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A Genome-Wide Scan of 1842 DNA Markers for Allelic Associations With General Cognitive Ability: A Five-Stage Design Using DNA Pooling and Extreme Selected Groups

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All measures of cognitive processes correlate moderately at the phenotypic level and correlate substantially at the genetic level. General cognitive ability (*g*) refers to what diverse cognitive processes have in common. Our goal is to identify quantitative trait loci (QTLs) associated with high *g* compared with average *g*. In order to detect QTLs of small effect size, we used extreme selected samples and a five-stage design with nominal alpha levels that permit false positive results in early stages but remove false positives in later stages. As a first step toward a systematic genome scan for allelic association, we used DNA pooling to screen 1842 simple sequence repeat (SSR) markers approximately evenly spaced at 2 cM throughout the genome in a five-stage design: (1) case-control DNA pooling (101 cases with mean IQ of 136 and 101 controls with mean IQ of 100), (2) case-control DNA pooling (96 cases with IQ >160 and 100 controls with mean IQ of 102), (3) individual genotyping of Stage 1 sample, (4) individual genotyping of Stage 2 sample, (5) transmission disequilibrium test (TDT; 196 parent-child trios for offspring with IQ >160). The overall Type I error rate is 0.000125, which robustly protects against false positive results. The numbers of markers surviving each stage using a conservative allele-specific directional test were 108, 6, 4, 2, and 0, respectively, for the five stages. A genomic control test using DNA pooling suggested that the failure to replicate the positive case-control results in the TDT analysis was not due to ethnic stratification. Several markers that were close to significance at all stages are being investigated further. Relying on indirect association based on linkage disequilibrium between markers and QTLs means that 100,000 markers may be needed to exclude QTL associations. Because power drops off precipitously for indirect association approaches when a marker is not close to the QTL, we are not planning to genotype additional SSR markers. Instead we are using the same design to screen markers such as cSNPs and SNPs in regulatory regions that are likely to include functional polymorphisms in which the marker can be presumed to be the QTL.

KEY WORDS: Intelligence; genome scan; quantitative trait loci (QTLs); DNA pool; association.

INTRODUCTION

General cognitive ability (*g*) is a highly heritable quantitative trait that varies from a low end of mild mental retardation to a high end of gifted individuals (Plomin, 1999a). One of the most consistent findings from individual differences research on human cognitive abilities and disabilities during the past century is that diverse cognitive processes intercorrelate. Despite the diversity of cognitive tests, individuals who perform

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well on one test tend to do well on other tests. In a meta-analysis of 322 studies that included hundreds of different kinds of cognitive tests, the average correlation among the tests was about 0.30 (Carroll, 1993). Principal component analyses indicate that *g* accounts for about 40% of the total variance of cognitive tests (Jensen, 1998). This overlap in cognitive abilities was recognized nearly a century ago by Charles Spearman (1904), who used *g* as a neutral signifier of general cognitive ability that avoided the many connotations of the word *intelligence.* Although a few critics remain (Gould, 1996), the concept of *g* is widely accepted by experts (Carroll, 1997; Neisser *et al.,* 1996; Snyderman and Rothman, 1987).

g is substantially heritable. There are more studies addressing the genetics of *g* than any other human characteristic. Dozens of studies, including more than 8000 parent-offspring pairs, 25,000 pairs of siblings, 10,000 twin pairs, and hundreds of adoptive families, all converge on the conclusion that genetic factors contribute substantially to *g* (Plomin *et al.*, 2001). Heritability estimates vary from 40% to 80% but estimates based on the entire body of data are about 50%, indicating that genetic variation accounts for about half of the variance in *g*. Most of the genetic variance for *g* is additive, which facilitates attempts to identify genes responsible for this heritability (Plomin *et al.,* 2001).

The most surprising finding from genetic research is that genetic influences on cognitive abilities almost exclusively involve *g*. Multivariate genetic analyses have consistently found that genetic correlations among cognitive abilities are very high—close to 1.0 (Petrill, 1997). This research provides clues for understanding how the brain works from an individual differences perspective. It suggests that there must be genetically-influenced mechanisms that affect performance across diverse cognitive tasks. Although this is not necessarily due to the influence of genetic variation on a single process, the multivariate genetic results indicate that the same genes affect different cognitive processes. That is, if a gene associated with a particular cognitive ability were identified, the same gene would be expected to be associated with other cognitive abilities as well. These genetic findings suggest that *g,* despite its complex nature, is a reasonable target for QTL research.

More than 100 rare genetic syndromes include mental retardation as a symptom (Wahlström, 1990) but the present study, called the IQ QTL Project, is the only study that has searched systematically for QTLs associated with *g*. Finding genes associated with complex quantitative traits like *g* requires power to detect QTLs of small effect size. Allelic association is able to detect QTLs of much smaller effect size than linkage, even QTL linkage (Plomin *et al.,* 1994; Risch, 2000; Risch and Merikangas, 1996; Risch and Teng, 1998). Allelic association with complex quantitative traits has been used primarily to investigate associations with candidate gene polymorphisms. In our earlier work, we genotyped 100 DNA markers in or near genes involved in brain functioning, primarily neurotransmitters, but no replicated associations with *g* were found (Petrill *et al.,* 1998; Plomin *et al.,* 1995). There was a suggestion of an association involving apolipoprotein E gene with the ε4 allele, which is associated with dementia, showing a lower frequency in the high *g* group. However, a follow-up study that included samples twice as large and two transcriptional regulatory region variants of the apolipoprotein E gene in addition to the usual polymorphism found little evidence for association (Turic *et al.,* 2001), although a recent study has reported an association (Deary *et al.,* in press). Our survey of 100 markers also included two markers for the cathechol-O-methyltransferase (COMT), a gene that has been reported to correlate with cognitive ability (Egan *et al.,* 2001). For both apolipoprotein E and COMT, results from the IQ QTL Project were in the same direction as that reported in the recent studies but the results were not significant, which may indicate problems of statistical power to detect QTLs of small effect size.

