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Analysis of Association at Single Nucleotide Polymorphisms in the *APOE* Region

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The discussion of the prospects of using a dense map of single nucleotide polymorphisms (SNPs) to identify disease genes with association analysis has been extensive. However, there is little empiric evidence to support this strategy. To begin to examine the practical issues surrounding this methodology, we identified 10 SNPs in the region immediately surrounding the apolipoprotein E locus (*APOE*), an established susceptibility gene for Alzheimer disease. Our goal was to examine patterns of allelic association to begin to investigate the question of whether *APOE* could have been identified using SNPs. Our strongest evidence of association was at the 2 SNPs immediately flanking *APOE*.

INTRODUCTION

One of the major goals of the Human Genome Project (HGP) has been to identify a set of easily typed markers spanning the genome (Collins and Galas, 1993). As a result, thousands of microsatellites have been mapped and are in widespread use in genetic studies. With the achievement of this aim, the newest endeavor of the HGP is to identify hundreds of thousands of single nucleotide polymorphisms (SNPs) across the genome. It is anticipated that, over the next 3 years, at least 100,000–200,000 SNPs will be identified by the HGP (Collins *et al.*, 1998). Through a separate initiative, the SNP Consortium (TSC) has been formed with the goal of generating 300,000 SNPs in the next 2 years, with at least half of the SNPs mapped by midyear 2001 (Marshall, 1999).

Although SNPs will have generally lower heterozygosity and, therefore, be potentially less informative than microsatellite markers, they have several advantages. SNPs are more abundant than microsatellites; they are estimated to occur, on average, every 750–1000 bp (Kwok *et al.*, 1996;Wang *et al.*, 1998). Analysis with SNPs may also be advantageous since they generally have a lower mutation rate than microsatellites. These properties make SNPs ideal for gene identification through association analysis since they increase the chance of finding a marker polymorphism in association with a disease allele. Association analysis attempts to identify disease genes by looking for associations between genetic marker alleles and a disease phenotype in case-control or family samples. Association studies can be more powerful for localizing genes than linkage analysis, particularly when the contribution of these genes to the disease is small, as would be expected for complex diseases (Risch and Merikangas, 1996). For association analysis to be useful, however, a dense map of markers will be required since associations can generally be found over only small distances, say no larger than 1 cM and often much smaller (Bodmer, 1986; Jorde *et al.*, 1993).

Although genome-wide association studies using SNPs have been proposed (Risch and Merikangas, 1996), there are many difficulties to be addressed before this strategy becomes feasible. A more immediate use of SNPs will be for association analysis in candidate genes or regions. SNPs may be used to examine candidate genes that have been implicated through their biological function or because they lie in a region identified by linkage analysis. Even when no obvious candidate gene exists in a region of linkage, SNPs may be useful for narrowing the region of interest. Regions identified by linkage analysis are often quite large, especially for complex disorders, easily spanning 10–20 cM or greater (Hauser and Boehnke, 1997). Since associations are found generally over much smaller distances, one strategy for identifying the dis-

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ease gene is to saturate the region with many closely spaced SNPs and test each for association with the disease. The assumption is that association will be concentrated only at those markers that are very close to the disease locus; hence, association analysis should help to narrow the region.

Arguments for the success of association analysis at SNPs in identifying disease genes have been mainly theoretical. The little empiric evidence that exists has shown mixed results. Some have identified relevant mutations with SNPs; others have not (reviewed by Pennisi (1998)). To test these ideas, we have initiated a SNP-mapping study around apolipoprotein E (*APOE*) on chromosome 19q, an established susceptibility gene for Alzheimer disease (AD). Chromosome 19q was first identified as harboring an AD susceptibility gene through linkage analysis in a series of multiplex AD families (Pericak-Vance *et al.,* 1991). The *APOE* gene was located in the region identified by linkage analysis and became a prime candidate for the AD susceptibility gene both by position and by function (Namba *et al.,* 1991; Strittmatter *et al.,* 1993a). Subsequent analysis of a functional polymorphism in the *APOE* gene showed significant association between the *APOE4* allele and AD in familial and sporadic AD (Strittmatter *et al.,* 1993a; Saunders *et al.,* 1993; Corder *et al.,* 1993). The *APOE4* allele has been shown to increase the risk for AD in a dose-related manner, while the *APOE2* allele has been found to have a protective effect (Corder *et al.,* 1994). These associations have been replicated in many studies and in many different populations (reviewed by Roses and Pericak-Vance (1997)).

