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ORIGINAL PAPER

# Testing for Linkage and Association Across the Dihydrolipoyl Dehydrogenase Gene Region with Alzheimer's Disease in Three Sample Populations

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**Abstract** Prior case–control studies from our laboratory of a population enriched with individuals of Ashkenazi Jewish descent suggested that association exists between Alzheimer's disease (AD) and the chromosomal region near the *DLD* gene, which encodes the mitochondrial dihydrolipoamide dehydrogenase enzyme. In support of this finding, we found that linkage analysis restricted to autopsy-proven patients in the National Institute of Mental Health–National Cell Repository for Alzheimer's Disease (NIMH–NCRAD) Genetics Initiative pedigree data resulted in point-wise significant evidence for linkage (minimum $p$-value = 0.024) for a marker position close to the *DLD* locus. We now report case–control replication studies in two independent Caucasian series from the US and Italy, as well as a linkage analysis from the NIMH–NCRAD Genetics Initiative Database. Pair-wise analysis of the SNPs in the case–control series indicated there was strong linkage disequilibrium across the *DLD* locus in these populations, as previously reported. These findings suggest that testing for association of complex diseases with *DLD* locus should have considerable statistical power. Analysis of multi-locus genotypes or haplotypes based upon three SNP loci combined with results from our previous report provided trends toward significant evidence of association of *DLD* with AD, although neither of the present studies' association showed significance at the 0.05 level. Combining linkage and association findings for all AD patients (males and females) results in a $p$-value that is more significant than any of the individual findings' $p$-values. Finally, minimum sample size calculations using parameters from the *DLD* locus suggest that sample sizes on the order of 1,000 total cases and controls are needed to detect association for a wide range of genetic model parameters.

**Keywords** Energy metabolism · Mitochondria · Gene · Alzheimer's disease · Polymorphism · *M*-test · Heterogeneity · Diagnostic error · Diagnosis · Misclassification

Special issue dedicated to John P. Blass.

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## Introduction

Genetic and biochemical evidence indicates that Alzheimer's disease [AD; (MIM104300)] is a complex disease or syndrome in which multiple independent or interacting cascades lead to a common disease end-point. Evidence from neuro-imaging indicates a correlation between early deficits in brain energy metabolism and the development of AD. Biochemical evidence shows that reduced activities of the α-ketoglutarate dehydrogenase complex (KGDHC) [1–3] and pyruvate dehydrogenase complex (PDHC) [1, 4, 5] in brain are strongly correlated with AD.

In an earlier report [6] we described positive association between markers in the gene for lipoamide dehydrogenase (*DLD*) and AD. Lipoamide dehydrogenase is a catalytic component of both KGDHC and PDHC. A recent genome scan study of late-onset AD (LOAD) families [7] identified a marker in linkage with AD, D7S2847, that lies within 5 cM of the *DLD* locus. Furthermore, inherited mutations in the *DLD* gene have been associated with metabolic diseases that often involve severe neurological symptoms [8–10].

In our earlier study [6], four common SNPs were identified and genotyped in a case–control series of 297 Caucasians from New York City, including 229 residents of a Jewish nursing home (Jewish Home and Hospital for the Aged; JHHA). Point-wise significant associations with AD were observed for either *ApoE*4 ($p < 10^{-6}$) or sex combined with *DLD* genotype ($p = 0.013$). The association with the *DLD* genotypes appears only in the male population in both the Caucasian series ($p = 0.0009$, $n = 83$) and the Ashkenazi Jewish subseries ($p = 0.017$, $n = 49$) [6].

The positive association between the *DLD* region and AD motivated us to examine other AD case–control cohorts to learn if this finding holds in other study populations. Two independent cohorts were genotyped for the identical DLD SNPs as those described in the previous study [6]. One was a series composed of unrelated cases and controls selected from the National Institute of Mental Health (NIMH) Alzheimer Disease Genetics Initiative and additional cases and controls from the National Cell Repository for Alzheimer's Disease (NCRAD). The second was a cohort of Italian patients with sporadic AD. In addition, the NIMH Alzheimer Disease Genetics Initiative cohort was also ascertained for linkage on Chromosome 7 by affected sibling pair analysis [11].

## Experimental procedures

### Linkage analysis

Phenotype and genotype data were used as provided in the NIMH Alzheimer Disease Genetics Initiative Database. A fuller description of the data is provided elsewhere [11]. Blacker et al. [11] stratified the pedigrees into subgroups depending upon age-at-onset. That is, there were ''Late-onset'' pedigrees (referred to hereafter as the ''Late'' subset of families), in which all affected members became affected after 65 years of age, and ''Not-late'' pedigrees (referred to hereafter as the ''NLate'' subset of families), in which at least one member became affected before age 65. The ''Total'' set of families consisted of the combined Late and NLate subsets of families. For the purposes of this work, we focused on the Late-onset pedigrees.

Multi-point genetic model-free linkage analysis of Chromosome 7 markers was performed using GENEHUNTER-PLUS (ASM program; exponential model) [12, 13]. We performed three analyses, called AP (autopsy proven), NAP (non-autopsy proven), and combined (AP + NAP), for the Tot, Late, and Nlate subsets, as previously described [14]. We then directly tested for locus heterogeneity using an extension of a test statistic first proposed by Morton [15] (see Sect. ''Statistical analyses'' below). In the event that we observed significant evidence for heterogeneity, we a priori used the linkage analysis results from the AP pedigrees. Our rationale was that such pedigrees are less subject to diagnostic misclassification. There were a total of 299 pedigrees in the linkage analysis of the Late age-of-onset pedigrees, of which 68 were AP and 231 were NAP [14].

### Patients and controls

*Data set 1*: Subjects were collected as part of the NIMH Genetics Initiative following a standardized protocol developed and tested by three sites [16]. Each site collected information from family members, medical records and direct examination of the subjects in order to reach a diagnosis of AD by NINCDS/ADRDA criteria [17, 18].