A problem with the candidate gene approach is that many of the thousands of genes expressed in the brain could be considered as candidate genes for *g*. Rather than examining candidate genes, allelic association can be made more systematic by using a dense map of markers. We took a first step in this direction by genotyping 47 microsatellite repeat markers on the long arm of chromosome 6 (Chorney *et al.,* 1998). We found a replicated association for a marker that happened to be in the gene for insulin-like growth factor-2 receptor (*IGF2R*), which has been shown to be especially active in brain regions most involved in learning and memory (Wickelgren, 1998). However, this result failed to replicate in an independent sample (Hill et al., 2001).

The problem with using a dense map of markers for a genome scan for QTLs of small effect is the amount of genotyping required. The number of markers needed for a complete genome scan based on linkage disequilibrium is a matter of some uncertainty

(Abecasis *et al.,* 2001; Kruglyak, 1999; Reich *et al.,* 2001), but it seems likely that at least 100,000 markers will be needed and this may be optimistic by an order of magnitude. These markers would need to be genotyped on large samples in order to detect QTLs of small effect size—an important reason for the failure to find replicable QTL associations for complex traits is that studies are underpowered to detect QTLs of small effect size (Cardon and Bell, 2001). For example, to detect a QTL of 1% heritability in an unselected sample with 80% power ($p < .05$), 800 individuals would need to be genotyped. However, in order to protect against false positive results caused by genotyping so many markers, much lower alpha values and much larger samples are needed, resulting in the need for tens of millions of genotypings.

In order to address these issues, we developed DNA pooling and a multi-stage replication design using extreme samples (Daniels *et al.,* 1998). DNA pooling greatly reduces the need for genotyping by pooling DNA from all individuals in a group and genotyping the pooled groups. Unlike DNA chips and other high-throughput approaches, genotyping costs for DNA pooling are independent of sample size. Using microsatellite repeat primers with fluorescent 5′ ends, an automated DNA sequencer produces an allele image pattern (AIP) for the pooled DNA in which the frequency of each allele is reflected in the height of its image and AIPs are compared between groups. Other approaches to DNA pooling have attempted to estimate absolute allelic frequencies for a single pool of DNA, which requires adjustments for technical problems such as differential amplification and stutter banding (e.g., Barcellos, *et al.,* 1997; LeDuc *et al.,* 1995; Perlin *et al.,* 1995). The essence of our approach to DNA pooling is that comparison of two groups requires only an estimate of relative allelic frequencies in which such problems are expected to cancel out. We focus on the difference in AIPs (ΔAIP) for the two groups seen when the AIPs for the two groups are overlaid (Daniels *et al.,* 1998). This is a sensitive method of detecting the largest differences in allele frequencies between samples as confirmed by individual genotyping (Daniels *et al.,* 1998).

DNA pooling, like most analyses, is more powerful when applied to selected samples. The logic of the QTL perspective is that greater power can be achieved by selecting more extreme individuals as well as by selecting larger samples of extreme individuals. The IQ QTL Project focuses specifically on high *g* rather than low *g* because quantitative genetic results suggest that heritability appears to be similar at the high end of the distribution of *g* as it is for the rest of the normal range of variation in *g* (Petrill *et al.,* 1998; Plomin and Price, 2001; Saudino *et al.,* 1994) In contrast, very low *g* functioning appears to be less heritable due to chromosomal abnormalities and idiosyncratic environmental events despite the well-known single-gene causes of severe mental retardation, which are rare (Plomin, 1999b). For this same reason, our control groups consisted of individuals of average *g* rather than low *g* individuals, although the design would have been more powerful if we had selected controls one to two standard deviations (*SD*) below the mean. It should be noted that although high *g* cases and average *g* controls appear to show similar heritability, this is a different issue from QTL association, which is based on allelic frequency differences between cases and controls.

We selected a high *g* group of 101 individuals with scores more than 2 *SD* above the mean (an IQ score $>$130), which would represent the 98th percentile of an unselected sample of 5000 individuals. Because greater power is needed to replicate results, we selected a replication high *g* group of 96 individuals of some of the brightest adolescents in the United States with estimated IQs greater than 160, which would represent the top .00003 of an unselected sample of three million. Although all subjects were white, it is nonetheless possible that QTL associations could be due to ethnic stratification. For this reason, replication was sought in a third sample consisting of 196 parent-child trios in which the offspring had estimated IQs greater than 160, which provides a within-family transmission disequilibrium test (TDT) that protects against population stratification as a possible source of QTL associations. We published preliminary proof-of-principle papers for a systematic search of chromosome 4 (Fisher *et al.,* 1999) and chromosome 22 (Hill *et al.,* 1999) using just the two case-control studies and with samples only half the size of the present study.

Our genome scan of 1842 markers used a five-stage design with these three extreme samples in order to attempt to balance false positives and false negatives by permitting a more lenient significance criterion in the first stage (which reduces false negatives for QTLs of small effect size) and then removing false positives in later stages. Using DNA pooling, markers that yielded nominally significant ($p < .05$) ΔAIPs in the first case-control sample (Stage 1) were replicated using the second case-control sample (Stage 2). Markers that survived Stage 2 were individually genotyped for the first sample (Stage 3) and markers surviving

Stage 3 were individually genotyped for the second sample (Stage 4). Markers surviving Stage 4 were individually genotyped for 196 parent-child trios for TDT analysis (Stage 5), which guards against population stratification. The approach was made even more conservative by requiring that, at each stage, a single allele showed a significant allelic frequency difference compared with all other alleles and that the same allele had to be replicated in the same direction in the replication case-control and TDT samples. For SSRs, this strategy is especially conservative because adjacent repeat alleles may form part of an evolutionarily related cluster.