Nearly all mammals tested, with the exception of *Homo sapiens,* have *APOE4* as the apolipoprotein E allele. This suggests that *APOE4* is the ancestral allele in humans, with mutations to alleles *APOE2* and *APOE3* occurring later (Weisgraber, 1994). The age of the *APOE4* allele indicates that there have been many opportunities for recombination, decreasing the area over which significant associations are expected to exist. It was known prior to this work that there was little association between the *APOE* alleles and microsatellite alleles in the distal direction (Pericak-Vance *et al.,* 1995). Therefore, it was suspected that *APOE* would be a rigorous, practical challenge for forming general parameters, techniques, and methodology for using SNPs for future disease association studies.

Ten SNPs were identified in a region of approximately 1.6 Mb immediately surrounding *APOE*. The SNPs were genotyped in samples of unrelated cases and controls and samples of AD sibships containing both affected and unaffected siblings. Our goal was to examine general patterns of association, both with the disease and between the markers themselves. Since the susceptibility locus was known in this case, it was possible to examine the association between the SNPs and the *APOE* alleles directly. Ultimately we want to determine whether we would have been able to identify *APOE* as a susceptibility locus for AD using association analysis with SNPs, and if so, under what conditions.

MATERIALS AND METHODS

Study subjects. We studied 1093 individuals, chosen to allow for both case-control and family-based association analyses. The case-control sample consisted of 270 AD cases with age of onset after age 59, mostly isolated (sporadic) cases collected at Duke University Medical Center Alzheimer Disease Research Clinic (DUMC ADRC), and 278 controls in the same age group, most of which were spouses of AD patients. A small number of controls were spouses of known non-AD dementia patients collected at the DUMC ADRC. To improve homogeneity, all individuals included in this study were Caucasians. The family sample consisted of 545 individuals in 183 sibships from Duke University, the National Institute of Mental Health, and Indiana University (Blacker *et al.,* 1997; Pericak-Vance *et al.,* 1997). Discordant siblings are required for the family-based association analyses, so we required that each sibship have two affected siblings and one unaffected sibling. Four of the sibships had only two individuals since one individual was removed from each for various reasons. To try to minimize potential misclassification of unaffected siblings, we selected the unaffected sibling that was oldest at age of exam from each sibship. This study focused on late-onset AD; thus, we required each affected individual to have age of onset no younger than 60 years. For the family data, all sampled affected family members, even if they were not selected from our database for this study, had to have age of onset after 59 years.

SNP generation. SNPs were generated by two different methods, which have been previously described (Lai *et al.,* 1998). For the proximal side of *APOE,* they were generated using truncated YAC fragments and *Alu–Alu* PCR products, which were sequenced and screened for polymorphisms. For the distal side of *APOE,* PACs were mapped and sequenced, and primers were constructed. For screening, seven random controls were sequenced for variation. SNPs detected were mapped and distances estimated between them using the (proximal region G3, distal region TNG) radiation hybrid panel. A map showing the 10 SNPs selected is given in Fig. 1.

SNP genotyping. The samples were completely genotyped at 10 SNP loci. Loci were chosen initially, if available, from each 100-kb "bin" away from the *APOE* locus. (The individuals had been genotyped at *APOE* previously.) A robotic liquid handling system (Packard MultiPROBE 204DT) was used to combine PCR reagents (1× Gibco PCR buffer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.04 μg/reaction primer, and 0.5 U/reaction Gibco *Taq* Platinum) and dispense 10 μl of master mix into 96-well microtiter plates containing 30 ng genomic DNA. Amplification was performed on MJ PTC200s using either a touchdown or a straight program for 40 cycles. Restriction enzyme was then added, and plates were incubated under conditions appropriate to the particular enzyme. Reactions were stopped by the addition of 60 μl of stop-dye (98% deionized formamide, 10 mM EDTA, pH 8.0, 0.025% xylene cyanol FF, 0.025% bromophenol blue). One microliter of each sample was loaded onto a 110-well 6% acrylamide denaturing gel. Gels were run on custom-made electrophoresis units (C.B.S. Scientific) for approximately 1 h and then fluorescently stained using SyberGold (Molecular Probes, Inc.). Samples were detected using a Hitachi FMBio II Scanner. Gel images were scored independently by two technicians. Data were then compared and entered for analysis.

As a quality control (QC) measure, since the subjects had little or no family structure, six individuals were duplicated per 96-well plate to help detect potential loading and reading errors (Rimmler *et al.,* 1998). All duplicated individuals were completely blinded to the technicians performing and reading the polymorphisms. QC samples were compared at the Center for Human Genetics Data Coordinating Center Group, and mismatches were reread or rerun in a continued blind fashion by the technicians.