The case control series studied was composed of unrelated cases and controls; only one subject was used (either case or control) from each family. Because linkage analysis of sibling pairs indicated evidence of linkage only in the autopsy-proven sib pairs and our original case–control study was limited to autopsied cases [6], we compared only autopsy-proven AD with controls in this series.

Data set 2: The second, Italian, series has been previously described [19]. All of the AD cases were clinically diagnosed following the Diagnostic and Statistical Manual of Mental Disorders criteria [20] and the Dementia Study Group of the Italian Neurological Society Guidelines for the diagnosis of dementia and AD [21].

Data set 3: The third set, JHHA, has been previously described and published [6]. While we do not perform any additional association analyses in this sample in this work, we use the results of the study when combining results across studies [see Sect. ''Combining *p*-values across independent studies to determine single *p*-value for association (Fisher combined *p*-value)''].

Summary counts of each of the different studies are presented in Tables 1 and 2.

## Genotyping

### *Case–control data*

Genotyping of the Exon 14 SNPs was done by primer extension and denaturing HPLC, as described in the earlier study [6]. The identifiers for these SNPs in the dbSNP database are as follows: 14A, rs8721; 14B, rs17154615; 14C, rs4564. The Exon 7 SNP [6], rs10263341 was genotyped using a custom TaqMan SNP genotyping assay and reagent kits provided by Applied Biosystems (Foster City, CA, USA).

### *Linkage analysis data*

As described by Blacker et al. [11], genotyping was performed at the Center for Inherited Disease

**Table 1** Counts of samples for linkage and association studies

| Study | Number of cases (affecteds) | Number of controls (unaffecteds) |
|---|---|---|
| NIMH–NCRAD (linkage) | 202 | NA[a] |
| NIMH–NCRAD (association)[b] | 135 | 197 |
| Italy | 242 | 247 |
| JHHA | 175 | 129 |

Sample counts for the populations considered in this study are provided. The JHHA counts come from our previous publication [6]

[a] Linkage analyses performed used only AP affected individuals

[b] The reason for the difference in numbers of cases for the linkage and association studies using the NIMH–NCRAD samples is that, only one AP case per pedigree was selected for the association study, while all AP cases in a pedigree were selected for the linkage study

**Table 2** Allele frequencies for three SNPs in *DLD* locus in sample populations considered in this study

| | | Minor allele frequency | |
|---|---|---|---|
| Sample | SNP | Cases | Controls |
| NIMH–NCRAD | 14A | 0.402 | 0.485 |
| | 14B | 0.144 | 0.133 |
| | 14C | 0.441 | 0.355 |
| Italy | 14A | 0.375 | 0.375 |
| | 14B | 0.119 | 0.163 |
| | 14C | 0.433 | 0.395 |
| JHHA | 14A | 0.425 | 0.378 |
| | 14B | 0.123 | 0.139 |
| | 14C | 0.356 | 0.385 |

Allele frequencies for the three *DLD* SNPs typed in the populations considered in this study are provided. The JHHA counts come from our previous publication [6]

Research using a modification of the CHLC version 9 marker set (381 markers, average spacing 9 cM, average heterozygosity 0.76, maximum gap 19 cM). We used the markers provided by NIMH database for Chromosome 7. A total of 20 microsatellite loci, at an average spacing of approximately 10 cM, were used in the linkage scan of Chromosome 7.

## Statistical analyses

### *Hardy–Weinberg and linkage disequilibrium studies with controls*

We performed a test of Hardy–Weinberg equilibrium (HWE) for each of the SNP loci in controls. Deviation from HWE in controls is often seen as resulting from some aberration of the data (e.g., genotype errors) [22–25]. We also determine whether each single-locus or three-locus diplotype frequency is significantly different between the NIMH–NCRAD and Italian data sets for controls.

Assuming that there is no significant evidence for differences between the two populations, we combine the SNP data in controls and compute linkage disequilibrium (LD) (as measured by $D'$ [26]) among all two-locus pairs. This computation is performed using the method implemented in the GOLD software [27] (see Sect. ''Electronic database information'').

### *M-test for heterogeneity in linkage analysis*

Here we provide a description of the *M*-test for heterogeneity that we applied in our previous work [14]. The text here is taken almost verbatim from that publication. The *M*-test is similar in purpose to the H-LOD statistic [28, 29] that is used when there is no a priori dichotomy. When *M* indicated that the data sets

were homogeneous, the linkage analysis using the combined pedigrees was used. A small $p$-value (<0.01) was considered to indicate linkage heterogeneity between linkage AP and NAP pedigrees. When $M$ indicated that the data sets are heterogeneous, we adopted the results for the AP pedigree data (a priori due to the more accurate diagnostic classification).

We directly test for locus heterogeneity using an extension of a test statistic first proposed by Morton [15]. The statistic is:

$$M(t) = \left( \sum_{i=1}^{2} [Z_i(t)]^2 \right) - [Z(t)]^2,$$

where $Z_i(t)$ is the multi-point Zlr statistic at position $t$ with families using the $i$th classification ($i = 1$ for AP linkage analysis; $i = 2$ for NAP linkage analysis) and $Z(t)$ is the multi-point Zlr statistic at position $t$ for the families in the combined linkage analysis; by combined, we literally mean that we concatenate the AP and NAP families together and perform linkage analysis for those data. Note that the Zlr statistic, developed by Kong and Cox [13], is a genetic model-free statistic that tests for increased allele sharing among affected relatives in general pedigrees. Under the null hypothesis that there is no increase in allele sharing among affected relatives, the Zlr statistic is normally distributed with mean 0 and variance 1. Similarly, under the null hypothesis that there is no linkage heterogeneity between AP and NAP families, $M(t)$ is asymptotically distributed as a central $\chi^2$ with 1 degree of freedom (df) [29] for two-point linkage analysis (not performed in this work). We use this distribution to compute $p$-values for $M(t)$. We note, however, that the $p$-values reported here should be viewed as approximate. As Ott [29] points out, multi-point linkage statistics are generally multi-modal and it is unclear whether asymptotic approximations hold as in two-point linkage.

