Loss of Apolipoprotein E Receptor LR11 in Alzheimer Disease

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Background: Genetic, epidemiologic, and biochemical evidence suggests that apolipoprotein E, low-density lipoprotein receptors, and lipid metabolism play important roles in sporadic Alzheimer disease (AD).

Objective: To identify novel candidate genes associated with sporadic AD.

Design: We performed an unbiased microarray screen for genes differentially expressed in lymphoblasts of patients with sporadic AD and prioritized 1 gene product for further characterization in AD brain.

Setting: Emory University, Atlanta, Ga.

Subjects: Cell lines were used from 14 patients with AD and 9 normal human control subjects.

Results: Six genes were differentially expressed in lymphoblasts of 2 independent groups of patients with probable AD and autopsy-proven AD. We hypothesized that 1 of the genes, termed low-density lipoprotein receptor relative with 11 binding repeats (LR11) (reduced 1.8- and 2.5-fold in AD lymphoblasts vs controls), might be associated with sporadic AD on the basis of its function as neuronal apolipoprotein E receptor. We found dramatic and consistent loss of immunocytochemical staining for LR11 in histologically normal-appearing neurons in AD brains. This reduction of LR11 protein was confirmed by quantitative Western blotting ($P = .01$).

Conclusions: There is loss of the microarray-derived candidate, LR11, in neurons of AD brains. This study shows that microarray analysis of widely available lymphoblasts derived from patients with AD holds promise as a primary screen for candidate genes associated with AD.

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Alzheimer disease (AD) is the most important cause of aging-related dementia, and its prevalence has risen dramatically with increases in the oldest segments of the population. While the mechanisms underlying the disease remain poorly understood, deposition of senile plaques composed of fibrillar aggregates of amyloid-$\beta$ (A$\beta$) peptide is believed to play an important pathogenic role. Converging lines of evidence also implicate apolipoprotein E (ApoE), low-density lipoprotein (LDL) receptors, and lipid metabolism in AD pathogenesis. Epidemiologic data link serum cholesterol level, dietary fatty acids, and exposure to certain lipid-lowering drugs to AD risk. Apolipoprotein E is the most abundant apolipoprotein expressed in brain, and a common polymorphism in the APOE gene represents the major genetic risk factor for sporadic, late-onset cases of AD. Other genetic association studies implicate members of the LDL receptor family, which bind ApoE, in AD pathogenesis. The mechanism underlying these associations is unclear. Apolipoprotein E binds A$\beta$ peptide and may modulate A$\beta$ fibrillization and amyloid deposition, or promote internalization of A$\beta$ through the LDL receptor–related protein (see reviews).

Previous studies have shown abnormal biochemical responses in extraneuronal tissues in sporadic AD, including platelets and lymphoblasts. In addition, most genes implicated in AD are ubiquitously expressed, and skin fibroblasts from individuals carrying a familial AD mutation secrete excessive amounts of A$\beta$ peptide. Therefore, physiologically relevant alterations in AD might be reflected in shared gene expression changes in neural and extraneuronal tissues.
sues. In this study, we used complementary DNA microarrays to screen for genes differentially expressed in lymphoblasts from patients with probable or definite AD. This novel strategy identified changes in 6 transcripts, including the lipoprotein receptor LR11 (LDL receptor relative with 11 binding repeats).19,20 On the basis of its function as a neuronal ApoE receptor and its expression in the brain, we hypothesized that LR11 might play a role in sporadic AD. To test this hypothesis, we examined LR11 protein expression in AD brains.

METHODS

SUBJECTS

Lymphoblast lines were obtained from healthy elderly control subjects and patients with AD who were all well characterized via annual assessments in the Alzheimer's Disease Center at Emory University, Atlanta, Ga. Informed consent was obtained in accordance with the regulations of the institutional review board at Emory University. The diagnosis of probable AD was made according to criteria of the National Institute of Neurological Disorders and Stroke and consensus of 2 experienced clinicians. (Multiple clinicians, including A.I.L. and J.J.L., participated in establishing the consensus diagnosis.) The pathologic diagnosis of definite AD was made by a neuropathologist according to criteria of the Consortium to Establish a Registry for Alzheimer's Disease.21 (Multiple neuropathologists, including M.G., were involved.) Cell lines were used from a total of 14 patients with AD and 9 normal human control subjects (Table 1).

