I coauthored a recently published research article describing a variable length, poly-T polymorphism in the TOMM40 gene, adjacent to apolipoprotein E (APOE) on chromosome 19, that accounts for the age at onset distribution for a complex disease, late-onset Alzheimer disease. These new data explain the mean age at disease onset for patients with the APOE4/4 genotype and differentiate 2 forms of TOMM40 poly-T polymorphisms linked to APOE, with each form associated with a different age at disease onset distribution. When linked to APOE3 (encoding the ε3 isoform of APOE), the longer TOMM40 poly-T repeats (19-39 nucleotides) at the rs10524523 (hereafter, 523) locus are associated with earlier age at onset and the shorter TOMM40 523 alleles (11-16 nucleotides) are associated with later age at onset. The data suggest that the poly-T alleles are codominant, with the age at onset phenotype determined by the 2 inherited 523 alleles, but with variable expressivity. Additional data will further refine the relationship between the length of the poly-T alleles and age at disease onset and determine if the relationship is linear.

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Genetics has progressed remarkably fast and probably has affected diagnosis and treatment in neurology as significantly as in any other area of medicine except, perhaps, oncology. New drug treatments are more difficult and take longer to discover and develop in the field of neurology.1 Much of the genetics of neurological diseases only recently has been learned, creating a long list of metabolic and structural mutations that seems to justify the point of view of “splitters” in the old wars of “lumping or splitting” similar clinical presentations. Several genetic mutations that explain most early-onset Alzheimer disease (AD) have been discovered.2 Estimates of the genetic load for late-onset AD (LOAD) owing to APOE4 (encoding the ε4 isoform of apolipoprotein E [GenBank AF261279]) range from 20% to 70%, with most investigators agreeing on approximately 50%.2

In the recent article describing the association between TOMM40 (GenBank AF043250) poly-T polymorphisms and age at LOAD onset, a phylogenetic approach was used to examine the region around the APOE gene. Two related discoveries were made: age at onset for this late-onset, complex disease is determined by a traditional form of mendelian inheritance at the poly-T locus in TOMM40. The age at onset phenotype complexity is introduced by each polymorphic, poly-T allele rather than a myriad of small effects from many other genes. The phylogenetic method was critical for identifying the age at onset-associated polymorphism. The TOMM40 gene is located next to the APOE gene in a region of strong linkage disequilibrium (LD). This means that through human evolution few recombination events have occurred between the 2 genes, and specific variations in each gene have been co-inherited. However, additional mutations have accumulated within the LD region that contains TOMM40 and APOE, providing genetic heterogeneity. The phylogenetic experiments and analyses leading to the discovery of the role of the TOMM40 523

Author Affiliations: Department of Neurobiology and Neurology and the Deane Drug Discovery Institute, Duke University, Durham, North Carolina.
The clinical implications of the review is to explain the methods used, the data, and the information of a statistically robust phylogenetic tree that enriches LOAD cases into one of the clades. There were 2 independent, case-control series that were analyzed. The samples for this analysis were provided by the Arizona Alzheimer Disease Research Center. There were 105 individuals, or 210 chromosomes, in the phylogenetic analysis with approximately 2 patients with LOAD for each cognitively normal, APOE genotype–matched, and age- and sex-matched control. The polymorphisms that were analyzed were identified by deep Sanger sequencing of the region of interest from all copies of chromosome 19 in the analysis. The data in Figure 2 are identical but with different information superimposed for explanatory purposes.

In Figure 2, beginning with the single nucleotide polymorphism 922, the vertical lines represent successive mutation events in the parent sequence that create branch points in the phylogenetic tree. The 2 alleles of the single nucleotide polymorphism, 922, most effectively distinguishes the 2 main branches of the phylogenetic tree; the A allele is shared by all sequences in clade A and the G allele is shared by all sequences in clade B. This is an unrooted phylogenetic tree; it has not been rooted by including an obvious ancestral sequence that would help to determine the absolute chronology of mutational events. Therefore, in this article, use of terms that imply timing refer to the apparent order of events according to this tree structure and not the absolute timing of mutational events. However, even in the absence of absolute chronology, comparison of divergent clades that enrich for a phenotypic variable, such as age at LOAD onset, provides an opportunity to screen the clades for mutations related to that disease. Some of the branch-point mutations of the phylogeny are unique to the subsequent branch (clade) and can be used to differentiate one clade from the other, whereas other mutations appear in more than 1 branch of the tree. Each horizontal line in Figure 2 indicates a sequence and a stack of horizontal lines indicates a sequence that is shared by multiple individuals. Figure 2 illustrates the point that mutations occur on chromo-