### *Association testing using case–control data*

When testing for association (see Sect. "Logistic regression"), we analyzed these data sets separately for three reasons: (1) we wished to treat these data as two independent replication studies for our previous association of DLD with AD; (2) these data were collected by different groups in different geographical areas; (3) analysis of the NIMH–NCRAD cohort was restricted to autopsy-confirmed cases in order to be more consistent with our original study [6]. We discuss combined results below (see Sect. "Discussion"). In contrast, control SNPs from the two data sets were combined when performing marker LD analysis (see Sect. "Hardy–Weinberg and linkage disequilibrium studies with controls").

### *Logistic regression*

We performed association analyses for cases (affected) and controls (unaffected) using genotype data from three SNPs genotyped in the *DLD* locus, hereafter abbreviated as 14A, 14B, and 14C. We had previously shown association of diplotypes from these SNP and one additional SNP (Exon 7) in a male Ashkenazic Jewish case–control population ascertained for AD [6]. We later discovered that Exon 7 is in complete disequilibrium with Exon 14C and therefore we removed it from these analyses.

As in our previous work [6], we did not construct haplotypes but rather used the three-locus genotype data. We did this so that our results would be robust to deviations from the assumption of HWE [30, 31]. From this point forward, we shall use the abbreviation "DLD diplotype" for DLD three-locus genotype. The three-locus genotype in the current study corresponds to the last three digits of the four-locus genotype in our previous study [6].

We performed a logistic regression using affection (affected or unaffected) as the response variable and the following measures as independent variables: gender, number of $\varepsilon_4$ alleles in an individual's APOE genotype, and the *DLD* diplotype for the individual. If we define variables in the following way: $x_1$ = gender (values are 1 for male, 0 for female), $x_2$ = number of $\varepsilon_4$ alleles in APOE genotype (values are 0, 1, or 2), $x_3$ = DLD diplotype (values are presented below; Table 3), then we model the data using a logistic regression model [32]:

$$\log\frac{p(\vec{x})}{1-p(\vec{x})} = \beta_0 + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\sum_{\substack{j=1 \\ j\geq i}}^{3}\beta_{i,j}x_i x_j, \quad (1)$$

where $p(\vec{x}) = \Pr(\text{affected}|\vec{x}) = \Pr(Y=1|\vec{x})$, the probability that an individual is affected, conditional on the vector $\vec{x}$ of the individual's covariate measures $[\vec{x} = (x_1, x_2, x_3)]$, and the terms $\beta_i$ and $\beta_{i,j}$ are the parameters of the regression (*regression coefficients*) that maximize the likelihood function

$$\prod_{k=1}^{n} p(\vec{x}_k)^{Y_k}(1-p(\vec{x}_k))^{1-Y_k}. \quad (2)$$

In the likelihood function (2), we assume that there are data for $n$ individuals, and the subscript $k$ refers to the $k$th individual. Also, the term $Y_k$ refers to the affection status of the $k$th individual ($Y_k = 1$ for affected; $Y_k = 0$ for unaffected).

After performing the regression analysis, we determine the significance of each independent variable in the model by means of a likelihood ratio test (LRT). For each variable in the regression equation (1), we obtain a value for the corresponding LRT. A large LRT value, or equivalently, a significant $p$-value, indicates that the regression coefficient is point-wise significantly different from 0. We perform all the above-mentioned methods using the S-Plus 6.1 Release for Windows software (http://www.insightful.com)

### *Coding for DLD diplotypes*

For a given *DLD* diplotype, we used the sequence ''abc'' of coded genotype at Exon 14A (a), Exon 14B (b), and Exon 14C (c) to express an individual's *DLD* diplotype. The coding for genotypes at each SNP is the same: ''2'' = more common homozygote in controls; ''3'' = heterozygote; ''4'' = less common homozygote in controls. The *DLD* diplotype for an individual is then just the ordered concatenation of the three SNP genotypes. For example, the diplotype 224 means that Exons 14A and 14B have genotype code 2 (more common homozygote), while Exon 14C has genotype code 4 (less common homozygote). Note that some cells have counts <5, and we discuss below the results of our application of the Fisher–Freeman–Halton exact test [33, 34] to these data. The list of *DLD* diplotypes that were observed in each of the association studies is presented in Table 3.

We observed several different *DLD* diplotypes in our data set. However, many diplotype counts were <5. We binned all diplotype counts in each data set (NIMH–NCRAD and Italian) with counts of <5 and recoded the binned diplotypes as ''XXX.'' Also, when we stratified by gender and affection, some diplotypes still had counts of <5. Therefore, as a confirmatory analysis for those factors of interest, we applied the Fisher–Freeman–Halton's exact test [33, 34] to our stratified data. These calculations were performed using the method implemented in the StatXact v.3.0 software.

### *Combining p-values across independent studies to determine single p-value for association (Fisher combined p-value)*

In some instances, we wish to combine the $p$-values across multiple independent studies. For example, we wanted a global $p$-value of association between AD and the *DLD* diplotypes across our previous study [6] and the two association studies presented here. Similarly, we wanted a global $p$-value of association