LYMOBASTS

Patient lymphocytes were immortalized by the Neitzel method22 in the Emory General Clinical Research Center. In brief, the lymphocytes were removed in the buffy coat layer after gradient separation (Histopaque-1077; Sigma-Aldrich Corp, St Louis, Mo). The lymphocytes were then incubated with Epstein-Barr virus (B95-8, American Type Culture Collection, Manassas, Va) in transforming medium consisting of RPMI-1640 (Gibco-BRL, Gaithersburg, Md), 20% heat-inactivated fetal bovine serum (Gibco-BRL), 2-µg/mL cyclosporine (Sandoz, Inc, Princeton, NJ), 2mMl-glutamine, and penicillin-streptomycin. After transformation, the lymphoblasts were maintained in RPMI-1640 medium supplemented with 15% heat-inactivated fetal bovine serum to a confluency of 80%. The mRNA was isolated according to a batch protocol (Oligoex; Qiagen) and quantified by spectrophotometry. Complementary DNA probe synthesis, hybridization with human UniGEM V complementary DNA microarrays, and signal analysis were conducted by Incyte Genomics (St Louis, Mo) as described.23 Transcript abundance for 7270 genes was assessed in experiment 1 and for 9374 genes in experiment 2. Because false-positive results are particularly high for low-intensity genes, a selective intensity filter (absolute fluorescence intensities ≥ 800) was applied to exclude genes with low hybridization signal intensities. Then, genes with an AD-to-control fluorescence intensity ratio (fold change) of 1.8 or greater were considered significant according to standard recommendations24 based on reproducibility data generated by Incyte Genomics, indicating that the level of detectable differential expression is 1.8-fold for UniGEM arrays.

NORTHERN ANALYSIS

In experiment 1, quantification of mRNA was additionally confirmed by Northern blot hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Northern hybridization analysis was performed with the use of formaldehyde to denature 500 ng of polyA RNA, followed by electrophoresis and transfer to a hybridization transfer membrane (GeneScreen; NEN Research Products, Boston, Mass). An in vitro transcription kit

Table 1. Characteristics of Subjects for Lymphoblast Cell Lines

<table>
<thead>
<tr>
<th>Patient No./ Sex/Age, y*</th>
<th>Diagnosis†</th>
<th>APOE Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental Group 1 (G1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5/F/76</td>
<td>Probable AD</td>
<td>4/4</td>
</tr>
<tr>
<td>2/F/77</td>
<td>Probable AD</td>
<td>3/4</td>
</tr>
<tr>
<td>5/M/71</td>
<td>Probable AD</td>
<td>3/2</td>
</tr>
<tr>
<td>4/F/79</td>
<td>Probable AD</td>
<td>3/4</td>
</tr>
<tr>
<td>5/F/76</td>
<td>Probable AD</td>
<td>4/4</td>
</tr>
<tr>
<td>6/F/77</td>
<td>Probable AD</td>
<td>3/4</td>
</tr>
<tr>
<td>7/M/82</td>
<td>Probable AD</td>
<td>3/4</td>
</tr>
<tr>
<td>8/F/NA</td>
<td>Probable AD</td>
<td>NA</td>
</tr>
<tr>
<td>Experimental Group 2 (G2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/M/76</td>
<td>Definite AD</td>
<td>4/4</td>
</tr>
<tr>
<td>2/M/73</td>
<td>Definite AD</td>
<td>3/4</td>
</tr>
<tr>
<td>3/M/68</td>
<td>Definite AD</td>
<td>3/3</td>
</tr>
<tr>
<td>4/M/85</td>
<td>Definite AD</td>
<td>3/4</td>
</tr>
<tr>
<td>5/F/62</td>
<td>Definite AD</td>
<td>4/4</td>
</tr>
<tr>
<td>6/F/83</td>
<td>Definite AD</td>
<td>3/4</td>
</tr>
<tr>
<td>Control Group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/F/81</td>
<td>G1, G2</td>
<td>3/3</td>
</tr>
<tr>
<td>2/F/80</td>
<td>G1</td>
<td>3/3</td>
</tr>
<tr>
<td>3/M/76</td>
<td>G1, G2</td>
<td>3/4</td>
</tr>
<tr>
<td>4/M/91</td>
<td>G1, G2</td>
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<td>5/M/57</td>
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<td>3/3</td>
</tr>
<tr>
<td>6/F/52</td>
<td>G1</td>
<td>NA</td>
</tr>
<tr>
<td>7/M/63</td>
<td>G2</td>
<td>NA</td>
</tr>
<tr>
<td>8/F/65</td>
<td>G1</td>
<td>3/4</td>
</tr>
<tr>
<td>9/7/72</td>
<td>G1, G2</td>
<td>4/4</td>
</tr>
</tbody>
</table>

Abbreviations: AD, Alzheimer disease; APOE, apolipoprotein E gene; NA, data were not available or missing.

*Mean ± SD age was 76.9 ± 3.3 years in group 1, 74.5 ± 8.8 years in group 2, 71.8 ± 13.1 years in control group G1, and 73.3 ± 12.3 years in control group G2. There were no significant differences in mean age between patient groups and their respective controls by 2-tailed t test (G1 patients vs controls, P = .33; G2 patients vs controls, P = .85).

†Definite AD was confirmed neuropathologically.

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with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif). 

