is user-friendly, and provides a complete analysis of TDT and sibTDT families. Other programs provide the TDT as an additional option to linkage analysis, for example Genehunter.[16] These programs also provide functions such as

Table 2: Web sites for association studies analysis methods

Rockefeller linkage site providing a complete listing of software for genetic linkage analysis	http://linkage.rockefeller.edu/soft/list.html
TDT/SibTDT	http://genomics.med.upenn.edu/spielman/TDT.htm
Genehunter	http://www.fhcrc.org/labs/kruglyak/Downloads/index.html
PDT	http://www.chg.duke.edu/software/pdt.html
ETDT	http://www.mds.qmw.ac.uk/statgen/dcurtis/software.html
Transmit	http://ftp-gene.cimr.cam.ac.uk/clayton/software/
EH	ftp://linkage.rockefeller.edu/software/eh/
Ehplus	http://www.iop.kcl.ac.uk/IoP/Departments/PsychMed/GEpiBSt/software.stm

permutation tests to assess the significance of the TDT statistic. The pedigree disequilibrium test (PDT) provides a test of association for extended families.[17]

Several extensions to the TDT have been developed. For a multi-allele marker with n alleles, the contingency table of Figure 1 is extended to have n columns and rows, where the entry in row i, column j shows the number of parents who transmit allele i in preference to allele j, and vice versa for row j, column i. Although such contingency tables can be analysed using standard statistical methods, the sparseness of data makes this difficult. Sham and Curtis[18] have developed a logistic regression approach, estimating a risk effect for each allele, and fitting models to the number of transmitted and non-transmitted alleles for a microsatellite marker, which is implemented in the program ETDT.

TDT can be applied to microsatellite markers

HAPLOTYPE ANALYSIS OF MULTIPLE SNPS OR MICROSATELLITE MARKERS

Association studies commonly test a series of SNPs within a candidate gene to determine whether any SNP increases risk of developing the disease. The analysis methods presented above for case control studies and the TDT assume that each SNP will be analysed independently, but some recognition of multiple testing is necessary. A Bonferroni correction to the p-values obtained from each SNP is overly conservative, since the SNPs may be in linkage disequilibrium, and therefore results from each test are not independent. Some TDT programs use permutation statistics to provide an overall assessment of significance (eg Genehunter).

Haplotypes of candidate gene SNPs can give greater power

Analysis methods based on single SNPs have limited power to detect a true genetic effect that requires a specific allele at several SNPs. This may be detected using haplotype-based methods, analysing all SNPs concurrently. Genehunter allows haplotype analysis of up to four SNPs. One of the most flexible programs for TDT-type analysis is Transmit.[19] This analyses either a single SNP or reconstructs haplotypes across SNPs, and also deals with multi-allele markers. Transmit estimates transmission probabilities of alleles to affected offspring, using parental and sibling genotypes where available, and it infers missing genotypes using Hardy–Weinberg equilibrium where necessary.

Haplotypes in case control studies can be analysed using EH or Ehplus[20,21] which estimate haplotype frequencies in a set of individuals. By performing the analysis on cases, on controls, and then on the pooled collection of cases and controls, a likelihood ratio test can be used to test for a difference in haplotype frequencies in cases and controls.

DISCUSSION

This paper provides an overview of association studies in genetics, focusing on two widely used study designs: the case

control study and the transmission disequilibrium test. The TDT was originally developed for use in family-based linkage studies, but its popularity is due primarily to its robustness against population stratification. However, population stratification may contribute only a small proportion of the false positive/non-replicated association studies, and case control studies are again popular for use in complex diseases.

TDT protects against population stratification but requires increased sample sizes

The major advantage of a case control study over the TDT is its increased power to detect genes of similar effect and therefore decreased sample sizes. The relative advantage of the case control study will depend on the underlying genetic model. For example, a mutation with a 10 per cent population frequency, and a multiplicative model where each copy of the mutation increases risk four-fold, requires 450 genotypes (150 trios) in a TDT study, but only 296 genotypes (148 samples in each group) for a case control study. Using the TDT therefore requires 51 per cent more genotyping than the case control study (using a significance level of 5×10^{-8} for a genome-wide association study, and a power of 80 per cent). These differences in sample size become critical when genotyping capacity limits the number of candidate genes/mutations we can test, and when the minor genetic effect due to any single mutation is at the boundary of what our study sample sizes can detect. However, the TDT provides a valuable confirmation that results from a case control study are indeed due to a true disease susceptibility locus. Sample sizes for case control studies can be calculated from standard statistical software, specifying the difference in allele or genotype frequencies between cases and controls. Several methods have been developed for calculating TDT sample sizes, including, most recently, a computer program TDT power calculator (TDT_PC) that allows diverse genetic models and family structures.[22]

Non-replication may reflect poor study design

A Medline search on 'genetic association study' reveals over 6,000 papers, many of these reporting associations that remain unconfirmed in further studies. For example, Altshuler *et al*.[23] analyse several polymorphisms that have previously been associated with increased risk of developing type 2 diabetes. Using case control and TDT studies, they replicate only 2 of the 13 results. The reasons for non-replication are diverse. The complex diseases may have many contributing genes of small to moderate effect. The power to detect this effect will be low in any single study and, unsurprisingly, non-replication occurs. The non-replication may be exacerbated by (1) studies that fail to interpret *p*-values based on the number of candidate genes or SNPs tested, (2) genetic mutations of differing frequency and effect across populations, (3) studies that ascertain patients using different clinical criteria, and (4) interactions with environmental effects that differ across populations.

Ignoring population stratification, any significant result from a disease association study has several possible interpretations. The tested SNP may be a true disease susceptibility mutation that directly affects risk. However, the SNP may merely be in linkage disequilibrium with the true mutation, and the significant result arises because the SNP allele and mutation are co-inherited in the population, rarely separated by recombinations. This remains a valuable result, because the mutation must lie within a short genetic region flanking the tested SNP. However, identifying the true mutation from a haplotype of SNP alleles that are in strong linkage disequilibrium is difficult, and may require functional studies. The third possibility is that the result occurs by random chance, and does not reflect any true disease mutation. This possibility increases when several genes or SNPs have been tested, or when cases have been stratified by clinical properties (such as severity or age of onset), unless the *p*-value has been corrected for multiple testing.

Association studies currently focus on a candidate gene, or a candidate region

Genome wide association studies will soon be feasible

containing a gene cluster.[5] However, genome-wide association studies will be feasible when we have a clearer understanding of genetic variation in the human genome, and when genotyping throughput has increased substantially. The basic analysis methods presented here will remain applicable, but the studies will provide enormous scope for testing haplotype effects or interactions across genetic regions.

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