**Table 3** *DLD* diplotype counts in NIMH–NCRAD and Italian series stratified by affection and gender

| Data set | | Male | | Female | | Combined | |
|---|---|---|---|---|---|---|---|
| Italy | Diplotype | Case | Control | Case | Control | Case | Control |
| | 224 | 16 | 7 | 22 | 26 | 38 | 33 |
| | 323 | 27 | 20 | 53 | 54 | 80 | 74 |
| | 324 | 7 | 5 | 5 | 8 | 12 | 13 |
| | 334 | 7 | 11 | 9 | 21 | 16 | 32 |
| | 422 | 17 | 12 | 26 | 27 | 43 | 39 |
| | 423 | 4 | 4 | 12 | 6 | 16 | 10 |
| | 433 | 7 | 18 | 19 | 15 | 26 | 33 |
| | 444 | 3 | 2 | 3 | 4 | 6 | 6 |
| | XXX | 2 | 1 | 3 | 6 | 5 | 7 |
| NIMH–NCRAD | 224 | 1 | 15 | 22 | 23 | 23 | 38 |
| | 323 | 10 | 28 | 30 | 40 | 40 | 68 |
| | 324 | 1 | 7 | 1 | 7 | 2 | 14 |
| | 334 | 4 | 10 | 9 | 15 | 13 | 25 |
| | 422 | 6 | 9 | 21 | 13 | 27 | 22 |
| | 423 | 2 | 3 | 4 | 3 | 6 | 6 |
| | 433 | 6 | 5 | 9 | 11 | 15 | 16 |
| | XXX | 1 | 1 | 8 | 7 | 9 | 8 |

The columns labeled ''224,'' ''323,'' etc., represent the coded three-locus diplotype counts for cases and controls, stratified by gender. For example, the diplotype code ''324'' refers to a diplotype in which the first genotype (14A) is 3 (heterozygote), the second genotype (14B) is 2 (homozygote 11), and the third genotype (14C) is 4 (homozygote 22). Also see Sect. ''Coding for *DLD* diplotypes.'' The column labeled ''XXX'' provides the total counts of three-locus diplotypes that were binned into one diplotype because their total counts were <5 in the overall sample

between AD and the males from these studies. To combine the *p*-values from these studies with the *p*-value from our previous association study of AD with DLD diplotypes [6] into a single global p-value for overall significance between AD and DLD diplotypes, we apply a method developed by R.A. Fisher. More specifically, when *p*-values from *n* independent studies are to be combined for a total inference in the form of a single *p*-value, R.A. Fisher's method (p. 99 in [35]) specifies that one should transform each value of *p*, which has a uniform distribution under the null hypothesis, into $c = -2 \ln(p)$, which has a Chi-square distribution with 2 *df*. The resulting *n c*-values are added together. Their sum represents a Chi-square variable with 2*n df*.

### *Point-wise and experiment-wise significance for p-values*

It is important to note that all of the *p*-values that will be reported in this work are ''point-wise'' *p*-values. That is, they report the results of one test applied to one data set. As has been well-established through research, performing multiple tests over multiple data sets will inflate the false positive rate, and researchers therefore need to distinguish between ''point-wise'' significance and ''experiment-wise'' significance [36]. We reiterate that, in this work, we only discuss point-wise significance.

### *Sample size requirements for genetic association testing with DLD locus*

One of the useful by-products of a LD analysis for a candidate gene study is an indication of the minimum sample size to detect association when applying case–control or pedigree-based association. The reason is that, one can estimate parameters such as inter-marker $D'$ or $\Delta^2$ [37], or average values of these parameters over all pairs. These values can then be used as input parameters into statistical power and sample-size calculators (e.g., Genetic Power Calculator [38], PAWE [39], PAWE-3D [40]). As noted with LD studies of other candidate genes [41, 42], inter-marker LD is often used as a surrogate measure for disease-marker LD.

It should be noted that baseline sample size requirements depend on multiple factors in addition to disease-marker LD measures. These factors include genotype relative risks [43], difference between disease and marker locus frequencies [44–49], and other factors. Gordon and Finch [50] provide an overview of these factors.

To provide some guidance on minimum sample size requirements to detect association for genes like *DLD*, we perform sample size calculations using the method implemented in the PAWE-3D webtool [40]. As noted on the webtool's homepage (see Sect. ''Electronic database information''), ''Power and sample size calculations are a critical part of the study design for genetic association analysis. Traditionally, statistical power for linkage or association analysis is computed by specifying genetic model parameters such as the disease allele frequency and the conditional probabilities Pr(affected|*j* copies of disease allele), where $j = 0$, 1, or 2 for a di-allelic disease locus. The conditional probabilities are often referred to as penetrances. Equivalently, one can specify the genotype relative risks [43] and the prevalence of the disease. While these values can usually be estimated with a high degree of accuracy for Mendelian disorders, they are typically unknown for complex diseases. One statistical method to deal with such uncertainty regards considering a range of values for parameters. One can then either report the 'worst-case scenario' (i.e., the smallest power or largest required sample size observed over the range) or median power and/or sample size values. One advantage is that researchers can observe a distribution of power values for the range of parameter values considered, including minimum, median, average, and maximum power.''

The significance level we choose for the sample size calculations is 0.01, which corrects for multiple tests that may be performed with a locus like *DLD*, where three SNPs were detected.

We use the following parameter specifications to compute the minimum sample size for the *DLD* locus: power = 0.9; ratio of controls/cases = 1; sampling distribution for parameters = beta distribution with mean 0.5 and variance 0.1; additive weights for linear trend test statistic; disequilibrium $\Delta^2$ range = (0.05, 0.5); genotype relative risk $R_1$ of heterozygote carrier range = (1.5, 3.5); genotype relative risk $R_2$ of homozygote range = (2.25, 15); disease allele frequency range = (0.2, 0.3); allele frequency of SNP marker allele in LD with disease allele range = (0.15, 0.45); disease prevalence range = (0.1, 0.25); misclassification of case as control probability range = (0.01, 0.10); misclassification of control as case probability range = (0.0, 0.10).