This multiple-stage strategy provides a better balance between false positives and false negatives than using a stringent alpha in a single study, which protects against false positives but also greatly increases false negatives for QTLs of small effect size. As will be explained later, the overall Type I error rate for the three studies is 0.000125, which protects against false positive results in our analysis of 1842 markers. Concerning false negatives, genotyping 1842 markers was meant only as a first step toward a complete genome scan for allelic association. As many as 100,000 markers would be required for a complete genome scan for allelic association relying on linkage disequilibrium.

METHODS

Samples

In overview, the project includes three samples: (1) an original sample of 101 high *g* cases and 101 average *g* controls was used for initial screening of the 1842 markers, (2) a replication sample of 96 high *g* cases and 100 average *g* controls, and (3) a within-family replication sample of 196 parent-offspring trios in which the offspring have high *g* scores. The samples were restricted to non-Hispanic, white children so that differences in marker allele frequencies between the groups were less likely to be due to ethnic differences. Subjects were excluded if at least one of the maternal and paternal grandparents was not white. The project was approved by the appropriate institutional review boards and informed consent was obtained from all participants.

The original case-control samples of 101 high *g* and 101 control individuals combined two samples used in our earlier work (Fisher *et al.,* 1999). The controls were children living in a six-county area around Cleveland, Ohio. They were selected for IQs between 90 and 110 from a larger group assessed using the Wechsler Intelligence Scale for Children, Revised (WISC-R; Wechsler, 1974). The mean IQ was 102.2 (6.3 SD; 52 male, 49 female; 13 years average age). A total of 51 of the children in the high *g* group were selected for IQs above 130 from the same group of children (136.0 mean IQ, 9.3 SD; 34, male, 17 female; 10 years average age). The remaining 50 high *g* individuals were obtained from the Study of Mathematically Precocious Youth (SMPY) in the United States (Lubinski and Benbow, 1994). The highest-scoring SMPY individuals were selected from the more than one million 7th and 8th graders who performed in the top 3% on a standardized test administered in their schools and were invited to take the Scholastic Aptitude Test (SAT) college entrance exam 4 years early before the age of 13. Fifty of the highest-scoring individuals were included in the high *g* sample (Fisher *et al.,* 1999). These individuals represent a selection intensity of about 1 in 30,000 as indicated by SAT scores 4 standard deviations above the mean (equivalent to IQ scores $>$160).

Replication case-control samples were obtained in a similar manner. The control group included 100 individuals with an average IQ of 102.4 (7.6 SD) selected from the Cleveland area in the same manner as the original control group, except that the subjects were adults (33 years average age; 65 female, 35 male) tested on subtests of the Wechsler Adult Intelligence Scale-III (Wechsler, 1997). The high *g* group ($N = 96$) was selected from the remaining highest-scoring individuals in the SMPY sample selecting preferentially for children whose biological mother and father were available for DNA collection in order to obtain parent-offspring trios. IQs estimated from SAT scores were again greater than 160.

The sample of 196 parent-offspring trios was also obtained from the SMPY study by identifying high *g* individuals whose biological mother and father were available. About one-third of these high *g* individuals were cases in the SMPY replication sample.

For all subjects in the original case-control groups, DNA was extracted from permanent cell lines established from blood samples. In the replication case-control and parent-offspring trio samples, DNA was extracted from cheek swabs (Freeman *et al.,* 1997), yielding an average of 50μg of DNA for each individual. The two types of DNA are not expected to differ in relation to the DNA pooling protocol.

Selection of Simple Sequence Repeat Markers

Primers for 2909 simple sequence repeat (SSR) markers on the autosomes were purchased from MWG-Biotech (Germany). These DNA markers were selected from the LDB sex-averaged summary map (*http://cedar.genetics.soton.ac.uk/public html*). Where positional order

differences occurred between physical and genetic maps, the physical map was given priority and the genetic distance was adjusted accordingly. The average marker interval is 1.5 cM with 80% of the genome within 1cM of a marker except for poorer coverage at telomeres. Primers for the 1842 markers that were successfully genotyped using standard PCR conditions described below can be found at *www.sgdp.org.uk.*

Amplification and Genotyping of Pooled DNA Samples

For DNA pooling, each individual DNA sample was diluted to 8 ng μL^{-1}. DNA quantification prior to pooling was performed in triplicate using the PicoGreen fluorescent assay and a Fluoroskan Ascent fluorometer. Two sets of pools, original and replication, were constructed. Each set consists of six separately prepared pools—three from the control groups of average *g* and three from high *g* individuals. Each of the three replicate pools from the original set of high *g* and control groups was amplified in duplicate resulting in six polymerase chain reaction (PCR) products.