**ANALYSIS OF THE SEQUENCING DATA: THE NEEDLE IN THE HAYSTACK**

Figure 1 shows the location of many single nucleotide polymorphisms and at least 4 poly-T tracts in TOMM40. Located in intron 6 is 523, the variable poly-T repeat that is associated with age of LOAD onset. A histogram of the frequency of the different-length poly-T alleles (in numbers of T nucleotides) in a case-control population is shown. Also indicated in Figure 1 is rs8106922 (hereafter, 922), which is the single nucleotide polymorphism that, in our analyses, anchors the split between 2 groups of evolutionarily related sequences that comprise the branches designated as clades A and B (Figure 2). The initial search for the potentially functional location in the LD region that comprises APOE, TOMM40, and APOC1 was guided by previous biological data. The goal was to see if one of the clades was enriched for patients with LOAD. Within this LD region, polymorphisms within a specific 10-kilobase piece of TOMM40 supported the formulation of a statistically robust phylogenetic tree that enriched LOAD cases into one of the clades. There were 2 patients for every control in the study shown in Figure 2. In clade A, however, the patient-control ratio was increased to 2:7, whereas in clade B the ratio decreased to 1:7 (Figure 2A). No other region that was analyzed supported a robust phylogenetic tree structure and did not enrich for patients into 1 clade. When the distribution of APOE genotypes across the clades was examined, almost all APOE4/4 resolved to clade A, whereas APOE3/4 and APOE3/3 were present in both clades. The data from the first case-control series analyzed in this manner were presented at the IX International Meeting on Human Genome Variation and Complex Genome Analysis (September 7, 2007). There was a strong hint that something was going on in this region, which happened to be located in the intronic sequence of TOMM40, not APOE.
somes in the context of earlier mutations, giving rise to sequence diversity even within regions of strong LD. The ovals in Figure 2A enclose mutations that introduce sequence diversity and divide the population into related, but distinct, clades. Terminal clades are enclosed by boxes in Figure 2B. Each terminal clade has a differential mutational history. Identifying a collection of phased mutations or polymorphisms within a region of strong LD, as represented in Figure 2, typically exceeds the capacity of genome-wide association studies. In addition, because many of these multiple mutations are uncommon, they are not adequately assayed in genome-wide association studies.

As described previously, clade A on the phylogenetic tree constructed from this region of TOMM40 was enriched for patients with LOAD. Further analysis showed that this enrichment was at least in part owing to an unequal distribution of individuals with the APOE4/4 genotype into clade A; 24.3% of the genotypes in clade A were APOE4/4, whereas APOE4/4 represented only 3.3% of clade B. The APOE3/4 genotype was evenly distributed between the 2 clades, and APOE3/3 was more prevalent in clade B.

An attempt was made to develop a complex algorithm, based on SNPs that differentiated clades A and B, which would estimate risk of LOAD. This proved difficult because some mutations were shared by multiple terminal clades, although on apparently different sequence backgrounds. The task became much simpler when the polymorphism that differentiated all the stacks of common haplotypes, enclosed by the boxes in Figure 2B, was identified. It was surprising to discover that each of these boxes was distinguished by the length of a polymorphic, poly-T locus, 523. Although the poly-T tract was approximately the same length for all haplotypes within each box, the average length of the poly-T tract was significantly different among the boxes. When the APOE background for each poly-T length allele was investigated, it became obvious why APOE4 had been identified as the risk gene for LOAD: APOE4 was virtually always connected to a long poly-T variant. Conversely, short poly-T variants were virtually always on APOE3 strands. However, some APOE3 alleles were connected to long poly-T variants.

Figure 3 shows histograms of the lengths of the 523 mutations on each APOE backbone for all individuals (patients with AD and controls) in this experiment. Looking first at the APOE backbones, all the poly-T lengths were greater than 19 T nucleotides and were typically 22 to 29 T nucleotides long. Looking next at the APOE3 backbones, there were clearly 2 separate poly-T length groups in relatively similar proportions. The short poly-T lengths were 11 to 16 T nucleotides, and the longer poly-T lengths were 29 to 39 T nucleotides. Thus, APOE3 alleles can now be characterized on the basis of being connected to either a long or short poly-T.

Further analyses demonstrated that all of the 523 short repeats always separated into clade B, whereas the long
poly-T length repeats, regardless of whether they were linked to APOE3 or APOE4, segregated to clade A. APOE4 occurred in clade A 97.8% of the time. Thus, looking at the precise distribution of poly-T lengths in geographically determined population studies of thousands of individuals will be much more informative than examining APOE status.

After confirming the phylogenetic structure of this region of TOMM40 and the distribution of the APOE and 523 genotypes on the tree, the association between 523 and the central endophenotype for LOAD, age at disease onset, was examined. This question was addressed using a separate population of patients with LOAD who had the APOE3/4 genotype and asking whether there was a difference in mean age at onset for carriers of the APOE3 long 523 vs APOE3 short 523 haplotype. The DNA and age at onset data for the well-characterized patients were obtained from the Duke Bryan Alzheimer Disease Research Center. All patients carried 1 APOE4 long 523 haplotype. Those patients who also carried a short 523 allele connected to APOE3 (n=5) had a mean age at onset of 78 years (Figure 4). Those patients who carried a second long 523 allele (n=29) connected to APOE3 had a mean age at onset of 70 years. This is similar to the age, first determined by Corder et al7 and verified during the last 17 years, at which 50% of APOE4/4 individuals have diagnosed disease. Analysis of the age at onset for a limited number of APOE3/3 patients suggests that there is earlier onset of cognitive impairment or LOAD for patients carrying long 523 alleles, consistent with observations for the APOE3/4 patients. The data discussed herein are from case-control cohorts rather than an epidemiologic study. Conclusions about population risk will have to wait for analyses of prospective, observational series of aged individuals. Several prospective studies of LOAD have been ongoing for 4 to 18 years and the plan...
The phylogenetic analysis of the APOE LD region indicates that each strand of the pair in every person has the capacity to add great phenotypic heterogeneity even within a relatively homogeneous group such as whites. In the case of LOAD, phenotypic complexity is measured as age at onset, obscuring the mendelian inheritance of the 523 locus and presenting as a late-onset, complex disease. The variable repeat that is inherited from each parent provides the phenotypic complexity measured by age at onset.