We briefly provide rationale for these settings here. Power is set to be high (0.9) because, from a design perspective, setting power to a high value at the design stage protects against issues such as genotyping errors [50] that may lead to power reduction. Also, equal numbers of cases and controls is optimal for statistical

power. We choose a beta distribution with mean 0.5 and variance 0.1 because we are primarily interested in sampling parameters from the midpoint of each of the ranges provided above. We choose the additive linear trend test as our statistic because of its false positive rate robustness to deviation from HWE in case and/or control populations [51]. The disequilibrium parameter values are chosen to be consistent with the findings of the *DLD* SNP LD analyses (see Sect. ''Results'' below). Genotype relative risks are chosen to cover a range of values; note that these parameter ranges include ApoE $\varepsilon_4$ effects, where the heterozygote relative risk $R_1$ is approximately 3 and the homozygote relative risk $R_2$ is approximately 10 [52]. Disease allele frequency ranges considered are those for more common variants. We note that we will require substantially greater sample sizes if the disease allele frequency is more rare (say <0.10). The range of SNP marker allele frequencies corresponds with ranges for SNPs selected for whole-genome association studies; that is, SNPs are usually filtered to be more polymorphic (minor allele frequency >0.10) [53]. The disease prevalence range given is consistent with LOAD, in that the prevalence increases as the population sampled is older. Finally, because it is well-documented that diagnostic misclassification may occur for Alzheimer's cases (and even controls) [54], we incorporate of range of phenotype misclassification parameter settings, to provide more realistic sample size calculations.

## Results

### Hardy–Weinberg and linkage disequilibrium studies with controls

Our goodness-of-fit tests for checking with control genotype counts in the three SNPs (14A, 14B, 14C) suggest that there is no evidence to reject the null hypothesis of HWE for any of the SNPs. The smallest *p*-value observed is 0.338 for 14B in the Italian data set (full results not shown).

Our LD analysis using the methods implemented in the GOLD software [27] is summarized in Table 4. Studying that table, we see that there is point-wise significant evidence for LD, as measured by the Chi-square statistic for each pair. In fact, the least significant *p*-value is 0.014, for the 14B–14C pair in the NIMH–NCRAD data. All other Chi-square values are ≤0.002. In particular, the Italian data set indicates highly significant evidence for all pairs (least significant *p*-value is $1.5 \times 10^{-10}$ for the 14A–14B pair). In addition, all pair-wise $D'$ values are estimated to be 1.0 for the Italian data set. The difference in LD measures and significances among the two data sets may be attributed to the fact that the Italian data set contains a more homogeneous population than the NIMH–NCRAD data set and therefore is expected to have higher inter-marker LD [55].

The pair-wise $D'$ values reported in Table 4 are in good agreement with the pair-wise $D'$ values derived from the HAPMAP project [56]. The Ex7-14A–B–C give $D' = 1$ for all pairs (Table 5). Furthermore, the pair-wise $D'$ values across the entire gene region for all 47 SNPs that were genotyped in the *DLD* gene ranges between 0.75 and 1 (data not shown). The Perlegen diplotype map of the *DLD* gene region indicates there are only 11 diplotypes for 24 Caucasians studied. These data taken together suggest that the SNPs that were genotyped in the current study are in strong LD with the entire *DLD* gene, and are sufficient to report all of the major diplotypes found in this region.

### Linkage analysis

Figure 1 presents the results of the linkage analyses for Chromosome 7 using the AP, NAP, and combined pedigrees. In addition, we present results for the *M*-test

**Table 4** Pair-wise linkage disequilibrium analysis for SNPs in *DLD* locus (controls)

| Data set | Marker 1 | Marker 2 | Delta[a] (bp) | $N$ | $\chi^2$ | $p_{val}$ | $\Delta^2$ | $D'$ |
|---|---|---|---|---|---|---|---|---|
| Italy | 14A | 14B | 10 | 248 | 41.06 | $1.5 \times 10^{-10}$ | 0.117 | 1 |
| | 14A | 14C | 190 | 248 | 153.86 | $2.5 \times 10^{-48}$ | 0.392 | 1 |
| | 14B | 14C | 180 | 248 | 45.85 | $1.3 \times 10^{-11}$ | 0.128 | 1 |
| NIMH–NCRAD | 14A | 14B | 10 | 197 | 9.38 | 0.002 | 0.047 | 0.573 |
| | 14A | 14C | 190 | 197 | 107.44 | $3.5 \times 10^{-25}$ | 0.411 | 0.890 |
| | 14B | 14C | 180 | 197 | 6.04 | 0.014 | 0.026 | 0.561 |

Results of graphical overview of linkage disequilibrium (GOLD) [27] linkage disequilibrium (LD) tests using our data and those from the HAPMAP project, are presented. Significant evidence for LD is determined using a Chi-square test. Estimates of inter-marker correlation $\Delta^2$ and disequilibrium $D'$ are also provided

*n* number of individuals used when performing pair-wise LD analysis

[a] Delta is the distance in nucleotides from the indicated SNP to the next SNP according to the map of Chromosome 7 on April 29, 2004

**Table 5** Pair-wise linkage disequilibrium values for SNPs in *DLD* locus from HAPMAP

| Marker 1 | Marker 2 | SNP1 | SNP2 | $\chi^2$ | $p_{\text{val}}$ | $\Delta^2$ | $D'$ |
|---|---|---|---|---|---|---|---|
| rs10263341 | rs8721 | Exon 7 | 14A | 58.67 | $9.0 \times 10^{-15}$ | 0.557 | 1 |
| rs10263341 | rs17154615 | Exon 7 | 14B | 7.737 | 0.0027 | 0.068 | 1 |
| rs10263341 | rs4564 | Exon 7 | 14C | 136.1 | $1.0 \times 10^{-31}$ | 0.967 | 1 |
| rs8721 | rs17154615 | 14A | 14B | 7.599 | 0.0029 | 0.048 | 1 |
| rs8721 | rs4564 | 14A | 14C | 61.25 | $3 \times 10^{-15}$ | 0.577 | 1 |
| rs17154615 | rs4564 | 14B | 14C | 8.013 | 0.0023 | 0.07 | 1 |

Results of graphical overview of linkage disequilibrium (GOLD) 27 linkage disequilibrium (LD) tests using our data and those from the HAPMAP project, are presented. Significant evidence for LD is determined using a Chi-square test. Estimates of inter-marker correlation $\Delta^2$ and disequilibrium $D'$ are also provided

results at each point. The results are presented in the form of *p*-values (-log transformed).