Rather than optimizing each primer pair, standard conditions were used for PCR amplification. Each PCR contained the following reagents: 48 ng pooled genomic DNA, dNTPs (1.2 mM each), 1 × *Taq* DNA polymerase buffer (Qiagen, with 2 mM $MgCl_2$), *Taq* DNA polymerase (Qiagen, 0.6 U), 1.4 pmol each primer and water to 12 µL. Pooled DNA was amplified using a modified touchdown PCR procedure (Rithidech *et al.,* 1997) as follows. The DNA was initially denatured at 95°C for 5 min; followed by 26 cycles at 95°C for 45 sec, 62°C for 45 sec subtracting 0.5°C for each subsequent cycle, and 72°C for 45 sec. A final extension was performed at 72°C (10 min). Using these standard optimizing conditions, 1588 of the 2909 markers (55%) yielded replicable amplification products in which at least four of each group's six replicate pools gave near-identical overlays. A second amplification protocol using *Taq* Gold (Perkin-Elmer) was applied for the 1321 markers that failed to yield a product in the initial PCR. This protocol included the same reagents as above except that Qiagen buffer and enzyme were replaced with 10× Taq Gold buffer, $MgCl_2$ solution (to 2.5 mM final concentration) and Taq Gold polymerase. The cycling parameters were as above, except for the initial denaturation step; this was increased to 9 min. A total of 259 markers were successfully amplified using the Taq Gold protocol, bringing the total number of scoreable markers to 1842 (63%).

Up to four fluorescently labelled (Hex, Fam, or Tet) markers were electrophoresed in each gel lane. Only markers whose products did not overlap (regardless of which dye they contained) were analyzed in the same lane. Due to differences in intensity of the three dyes, Fam-labelled markers were diluted 10-fold, Tet-labelled markers were diluted 5-fold and Hex-labelled markers were diluted 3-fold. Diluted pools of PCR products (1.5 µL) were mixed with loading dye (1.5 µL) and GS500 size ladder (0.5 µL, Perkin-Elmer). These mixes were loaded, typically on denaturing gels and run on ABI 373A and 377 sequencers.

Allele image patterns (AIPs) were generated on an ABI DNA sequencer for each group's six PCR products for each marker. The six unmodified AIPs for each group (high g, or control) were overlaid using GENOTYPER software and imported into DeBabelizer. The consensus AIP was taken to represent the relative allele frequencies of the marker. In order to compare the results of pooled genotyping of the original high *g* and control groups, we measured the total area that was not shared by the two superimposed consensus AIPs and expressed this as a fraction of the total shared and non-shared area according to the method of Daniels *et al.* (1998). This test statistic is called ΔAIP. Markers were tested for significant differences in ΔAIPs between groups using a program that simulates allele patterns from the ΔAIP scores, heights of the peaks, and the number of peaks (Daniels *et al.,* 1998).

Individual Genotyping

For individual genotyping, PCR was performed using the same protocol as described for pooled DNA, except that 30 ng DNA was used per individual. The significance of the target allele (described later) was tested using Pearson χ^2 comparing the frequency of that allele against all other alleles.

Five-Stage Design

A five-stage design with three independent samples was used in order to provide a balance between false positives and false negatives in the search for QTLs of small effect size.

Stage 1 (Original Case-Control Sample—Pooled DNA Samples)

For markers yielding ΔAIPs with $p < .05$, the allele that showed the largest difference between the high *g* and control group was identified by using an allele-

specific test in order to establish an allele-specific directional hypothesis that could be tested in subsequent stages. The allele-specific test incorporates an algorithm to correct for differential amplification and stutter for di-, tri- and tetranucleotide repeats derived empirically. The height of the target allele's peak for each group was converted to a ratio of the total of all the AIP peak heights so that the score represented the number of alleles in that peak (Fisher *et al.,* 1999). Pearson χ^2 with a 2×2 contingency table was used to compare the frequency of the target allele versus all other alleles for the high *g* and control groups. This allele-specific test is useful because significant ΔAIPs can be caused by small differences between groups for several alleles or by a relatively large difference for a specific allele. Unless a marker shows a significant difference for a specific allele in the original sample, it is unlikely that the marker will be significant in subsequent stages. For this reason, markers survived to Stage 2 only if they met two criteria in the original sample: a significant ($p < .05$) ΔAIP and a significant ($p < .05$) specific allele difference between the two groups.

Stage 2 (Replication Case-Control Sample—Pooled DNA Samples)

The allele-specific directional hypothesis from Stage 1 was tested in the replication case-control sample using pooled DNA. A one-tailed test of significance was used because the target allele was required to show allele frequency differences between groups in the same direction as in Stage 1 ($p < .05$).

Stage 3 (Individual Genotyping of Original Case-Control Sample)

For markers surviving to Stage 3, subjects in the original case-control sample were genotyped individually. The allele-specific directional hypothesis was tested for significance ($p < .05$, one-tailed) using Pearson χ^2 comparing that allele against all others.

Stage 4 (Individual Genotyping of Replication Case-Control Sample)

Individual genotyping of the replication case-control samples was conducted for surviving markers. The allele-specific directional hypothesis was tested for significance ($p < .05$, one-tailed) using Pearson χ^2 comparing that allele against all others.

Stage 5 (Individual Genotyping of Parent-Offspring Trios)

Finally, surviving markers were genotyped individually for the parent-offspring trios. The number of transmitted and nontransmitted target alleles were tested using the transmission disequilibrium test (TDT; Ewens and Spielman, 1995) and the haplotype relative risk (HRR; Terwilliger and Ott, 1992). We were not able to use quantitative TDT (Allison, 1997) because the offspring were so highly selected that variance within the group is restricted (Page and Amos, 1999). The within-family comparison of parent-offspring trios controls for possible effects of population stratification.

We also assessed population stratification using a DNA pooling version of the genomic control method (Pritchard and Rosenberg, 1999). Typically, the genomic control method investigates stratification by analyzing linkage disequilibrium between unlinked markers across the genome. However, because the markers that we genotyped individually in Stage 3 were selected for showing possible differences between cases and controls, we extended the method using allele frequencies estimated from pooled DNA data for the original sample for 60 markers on chromosome 1 with intervals greater than 1cM. The effort involved in estimating allele frequencies led us to present the results in this paper for markers on chromosome 1, although we are continuing to analyze all of the markers throughout the genome for a more thorough analysis of genome control for pooled DNA.