Statistical analyses. In the case-control sample, we used the standard χ^2 test for equality of allele proportions in cases and con-

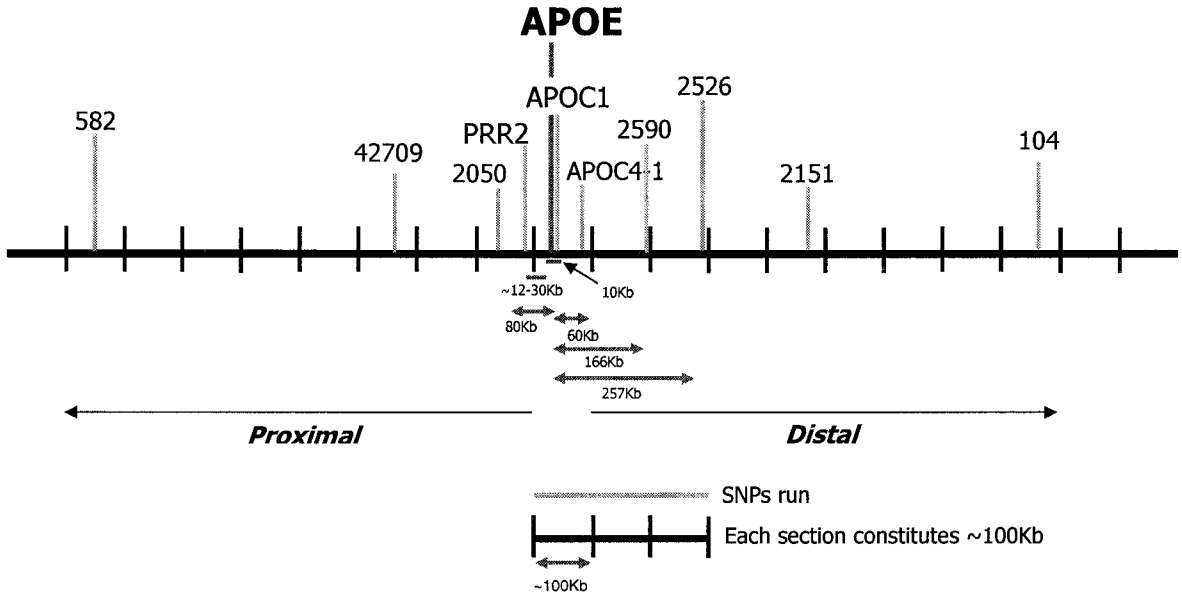

FIG. 1. Map of region surrounding *APOE* (19q13.2).

trols (see, e.g., Schlesselman (1982)). In the family sample, two statistics were used to test for association: the Sib-Transmission/Disequilibrium test (S-TDT) of Spielman and Ewens (1998) and the Sibship Disequilibrium test (SDT) of Horvath and Laird (1998). To guarantee a valid test of association for the S-TDT, we selectively sampled a single sib pair from each family, choosing the pair with the fewest marker alleles in common as suggested by Curtis (1997). For testing at *APOE*, having three alleles, a global multiallelic test statistic was used (Monks *et al.*, 1998) for the S-TDT. For both the S-TDT and the SDT, χ^2 approximations were used to calculate *P* values.

Tests for allelic associations between pairs of markers were conducted in the control sample. We used a likelihood ratio test with the EM algorithm, which allows for unknown phase of double heterozygotes (Weir, 1996). Since the test examines only biallelic loci, comparisons were made for each *APOE* allele (*APOE2, 3,* and *4*) versus the rest. For all analyses, χ^2 approximations were used to determine *P* values. Although many tests have been conducted in this analysis, since this work is exploratory in nature, we did not adjust for multiple tests.

Linkage analyses were conducted in the complete family data set using the means test from SIBPAL (SAGE, version 2.1; Louisiana State University Medical Center, 1994), which provides nonparametric tests for linkage that use discordant sib pairs as well as affected sib pairs. Since genetic distances between SNPs were not known, only two-point linkage analyses were conducted.

RESULTS

Marker/Disease Associations

We tested for association between the SNPs and AD with both a case-control test, using unrelated individuals, and family-based tests, using affected and unaffected siblings. Table 1 gives the allele frequencies in cases and controls and the results from the χ^2 case-control tests for allele frequency differences in cases with late-onset AD and age-matched controls. Some evidence of allelic association with AD ($P < 0.05$) was found with the case-control test for three of the SNPs: *2151, APOC1,* and *PRR2*. The association at *APOC1* was highly significant, with the positively associated allele occurring about twice as frequently in cases as in controls. The association at *APOC1* has also been identified in other studies (Yu *et al.*, 1994). In Table 1, we also show the result of the test for association between the alleles at *APOE* with AD in the cases and controls. As was expected, the result is highly significant.