From Fig. 1, we see that the *M*-test *p*-values are <0.05 (-log transformed values >1.30) for two regions on Chromosome 7. The first region ranges from approximately 0 to 30 cM. In that region, heterogeneity is due significant linkage in the NAP pedigrees with no evidence for linkage in the AP pedigrees. As noted above and in our previous publication [14], in the event of evidence for heterogeneity, we test for linkage using the AP pedigrees. Therefore, we conclude that there is no significant evidence for linkage of AD in this region of Chromosome 7.

The second region ranges from approximately 105 to 115 cM. This region contains the *DLD* locus (approximately 109 cM). In this region, heterogeneity is due to significant linkage in the AP pedigrees with no evidence for linkage in the NAP pedigrees. The maximum Zlr statistic in the AP pedigrees is 1.97 [*p*-value = 0.024; -log(*p*-value) = 1.61] at position 109 cM. While this is not a test of association, it is interesting to note that we observe evidence for linkage near the *DLD* locus.

It is interesting to note that the peaks of our AP + NAP *p*-values (-log transformed) correspond with those of Blacker et al. [11], from which the linkage data were taken. While Blacker et al. did not observe genome-wide significance on Chromosome 7 in their study, they did have a relative peak near position 100 cM (not far from the *DLD* locus) in the Late subset of families.

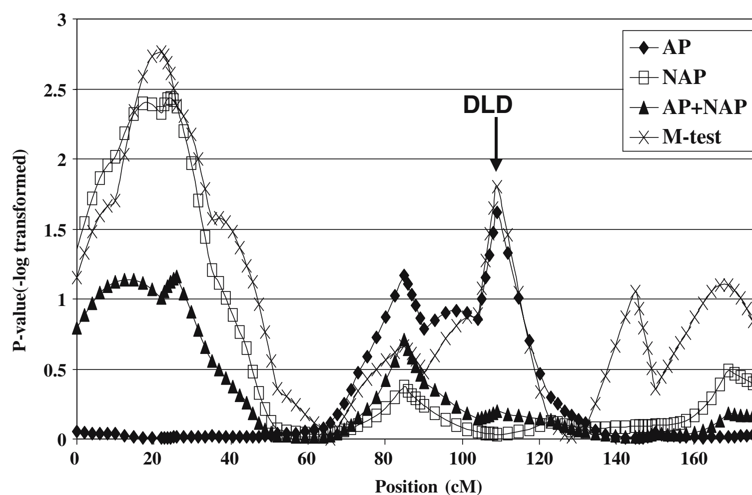


**Fig. 1** *p*-Values (-log transformed) for Zlr statistics and *M*-test on Chromosome 7 in NIMH late-onset pedigrees using AP, NAP, and combined (AP + NAP) pedigrees. Note that the peak at ~20 cM is due exclusively to signal from NAP pedigrees, with no support from AP pedigrees. In this figure, we present *p*-values (-$\log_{10}$ transformed) for AP, NAP, and combined (AP + NAP) Zlr scores at each position along the chromosome where GENEHUNTER-PLUS computed a Zlr score and the corresponding transformed *p*-value for the *M*-test at that position. The following symbols are used to represent the log-transformed *p*-values for each score: *filled diamond* AP Zlr, *open square* NAP Zlr, *filled triangle* combined Zlr, *cross symbol M* statistic

Association testing using case–control data

Association testing for *DLD* was repeated in two independent cohorts. The NIMH–NCRAD collection included only autopsied AD patients in order to more closely resemble the population used in our original study [6]. The Italian collection was composed of clinically diagnosed AD. We chose to test association in this cohort even though it lacked autopsy-confirmed AD cases because it had other advantages that might outweigh the power losses due to less accurate diagnosis. First, the group was more ethnically homogenous, which reduces background heterogeneity. Second, it was the size of the cohort increased the likelihood of observing evidence of a positive association.

We present the LRT values for each of the factors (i.e., regression coefficient) in Eq. 3 and for each population (NIMH–NCRAD, Italian) in Table 6. As mentioned above, the LRT statistic tests whether the coefficient for the corresponding factor is point-wise significantly different from 0; that is, it determines whether a particular factor significantly (in a statistical sense) contributes in an additive way to the affection status of an individual. Note that, under the null hypothesis that the corresponding factor is 0.0, the LRT is distributed as a central $\chi^2_m$ ($m$ = degrees of freedom). Degrees of freedom for each factor are provided in the third column of Table 6.

We see from the results of Table 6 that the factors that most significantly (point-wise) determine whether an individual is affected with Alzheimer's are dataset dependent. For the Italian dataset, the number of $\varepsilon_4$ alleles in a person's APOE genotype ($p$-value = $4 \times 10^{-19}$) is the only factor, at the 0.05 significance level, that significantly determines whether a person has Alzheimer's. Two other factors that tend toward point-wise significance, while not achieving it, are (1) the interaction between an individual's *DLD* diplotype and gender ($p$-value = 0.108), and (2) an individual's gender ($p$-value = 0.115).

In the NIMH–NCRAD data set, the factors that most significantly (point-wise) determine whether a person has Alzheimer's are (in order of ascending $p$-values): (1) the number of $\varepsilon_4$ alleles in a person's APOE genotype ($p$-value = $2 \times 10^{-6}$); (2) a person's gender ($p$-value = 0.001); (3) the individual's three-locus *DLD* diplotype ($p$-value = 0.069); and (4) the interaction between an individual's three-locus *DLD* diplotype and the number of $\varepsilon_4$ alleles in a person's APOE genotype ($p$-value = 0.07). While the last two factors are not point-wise significant at the 0.05 level, they tend toward point-wise significance. We additionally note that the interaction between an individual's *DLD* diplotype and their gender has a $p$-value = 0.157.