Power

Power analyses indicate that the multiple-stage design is robust to false positives and that the use of extreme selected sampling provides power to detect QTLs of small effect. Our data simulation and analytic power analyses systematically varied effect size, QTL and marker allele frequency, and degree of linkage disequilibrium (D', the proportion of maximum disequilibrium possible that is observed, with $D' = 1.0$ when linkage disequilibrium is complete and allele frequencies for the marker and QTL are equal). For the original and replication case-control comparisons, we assumed a normally-distributed trait with a sibling correlation of 0.4. Cases were selected to be 2.5 *SD* above the mean for the original sample and 5 *SD* above the mean for the replication sample. Controls were selected to be $\pm.67$ *SD* from the mean.

For the original case-control sample, power estimates to detect QTLs that account for 5%, 2.5%, and 1%

of the variance are, respectively, 100%, 93%, and 56%, with alpha set at 0.05 when D' is 1.0 and marker and QTL frequencies are 0.25. As in all such analyses, power drops off sharply as linkage disequilibrium between the marker and QTL decreases. When D' drops to 0.50, power estimates are 73%, 42%, and 19%, respectively, for effect sizes of 5%, 2.5%, and 1%. Lower marker and QTL frequencies and especially greater differences in their frequencies also attenuates power. The replication case-control sample yields even greater power because the high *g* group is more highly selected. Power estimates for the replication study to detect QTLs that account for 5%, 2.5%, and 1% of the variance are 100%, 100%, and 98%, respectively, when D' is 1.0. When D' is 0.50, power estimates are 100%, 92%, and 54%, respectively.

Power simulations for the parent-offspring trios were conducted for 196 trios with offspring selected to be 4 *SD* above the mean. Power estimates to detect QTLs that account for 5%, 2.5%, and 1% of the variance are 100%, 100%, and 97%, respectively, for $D' = 1.0$. When D' is 0.50, power estimates are 100%, 88%, and 40%, respectively.

The rationale for the multiple-stage design is to increase the likelihood of detecting QTLs of small effect by using nominal alpha levels which permit false positive results in the early stages but remove them sequentially in subsequent stages. The overall Type I error rate with .05 alpha for the three samples is .000125 (that is, $.05^3$). Using an alpha of .05 implies that the number of false positive findings expected for 1842 markers are 92 for the first case-control sample, 5 for the second case-control sample, and 0 for the third sample of parent-offspring trios. Fewer false positives are actually expected because markers must pass three additional hurdles. In the first stage, not only must the overall difference in allele image patterns (ΔAIP) reach a simulated *p* value of .05 (Daniels *et al.,* 1998) but also one specific allele must show a significant effect. Thus, markers that show a significant ΔAIP are rejected if the ΔAIP is due to small differences among several alleles. Second, at each subsequent stage, the same allele was required to yield a significant effect in the same direction. Third, individual genotyping in Stages 3 and 4 must confirm the results of DNA pooling in Stages 1 and 2.

Results

Fig. 1 presents DNA pooling results for one of the markers (*D2S427*) for the original and replication samples. Because the DNA is pooled, the AIPs show all alleles for this tetranucleotide repeat marker, rather than just one or two alleles which would be seen when individuals rather than pools are genotyped. The relative height of each allele is used as an index of its frequency. The overlaid AIPs for the high *g* group and the control group indicate that differences between the AIPs for the two groups are due primarily to allele 2 for both the original and replication samples.

Fig. 2 shows individual genotyping results for the same marker (*DS2427*). Comparing Fig. 2 with Fig. 1 illustrates that pooled genotyping successfully screens for differences in allele frequencies between two groups. An allele-specific test for the individual genotyping reached our nominal level of significance ($p = .048$) in the original sample but not in the replication sample ($p = .149$).

Stage 1

ΔAIP and simulated *p* values for the 1842 markers for the original case-control sample are available at *www.sgdp.org.uk.* Of the 1842 markers, 229 markers (12%) yielded ΔAIPs with simulated $p < .05$; 92 markers were expected to be significant by chance alone with $p < .05$. Of the 229 significant markers, 108 also yielded an allele specific test (AST) with $p < .05$ (also available at *www.sgdp.org.uk*).

Stage 2

These 108 markers were tested for replication using DNA pooling in Stage 2. Eight markers were significant ($p < .05$), whereas 5 of the 108 markers were expected to be significant by chance. Of these 8 markers, 6 markers were significant in the same direction as the original sample. The AST chi-squares and *p* values for these 108 markers in the original and replication samples can also be found at *www.sgdp.org.uk.*

Stage 3

These 6 markers were genotyped individually for each high *g* and control individual in the original sample (see Table I). Individual genotyping generally confirmed the results of DNA pooling in that all of the 6 markers showed differences between the groups for the same allele in the same direction as found in DNA pooling. Although we will explore all of these markers in the future, we maintained strict criteria for significance for the genome scan in order to remain within the boundaries of our projected Type I error rate. The allele specific directional test reached our nominal significance level of $p < .05$ for 4 markers (*D2S427, D4S2460, D6S277, D14S65*).