Since the results for the family-based tests were qualitatively similar to those from the case-control test, we summarize those results in the text only. The family-based tests had *P* values less than 0.05 at two of

TABLE 1

Comparison of SNP Alleles in Controls and Cases

Marker[a]	Controls[b]	Cases[b]	*P* value
104	62 (0.13)	50 (0.10)	0.21
	426 (0.87)	442 (0.90)	
2151	222 (0.41)	249 (0.48)	0.017
	322 (0.59)	269 (0.52)	
2526	174 (0.44)	186 (0.44)	0.87
	224 (0.56)	234 (0.56)	
2590	158 (0.30)	135 (0.27)	0.23
	368 (0.70)	371 (0.73)	
APOC4-1	248 (0.48)	245 (0.50)	0.46
	272 (0.52)	245 (0.50)	
APOC1	107 (0.22)	200 (0.45)	3.50×10^{-13}
	375 (0.78)	248 (0.55)	
APOE	21 (0.07)	12 (0.04)	1.40×10^{-10}
	259 (0.78)	141 (0.56)	
	50 (0.15)	101 (0.4)	
PRR2	224 (0.46)	277 (0.54)	0.005
	268 (0.54)	233 (0.46)	
2050	177 (0.33)	182 (0.35)	0.58
	355 (0.67)	340 (0.65)	
42709	168 (0.36)	177 (0.41)	0.10
	298 (0.64)	251 (0.59)	
582	252 (0.48)	248 (0.49)	0.86
	272 (0.52)	262 (0.51)	

[a] One row for each allele at a marker.
[b] Allele count (frequency).

the SNPs. As with the case-control test, *APOC1* gave the most significant result ($P = 0.002$) and was replicated with both the S-TDT and the SDT. The SDT detected association with $P = 0.04$ at SNP *582*, but the S-TDT failed to reach significance at the 0.05 level. *APOE* itself gave highly significant evidence of association with AD in the family sample for both of the tests (S-TDT, $P = 2.4 \times 10^{-6}$; SDT, $P = 2.1 \times 10^{-5}$).

Marker/Marker Associations

Testing for allelic association between the SNPs and *APOE* directly did not reveal any additional associations not previously identified by the marker/disease analyses (data not shown). We found strong association between the *APOC1* alleles and each of the *APOE* alleles ($P < 0.0001$), with the low-frequency allele at *APOC1* occurring almost exclusively with alleles 2 and 4 of *APOE*. However, no significant associations with the *APOE* alleles were detected at any of the other SNPs. Additionally, little association was identified between the SNPs themselves. Of the 45 pairwise comparisons, only four had *P* values less than 0.1, with the smallest *P* value being 0.02 between SNPs *2590* and *42709* (data not shown).

Linkage Analysis

For comparison with the association analyses, linkage analyses were conducted in the sibships. To summarize the results, linkage analysis using the means test (SIBPAL) produced *P* values less than 0.05 for two of the SNP loci: *APOC1* ($P = 0.007$) and *PRR2* ($P = 0.029$). The results at *APOE* were highly significant with a *P* value less than 0.0001.

DISCUSSION

SNPs have been postulated to be the next level of genotyping marker. While much discussion has gone into the technical aspects of detecting these polymorphisms, only recently has any work been directed toward exploring the practical issues of their use. We believe that the best approach for this work is to use a known susceptibility gene, such as *APOE*, as a model. Our primary goals in this initial analysis were to characterize the association at SNPs around *APOE* and to begin to investigate the question: Could we have found *APOE* using SNPs?

To summarize the results presented here, we detected strong evidence of association at *APOC1*, lying only about 10 kb distal to *APOE*, and more moderate evidence of association at *PRR2*, approximately 12–30 kb on the proximal side of *APOE*. Thus, the region identified by markers having our strongest results for the association analysis spans no more than about 40 kb and contains the *APOE* gene. This is not to imply that this is the extent of the region of association. It is possible that more distant markers may be in linkage disequilibrium with *APOE*. The next closest markers to *APOE* that we tested, *APOC4-1* and *2050*, approximately 60 and 80 kb away, respectively, showed no evidence of association; however, we did find some evidence of association at *2151*, which is approximately 440 kb distal to *APOE*.