The APOE $\varepsilon_4$ effect has been well-documented [52, 57] and extensively replicated [54]. The interaction between an individual's *DLD* diplotype and their gender is of interest to us because of the observation in our previous study [6] that Alzheimer's appeared to be associated with a person's *DLD* four-locus diplotype in Ashkenazic Jewish males. To investigate the interaction more thoroughly, we applied Fisher–Freeman–Halton's Exact test to the male and female stratified *DLD* three-locus counts in the Italian and NIMH–NCRAD data sets (Table 3; see Sect. "Exact test results" immediately below).

**Table 6** Likelihood ratio test results for logistic regression analysis

| Data | Factor | LRT | *df* | $p_{val}$ |
|---|---|---|---|---|
| Italy | *AE4* | *84.913* | *2* | *$4 \times 10^{-19}$* |
| | Sex | 2.48 | 1 | 0.115 |
| | *DLD* | 9.994 | 8 | 0.265 |
| | AE4 × sex | 2.028 | 2 | 0.363 |
| | AE4 × *DLD* | 9.347 | 15 | 0.859 |
| | Sex × *DLD* | 13.119 | 8 | 0.108 |
| NIMH–NCRAD | *AE4* | *26.336* | *2* | *$2 \times 10^{-06}$* |
| | *Sex* | *10.309* | *1* | *0.001* |
| | *DLD* | 13.146 | 7 | 0.069 |
| | AE4 × sex | 1.000 | 2 | 0.607 |
| | AE4 × *DLD* | 19.865 | 12 | 0.070 |
| | Sex × *DLD* | 10.6 | 7 | 0.157 |
| JHHA | *AE4* | *24.810* | *1* | *$6 \times 10^{-07}$* |
| | Sex | 0.5711 | 1 | 0.449 |
| | *DLD* | 15.742 | 11 | 0.151 |
| | Sex × *DLD* | *22.486* | *10* | *0.013* |

The first column of this table indicates what data set is being considered: Italian or NIMH–NCRAD. In the second column, the abbreviations for each of the factors is as follows: sex = gender (values are 1 for male, 0 for female); AE4 = number of $\varepsilon_4$ alleles in APOE genotype (values are 0, 1, or 2); *DLD* = *DLD* diplotype at *DLD* locus (values are presented in Table 2). Also, the symbol "×" signifies the interaction term between two factors. Under the null hypothesis that the coefficient corresponding to a particular factor is 0, the LRT statistic has an asymptotic central $\chi^2$ distribution with degrees of freedom (*df*) equal to the number provided in the third column. The asymptotic $p$-value corresponding to a chosen factor's LRT statistic with corresponding *df* is provided in the last column of Table 4. All factors that are significant at the 0.05 level are printed in italics

Exact test results

When we stratified the data by affection and gender, some *DLD* diplotype counts showed cell counts <5 (Table 3). Therefore, we applied Fisher–Freeman–Halton's exact test [33, 34] to the male and female data in Table 3. Results of those analyses may be

found in Table 7. We make several observations regarding this table. The first is that none of the results is significant at the 0.05 level. Our second observation is that, for each data set, the *p*-value for males is more significant than for females.

We applied the Fisher combined *p*-value method to *p*-values 0.0009, 0.156, and 0.115, obtained from each of our association tests of *DLD* in Alzheimer's male cases and male controls ([6] and Table 7). We use Fisher's method as implemented in the *PVALUES* program [29] (see Sect. ''Electronic database information''). Our resulting global *p*-value is 0.0011, suggesting that the *DLD* locus is associated with AD in males. We comment that these results must be viewed with significant caution. For example, diplotype 224 appears to have a different pattern among females than males in both groups. When one focuses on males, for the Italy sample there is a 16 case to 7 control split, while for NIMH–NCRAD, the split is 1 case to 15 controls. The two populations appear to have very different distributions and splits. Using Fisher's method to combine *p*-values does not strengthen the original *p*-value (the Fisher combined *p*-value method is 0.0011 compared to the original *p*-value of 0.0009).

## Combining linkage and association results

Fisher's combined *p*-value method provides us with a technique to combine linkage and association results for our *DLD* analyses. The spirit of the results presented here is similar to that of meta analyses for whole genome scans [58, 59], where results are combined across different methods and samples through investigation of the *p*-values across studies.

Taking the most significant point-wise *p*-value for the AP linkage analysis ($p = 0.024$; Fig. 1), and combining it with the *p*-values of the association studies for *DLD* (0.265, 0.069, 0.151; Table 3) using Fisher's combined *p*-value method, we compute an overall *p*-value across the linkage and association studies of 0.013. It is interesting to note that the overall *p*-value, according to Fisher's method, is more significant than any of the individual *p*-values, suggesting that the region encompassing the *DLD* locus may contain a susceptibility gene for Alzheimer's. It is important to emphasize, again, that this is an exploratory method, and the *p*-value of 0.013 should not be interpreted as being *the p*-value for combined linkage and association testing, but rather a suggestive trend toward evidence for linkage and association, given that it is more significant than any of the individual *p*-values.