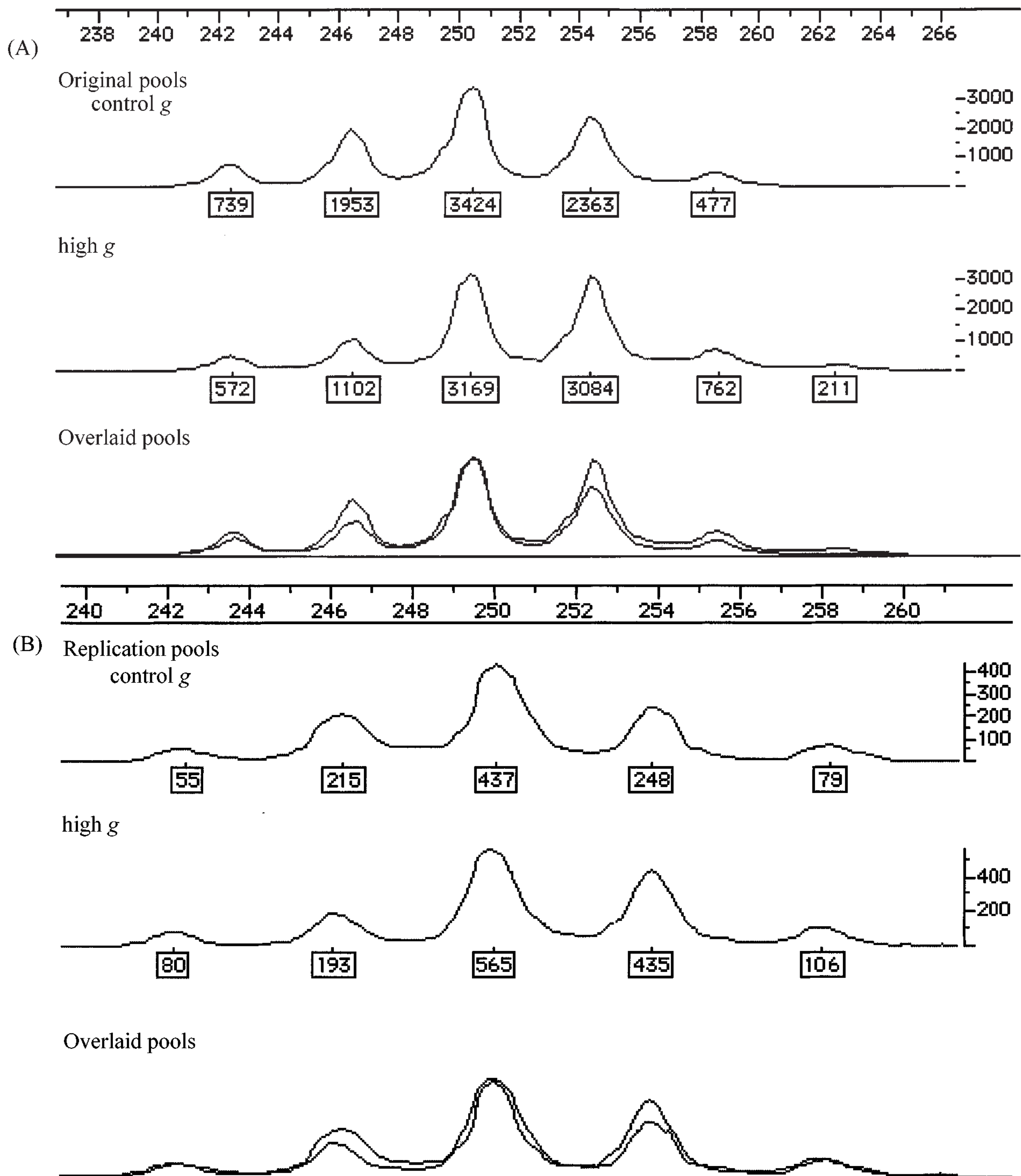

Fig. 1. DNA pooling results in the original and replication case-control samples for a tetranucleotide marker (*D2S427*) for the control *g* group (top), high group (middle), and their overlaid images (bottom), as viewed using Genotyper software. This software scales the height of each of the image patterns to a common height automatically. The numbers above the allele image patterns (AIP) represent the size in bp of the marker's alleles. The numbers below and to the right of the AIPs represent peak heights in fluorescence units. ΔAIP was calculated from the overlaid images by measuring the total area that was not shared by the two images irrespective of how many times the curves from the two pools crossed. This was then expressed as a fraction of the total shared and non-shared area (Daniels *et al.,* 1998). The ΔAIP simulated p values that test an overall difference in allele frequencies between the groups is 0.026 for the original sample and 0.003 for the replication sample. The allele-specific test for allele 2 (246bp) yielded $\chi^2 = 6.97$ ($p = .008$) for the original sample and $\chi^2 = 5.45$ ($p = .010$) for the replication sample. Fig. 2 shows the results of individual genotyping for this marker.

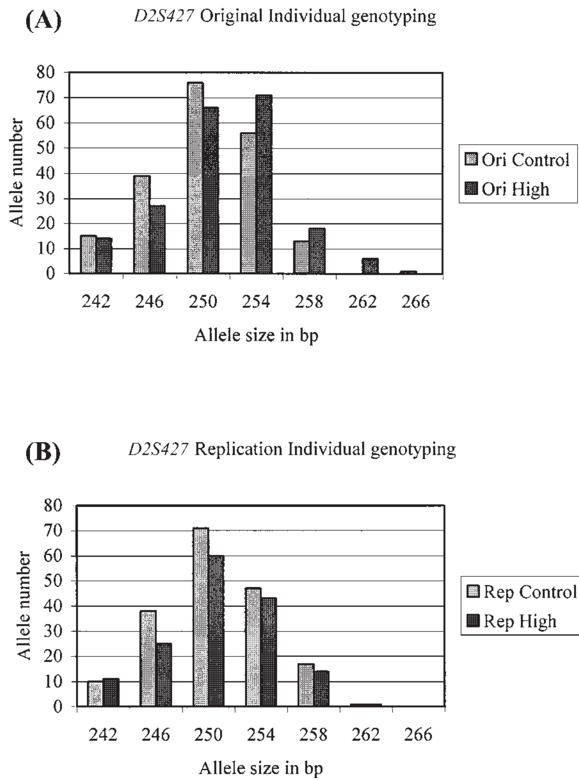

Fig. 2. Individual genotyping results for *D2S427* in the original (**A**) and replication (**B**) case-control samples. The allele-specific test of allele 2 yielded $\chi^2 = 2.76$ ($p = .048$) in the original sample and $\chi^2 = 1.08$ ($p = .149$) in the replication sample.