The family-based tests of association were consistently less significant than the case-control tests. This was expected on the basis of our power simulations (data not shown), which showed that the case-control test is expected to be more powerful than either of the family-based tests for the sample sizes used in this study. Consequently, larger samples will be required for family-based tests than will be needed in case-control tests, but there are clear advantages to using the family-based tests that may outweigh the additional cost in obtaining more individuals. It is well known that case-control tests may detect associations in stratified or admixed populations that represent differences in risk for the disease and allele frequencies in the strata, rather than represent biologically meaningful associations (Ewens and Spielman, 1995). The family-based tests used here are valid tests of linkage disequilibrium. That is, they test simultaneously for both allelic association and linkage. Additionally, the families may also be used in standard linkage analyses, such as the affected sib-pair tests that were used here. So the same families can be used for different types of analysis; separate samples for linkage and association studies are not required.

The fact that, with the exception of *APOC1*, little association was identified even in this small region is not surprising. The polymorphism associated with increased risk for AD, *APOE4*, is relatively common in the population, having a frequency of approximately 0.15 (Menzel *et al.*, 1983). This suggests that it is a relatively old polymorphism and is likely to be in, or close to, linkage equilibrium with most marker polymorphisms. Significant levels of association will likely be concentrated at markers very close to *APOE*. Therefore, current endeavors of the SNP Consortium and the HGP to develop hundreds of thousands of SNPs in the next few years would appear to be appropriate and are supported by this initial work.

The results from the linkage analyses agree quite well with the results from the association analyses. Like the association tests, the strongest result for the linkage tests, among the SNPs, was at *APOC1*, and marginal evidence was detected at *PRR2*. The fact that stronger evidence for linkage was not detected at more markers may be a result of the affected sib-pair structure of the families used in these analyses. In the original report of linkage to 19q, a marker BCL3, less than about 200 kb from *APOE*, showed strong evidence of linkage to AD genes in a sample of 32 larger extended families (Pericak-Vance *et al.*, 1991). It may also be a reflection of the relatively low informativity of SNPs compared to more polymorphic markers traditionally used in linkage analyses.

To address the issue of whether we would have been

able to identify *APOE* as a susceptibility locus for AD using SNPs, we need to put the question into context. The situation that we can address directly with this experiment is that of fine-mapping. In particular, the question of identification may more specifically be phrased: if a region, previously known to be linked to an AD gene, is saturated with SNPs, would we be able to localize the gene further? With the prior evidence of linkage, the results for association at *APOC1* and the more marginal results at *PRR2* and *2151* provide strong support for the existence of an AD susceptibility gene in this relatively small region. It is encouraging that the two markers closest to *APOE* (*APOC1* and *PRR2*) show the strongest evidence of association. The region bounded by these two markers is less than about 50 kb. Thus, the association analysis at the SNPs does refine the position of the susceptibility gene.

Even with the region narrowed, there is still the challenge of identifying the actual gene involved. The strong evidence for association at *APOC1* would have surely made *APOC1* itself a very strong candidate gene for AD. This result underscores the fact that even strong associations do not guarantee that the marker is in the disease gene. Although there is strong association with *APOC1* alleles and AD reported in this and other studies, no biological or functional involvement in AD susceptibility has been demonstrated for *APOC1*. Instead, this association is the result of linkage disequilibrium with the *APOE* mutation, which was detected in this and other studies (Lauer *et al.*, 1988; Poduslo *et al.*, 1995). Similarly, early studies reported association between AD and a polymorphism in the *APOC2* gene, located about 32 kb distal to *APOE*, but this association was not widely replicated across studies (Saunders *et al.*, 1993; Schellenberg *et al.*, 1987). In addition to the wide replication of findings of association with *APOE* and AD, several lines of evidence support a functional role of the *APOE* gene in AD (Strittmatter *et al.*, 1993a,b; Schmechel *et al.*, 1993). Thus, it was the convergence of positional and biological evidence followed by the demonstration of association with the functional polymorphism itself that ultimately led to the identification of the role of *APOE* in AD.

Finally, we want to emphasize that the strength of the effect of the *APOE4* allele was a critical factor in our findings here. The ability to detect allelic associations in case-control and family-based tests of association depends not only on the level of the allelic association, but also on the strength of the effect of the alleles at the disease locus on the susceptibility. The effect of the *APOE4* allele on susceptibility for AD is considered to be strong, with risk increasing by a factor of 2–4 for each *APOE4* allele carried by an individual (Corder *et al.*, 1993). For genes with a smaller effect, the power to detect a given level of allelic association will be lower, and detectable levels of association generally will span a smaller region.

This work has only begun to address some of the issues involved in using SNPs in large-scale association studies. We are continuing to build upon this initial work, to map the area of association around *APOE*, and to develop methodologies and techniques to use these important markers in the future.

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