These new data inspire a new question: Is APOE the risk gene or is the disease-causing locus the 523 poly-T in TOMM40? Genetic parsimony and Occam’s razor would rest with 523 as the risk allele and APOE4 being identified because it is almost always connected to a long 523 poly-T allele. The genetic inheritance of the disease would now seem to be clearer than the biology.

For a drug discovery program, more than gene identification is relevant; critical for LOAD is to understand its pathogenic mechanisms and triggering events. It is known that apoE protein interacts with the mitochondrial outer membrane and that different apoE isoforms interact differently with and cause differential effects on the dynamics and function of neuronal mitochondria, leading to differences in neurite outgrowth.8-11 There is, therefore, likely also a role for APOE in disease pathogenesis. The proteins apoE and Tom40 are implicated in mitochondrial toxicity early in the proposed pathogenic process of AD, and perturbation of the normal activities of these proteins can explain the formation of amyloid plaques and other toxic protein aggregates. The so-called amyloid cascade exists and may exacerbate pathogenesis once initiated. However, early toxicity is likely initiated by apoE-Tom40 protein interactions, causing the release of cytochrome C from damaged mitochondria with subsequent apoptosis and initiation of the amyloid cascade.12,13 Targeting the long-term poisoning of mitochondria and increased apoptosis would seem to be a good way to delay the age at onset and interrupt the course of AD: the best place to turn off the water in a cascade is to block the source.

The variable-length, poly-T polymorphism appears to be stably inherited through human evolution rather than representing a site for sporadic, recurrent, contemporary mutational events. The phylogenetic approach identifies stable length mutations that occur on different evolutionary mutational backgrounds. The situation is, therefore, unlike the dominant triplet repeat diseases that have variable expressivity, such as myotonic muscular dystrophy, where the number of repeats is unstable through successive generations. Thus, it would seem that the 523 polymorphism could be used in a LOAD age at onset predictive test. On the basis of the age of the individual (between the ages of 60 and 87 years) and his or her 523 genotype, it is proposed that a risk estimate (ie, high or low) can be made for the onset of mild cognitive impairment symptoms and conversion to AD during the next 5 to 7 years (ie, not lifetime risk).

Replication and validation of the relationship between 523 and age at LOAD onset are necessary before the marker is used in clinical practice. For that reason, a prospective clinical study has been designed that combines validation of the genetic marker with assessment of the efficacy of a safe drug for delay of disease onset.14 A number of considerations regarding this clinical study have been discussed with scientists at the Food and Drug Administration via the Voluntary Exploratory Data Submission mechanism. Discussions with pharmaceutical companies have already been initiated by Zinfandel Pharmaceuticals Inc (Durham, North Carolina), a “virtual” drug development company organized to design, accelerate, and manage the clinical trial. During this prospective study, the poly-T assay will be developed into a Food and Drug Administration–qualified test. Even with the improved estimate of age at onset risk provided by the genetic marker, a study of this nature will take 5 or more years to complete.

Although the choice was made not to commercialize this test for clinical use until after validation, the test will be available for sponsored academic research. In parallel, licenses for commercial studies will be negotiated because the marker will have application in stratification of results from ongoing clinical trials of AD interventions and prospective stratification of studies. Given the range of APOE allele frequencies across ethnic groups, it is likely that different ethnic groups will have different allele frequencies for the poly-T variant as well. Ethnic diversity is not a problem if anticipated, and primary phylogenetic studies will be necessary to describe any differences in evolution of the LD region and to calculate the allele frequencies.

I anticipate, especially based on my early 1990s experience with the spirited debate around the diagnostic use of APOE genotyping in AD, that there will be differences of opinions on various ethical, legal, and social issues. I have therefore already spent more than a year in consultation with a panel of external, worldwide ethical, legal, and social issues experts who helped me develop the clinical plan.

There are many unanswered questions that will be addressed in the coming years, and one of them refers to the genetics of other so-called complex diseases. It is reasonable to consider that other small structural polymorphisms not detected by genome-wide association studies could be responsible for the variable expression of other complex diseases. These loci could be identified using targeted phylogenetic strategies. The work described in this review demonstrates the complexity of the genotype-phenotype relationship and is a reminder that we researchers are just beginning our journey to understand the human genome.

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Correspondence: Allen D. Roses, MD, Deane Drug Discovery Institute, 1 Science Dr, Ste 342, Campus Box 90344, Durham, NC 27708 (allen.roses@duke.edu).
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