Stage 4

Allele-specific results for the four markers were also in the expected direction in the case-control replication sample for the AST. The results reached significance for two of the markers (*D4S2460* and *D14S65*).

Stage 5

D4S2460 and *D14S65* were genotyped individually for the 588 individuals in the 196 parent-child trios for TDT analysis. The number of informative trios for the TDT analysis was 147 for *D4S2460* and 138 for *D14S65*. Neither marker was significant; indeed, both markers yielded TDT results in the wrong direction. That is, in the two case-control samples, the target allele (allele 5 for *D4S2460* and allele 12 for *D14S65*) yielded a lower frequency in the high *g* group than in the control group. Thus, in the TDT analysis, alleles other than the target alleles would be expected to be preferentially transmitted (that is, the target allele should be more often not transmitted). HRR analysis yielded results similar to the TDT analysis for both markers.

Genomic Control

One reason why the TDT might fail to replicate the case-control results for *D4S2460* and *D14S65* is population stratification. If population stratification were a factor in these samples, then allele-frequency differences would be expected between cases and controls for many markers throughout the genome (Pritchard and Rosenberg, 1999). However, it has not been sufficiently appreciated that for complex traits a QTL perspective would also predict small frequency differences between cases and controls for many markers. We have already shown that for the 1842 autosomal markers, 12% showed simulated p values $< .05$ when 5% were expected to be significant by chance alone, which could be taken as evidence of many QTLs or of population stratification. Nonetheless, we applied the method suggested by Pritchard and Rosenberg (1999).

Table I. Individual Genotyping Results for Six Markers Surviving to Stage 3

	Stage 3				**Stage 4**				**Stage 5**			
	Original case-control sample Frequency (%) of target allele				Replication case-control sample Frequency (%) of target allele				Parent-offspring trios (TDT)			
Marker (target allele)	High *g*	Control *g*	χ^2	*p*	High *g*	Control *g*	χ^2	*p*	Transmitted (%)	Non-transmitted (%)	χ^2	*p*
D2S427 (2)	27 (13.4)	39 (19.5)	2.76	.048	25 (16.2)	38 (20.7)	1.08	.149				
D4S2460 (5)	56 (28.9)	77 (40.1)	5.40	.010	50 (29.4)	73 (38.0)	2.98	.042	109 (55.3)	85 (43.1)	*	
D6S277 (5)	50 (29.4)	22 (15.1)	9.18	.001	47 (25.8)	46 (24.5)	0.09	.382				
D14S65 (12)	81 (40.1)	97 (48.5)	2.87	.045	69 (36.3)	89 (47.8)	5.3	.012	102 (37.0)	84 (30.4)	*	
D16S687 (4)	15 (8.0)	23 (12.4)	1.97	.080								
D18S1110 (5)	53 (30.5)	59 (37.8)	1.99	.079								

*Results in wrong direction for allele-specific directional test.

A novel aspect of our application is that allelic frequencies were estimated from DNA pooling. We estimated allele frequencies using pooled DNA data for the 60 markers on chromosome 1 for the Stage 1 sample of cases and controls for reasons described earlier. We summed χ^2 comparing the most frequent allele to all other alleles across these 60 markers. This yielded a nonsignificant result ($\chi^2 = 38.4$, $df = 60$, $p = .986$), suggesting that population stratification does not contribute importantly to differences between cases and controls as indexed by the Pritchard and Rosenberg index.

DISCUSSION

In this first step towards a systematic genome scan for allelic association, 1842 simple-sequence repeat markers yielded two markers (*D4S2460* and *D14S65*) that met our multiple criteria in two independent case-control samples, but these two markers did not replicate in the TDT sample. The criteria for replication used in this study were conservative if not extreme. We are not aware of any other study that has demanded replication in three samples using two different designs (case-control and parent-offspring trios). However, a conservative approach seems warranted given problems in the literature with failure to replicate QTL association results (Cardon and Bell, 2001). Although our multiple-stage design with three extreme selected samples attempts to balance false positives and false negatives in an effort to detect QTLs of small effect size, the sample sizes and number of markers genotyped described in the present paper tips the balance very much in favor of avoiding false positives than false negatives. The design protects against any false positive results in that the overall Type I error rate is .000125 using a nominal p value of .05 at each stage. However, as discussed below, the present study has at best skimmed the surface for possible QTLs of small effect size. Thus, the present results should not be taken as an indictment of the QTL approach, but rather as a warning of the exorbitant demands of power to detect QTLs of small effect size.

Concerning *D4S2460* and *D14S65,* it is possible that these markers were significant in the two case-control samples, but not the TDT sample, because of ethnic stratification. However, we doubt this possibility for two reasons. First, all subjects were white and ethnic stratification is unlikely to account for case-control differences unless the cases and controls differ substantially in ethnicity. Second, our genomic control analysis of 60 markers using pooled DNA data showed no evidence of ethnic stratification.