**Table 7** Fisher exact test results

| Data set | Sample | Exact test value | *df* | $p_{val}$ |
|---|---|---|---|---|
| Italy | Male | 11.59 | 8 | 0.156 |
| | Female | 8.75 | 8 | 0.364 |
| NIMH–NCRAD | Male | 10.88 | 7 | 0.113 |
| | Female | 8.72 | 7 | 0.271 |

Results of Fisher–Freeman–Halton's exact test [33, 34] applied to the contingency table data from Table 2 are presented. The value of Fisher–Freeman–Halton's exact test and the corresponding *p*-value for each sample within each data set is computed using the method employed in StatXact software version 3.0 (see Sect. ''Electronic database information'')

## Linkage disequilibrium patterns for *DLD* locus

Based on the results of the inter-marker LD for the three SNPs considered in the *DLD* locus, there is significant evidence for inter-marker LD. In particular, the average $\Delta^2$ value over all six values in Table 4 is 0.19. This average suggests that sample sizes must be increased by a factor of 1/0.19, or approximately 5.25, when using these SNPs for association testing to achieve the same power as if one were testing the disease locus [60]. Similarly, Table 5 reports the inter-marker LD measures for the HAPMAP *DLD* data [56, 61, 62]. We compute an average $\Delta^2$ value of 0.38 over all six values in Table 5. Therefore, one needs sample size increases on the order of 1/0.38, or approximately 2.62, when using the HAPMAP SNPs for association testing to achieve the same power as if one were testing the disease locus [63].

## Sample size requirements for genetic association testing with *DLD* locus

We present the results of the minimum sample size calculations for 0.90 power at the 0.01 significance level using the PAWE-3D webtool with parameter specifications as noted above (see Sect. ''Sample size requirements for genetic association testing with *DLD* locus'') graphically in Fig. 1. We also provide percentiles of the distribution in Fig. 2 and Table 8. From Table 8, we see that, minimum sample sizes needed to detect association at the 0.01 level are larger than what we have collected in the individual studies, although combining all samples together (e.g., adding cases and controls in Tables 1 and 2 for a total of 1,125) provides a minimum sample size in the range of the median sample size (934; Table 8) needed to detect genetic association with the parameters considered. We provide further comment on these results below (Sect. ''Discussion'').

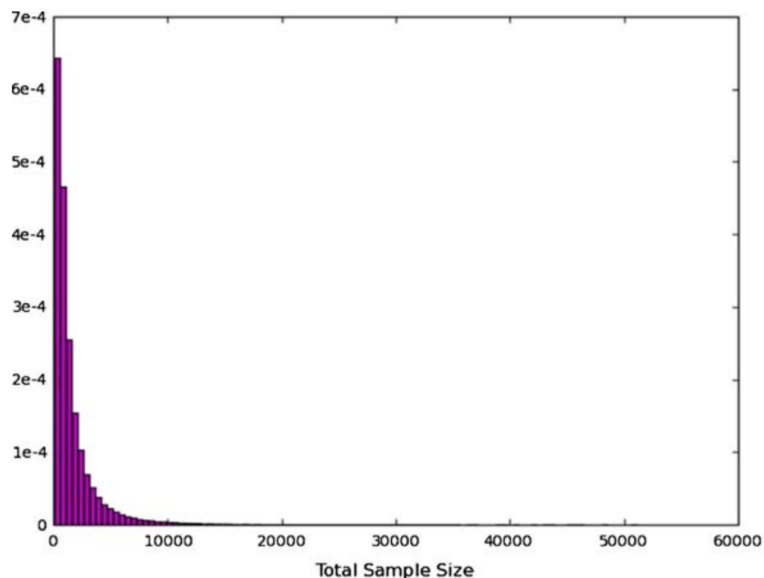


**Fig. 2** Histogram of minimum sample size calculations using PAWE-3D webtool. In this figure, we present the histogram of minimum total sample size (cases + controls) needed to detect association at the 0.01 significance level with a power of 0.90 assuming equal numbers of cases and controls. The figure is created using the method implemented in the PAWE-3D webtool (see Sect. ''Electronic database information''). Parameter setting ranges are described in Sect. ''Sample size requirements for genetic association testing with *DLD* locus''

**Table 8** Percentiles of minimum sample size calculations to detect association using PAWE-3D webtool (see Sect. ''Electronic database information'')

| Percentile | Minimum total sample size |
|---|---|
| 0 | 96 |
| 10 | 310 |
| 25 | 496 |
| 50 (median) | 934 |
| 75 | 1,918 |
| 90 | 3,758 |
| 100 | 51,140 |

Various percentiles of the minimum total sample size (cases + controls) needed to detect association at the 0.01 significance level with a power of 0.90 assuming equal numbers of cases and controls, are presented. The table is created using the method implemented in the PAWE-3D webtool (see Sect. ''Electronic database information''). Parameter setting ranges are described in Sect. ''Sample size requirements for genetic association testing with *DLD* locus''

## Discussion

We have performed linkage and association analysis of diplotypes in the *DLD* gene with Alzheimer's in the NIMH–NCRAD and Italian data series, controlling for Gender and ApoE $\varepsilon_4$ status. Perhaps the most interesting finding in this work is that, when we combine linkage and association $p$-values across all studies (NCRAD–NIMH, Italy, JHH), we compute a $p$-value that is more significant than any of the individual $p$-values (Sect. ''Combining linkage and association analyses'').

Our LD findings are that the three loci studied (14A, 14B, 14C) show highly significant $(p \ll 0.01)$ evidence for pair-wise association in controls in both series, the stronger findings being in the Italian series, which consists of data from a more genetically homogeneous population. These findings further suggest that genetic association studies that involve the *DLD* region should have good power to detect association as long as the baseline power (assuming one is typing the actual disease variant) is high [50], since the inter-marker LD observed here is rather low (0.2–0.4, on average).

While we realize that what we say here is speculative, the results of our sample size calculations (Fig. 2; Table 8) suggest that the reason that no single association study provides significant evidence for association with AD *may be* that the sample sizes for each individual study are too small. We repeat the point that, when we combine the linkage and association study results for *DLD* together, we see a $p$-value that is more significant than any of the individual $p$-value results for *DLD*. We comment that, only with sufficiently large sample sizes will we be able to rule out whether the *DLD* gene is in fact associated with AD.

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M.D. Electronic database information SNPbrowser software: www.allsnps.com/snpbrowser/ HAPMAP: www.hapmap.org GOLD: http://www.sph.umich.edu/csg/abecasis/GOLD/ PAWE-3D: http://linkage.rockefeller.edu/pawe3d/

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