What is responsible for the failure to find replicable QTLs that survive our stringent criteria? An important part of the answer is that many more markers are needed for a genome scan for allelic association relying on linkage disequilibrium. Compared with the 300 markers needed for a genome scan for linkage, 1842 markers seems like a lot of markers; however, 100,000 markers may be needed to exclude QTL association. The problem for allelic association analysis is that power drops off precipitously when a marker is not very close to the QTL. When D' is 1.0, the power to detect a QTL with 5%, 2.5%, and 1% heritability is, respectively, 100%, 93%, and 56% for the original case-control sample; 100%, 100%, and 98% for the replication case-control sample; and 100%, 100%, and 97% for the TDT sample. However, when D' is 0.50, rather than 1.0, the power estimates decline to 73%, 42%, and 19%, respectively, for the original case-control sample; 100%, 92%, and 54% for the replication case-control sample; and 100%, 88%, and 40% for the TDT sample. Our data simulations, which agree with empirical linkage disequilibrium data from the human genome sequence, indicate that with our 2 cM SSR map, D' falls to 0.50 at 700 kb for new mutations (100 generations old) and 70 kb for ancient mutations (1000 generations old). Thus, our simulations suggest that our 2 cM screen may provide as much as 70% coverage with D' of 0.50 for new mutations but less than 10% coverage for old mutations.

If the 50% heritability of *g* is due to 50 QTLs with the average effect size of 1% and if we have only 50% coverage with $D' = 0.5$, then we would have been lucky to detect one QTL. Thus, although the design attempts to balance false positives and false negatives in the quest for QTLs of small effect size, it nonetheless does a much better job of protecting against false positives than false negatives. The present study is novel in that it is to our knowledge the first attempt to begin to screen the genome systematically for allelic association, even though it is merely a first step in that direction; it uses DNA pooling as an efficient method to screen many markers; and it uses extreme selected groups to boost power to detect QTLs of small effect. However, the study was begun in 1998, before the attenuation of power of indirect association based on linkage disequilibrium was understood.

A specific problem with the present design is that its power at the first stage is only moderate to detect a QTL with 1% heritability even when D' is 1.0. One direction that we are pursuing in order to decrease the number of false negative results at this first stage is to

relax the nominal level of significance even more at Stage 1 to $p = .10$ which halves Type II error. Although this doubles the overall Type I error rate (.00025), the design still provides adequate protection against false positive results in a scan of 2000 markers. Although it might seem reasonable to use our Stage 2 sample for initial screening because the Stage 2 sample is more powerful than the Stage 1 sample due to its much more extreme selection, we were not able to do this for two practical reasons. First, our Stage 2 sample was not complete until well into our genome scan. Second, we have permanent cell lines for the Stage 1 sample but not for the Stage 2 sample. One consolation is that there is an advantage in using the powerful sample for replication because replication requires greater power than an original finding.

It should be mentioned that the use of DNA from permanent cell lines for the Stage 1 sample and DNA from cheek swabs for the Stage 2 sample could be a source of differing results between the two stages. As mentioned earlier, we do not expect DNA pooling results to differ for the two sources of DNA but we are currently exploring this issue. Even if the two sources of DNA did yield differences, the comparison between cases and controls would nonetheless be safe because both cases and controls within each stage use the same source of DNA.

With the recent mining of single nucleotide polymorphisms (SNPs), soon it will be possible to conduct a genome scan using 100,000 evenly spaced common SNPs. However, the pattern of linkage disequilibrium depends upon several factors such as marker type, allele frequency, mutation and recombination, and recent data suggest that it is highly variable between different regions of the genome (Zavattari *et al.,* 2000) and in different ethnic groups (Reich *et al.,* 2001). In addition, it is clear from many studies that whereas the average strength of linkage disequilibrium declines with increasing physical distance, many pairs of closely adjacent markers show little or no LD (e.g., Abecasis *et al.,* 2001). For this reason, we believe that the most appropriate strategy at the present time is to focus on potentially functional polymorphisms. However, rather than focusing on a few candidate genes or gene systems, we can look forward to a systematic search using all functional polymorphisms in coding sequences (cSNPs) and in regulatory regions. These tens of thousands of SNPs can be genotyped using high-throughput techniques such as DNA pooling, which has been extended to SNPs (Hoogendoorn *et al.,* 2000). In the meantime, it is possible to use nonsynonymous cSNPs as well as functional SNPs in regulatory regions as they become available. For example, focusing on 225 genes on chromosome 21, a total of 337 cSNPs were suggested using bioinformatic approaches of which 78% have been confirmed (Deutsch *et al.,* 2001), although caution is warranted, because only about 15% of SNPs in some databases have been shown to be polymorphic in any population (Marth *et al.,* 2001). Although the problem remains that a QTL association with a functional SNP might in fact be due to another nearby SNP, it is a reasonable assumption that a functional SNP *is* the QTL (that is, $D' = 1.0$), which greatly increases the power of QTL association. We are now using such markers with our five-stage design in order to identify QTL associations that meet our strict criteria for significance.

A gloomier prospect is that QTLs for *g* account for less than 1% of the variance. Although we do not know the distribution of effect sizes for *g* or any other complex trait, if QTL heritabilities are less than 1% or if QTLs interact epistatically, it will be difficult to detect them reliably. Nonetheless, the convergence of evidence for the strong heritability of *g* from family, twin, and adoption studies convinces us that *g*-relevant DNA polymorphisms exist and that most genetic effects are additive. The solution, of course, is that we will need to increase the power of our designs in order to track down the QTLs responsible for the heritability of *g,* even if the QTL heritabilities are less than 1%. DNA pooling will be useful in this context because it costs no more to genotype 1000 individuals than 100 individuals.

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