The intensity of bands was quantified 


ized for protein loading by reprobing blots with anti–14-3-3 

dak Image Station 440cf (Eastman Kodak, New Haven, Conn) 

were captured and band intensities measured by means of a Ko-

nescence (Renaissance; Perkin-Elmer, Boston, Mass). Images 

at room temperature with secondary antibodies conjugated to 

Bcl2-A1 

phosphate dehydrogenase (GAPDH) is similar in both samples. 

(MEGAscript; Ambion, Inc, Austin, Tex) was used for [α phospho-

Bcl2-related protein A1 (Bcl2-A1) and interferon-γ receptor (IFNγ-R) are decreased in AD samples. Hybridization signal for glyceraldehyde-3-

Bcl2-A1 

DIN 32/5′-triphosphate ([γ-32P]dUTP) 

receptor mRNA were decreased, while hy-

primary antibody showed negligible staining. 

WES TERN BLOTTING 

Tissue samples from frontal cortex of 3 human controls and 6 

with lymphoblast mRNA from 6 patients with definite AD was compared with lympho-

From this initial microarray results with an independent method, 

fold changes observed for each of these genes by microarray 

positive staining (−2.1, −2.4, and +3.3, respectively). Hy-

primed normal controls (Table 1). In the definite AD 

mRNA levels of selected genes were assessed by North-

mRNA were below significance threshold (a complete list of differ-

were decreased by 1.8-fold or greater, expression of 3 was increased by 1.8-fold or 

and 7252 mRNAs were below significance threshold (a complete list of differently expressed genes is 

confirmed definite AD were compared with lympho-

LR11, is an ApoE recep-

SECONDARY MICROARRAY SCREEN 

IN LYMPHOBLASTS OF PATIENTS 

WITH DEFINITE AD 

We sought to validate our initial results and narrow the 

of the microarray screens is available from the authors). Down-regulation of 5 genes and up-regulation of 1 gene was confirmed in 

SECONDARY MICROARRAY SCREEN 

IN LYMPHOBLASTS OF PATIENTS 

WITH PROBABLE AD 

To screen for genes differentially expressed in lympho-

mRNA was increased 

LR11 domain.28 Blots were rinsed and incubated for 1 hour 

at room temperature with secondary antibodies conjugated to 

horseradish peroxidase (Pierce) and visualized by chemilumi-

emobilon-P; Millipore, Billerica, Mass). Blots were blocked in 

5% nonfat milk–Tris-buffered saline at room temperature for 

30 minutes, then probed overnight at 4°C with a polyclonal an-

mRNA from 6 patients with definite AD was compared with lymphoblast mRNA from 6 age-, sex-, and race-

nerm hybridization in the probable AD and control groups. 

Hybridization signals for Bcl2-related protein A1 and in-

receptor mRNA mRNAs were decreased during microarray experiments. In the first experimental set, im-

mortalized lymphoblasts from 7 patients with a clinical 

diagnosis of probable AD and 1 patient with autopsy-

confirmed definite AD were compared with lymphoblasts from a group of 8 age-, sex-, and race-

lymphoblast lines (Table 1). Of 7270 genes analyzed, 

expression of 15 mRNAs was decreased by 1.8-fold or greater, expression of 3 was increased by 1.8-fold or 

and 7252 mRNAs were below significance threshold (a complete list of differently expressed genes is 

confirmed definite AD were compared with lymphoblasts from a group of 8 age-, sex-, and race-

were analyzed. 

Table 2 

Comparison of candidate genes by analyzing samples from an in-

dependent group of patients with autopsy-confirmed dia-

nosis of AD. In this second experiment, lymphoblast 

mRNA from 6 patients with definite AD was compared with lymphoblast mRNA from 6 age-, sex-, and race-

matched normal controls (Table 1). In the definite AD 

group, of 9374 genes analyzed, mRNA expression of 108 was decreased by 1.8-fold or greater, expression of 7 was 

increased by 1.8-fold or greater, and 9259 mRNAs were 

below significance threshold (a complete list of differenti-

ally expressed genes identified in the microarray 

primary antibody showed negligible staining. 

IMMUNOHISTOCHEMISTRY 

Blocks of frontal cortex from 13 patients with AD and 7 con-

were then treated with hydrogen peroxide, washed in 

Tris buffer, blocked with normal serum, and incubated with 

anti–LR11 antibodies overnight at 4°C. On day 2, sections were 

inked with biotinylated secondary antibody followed by avi-

in the probable AD and control groups. 

Hybridization signals for Bcl2-related protein A1 and inter-

feron-γ receptor mRNA were decreased, while hy-

Figure 1. Technical confirmation of microarray results for 3 genes 

differentially expressed in lymphoblasts from patients with probable 

Alzheimer disease (AD) by Northern blotting. Compared with reference 

control RNA, hybridization signal for IgG3 subtype is increased, and signals 

for Bcl2-related protein A1 (Bcl2-A1) and interferon-γ receptor (IFNγ-R) are 

decreased in AD samples. Hybridization signal for glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) is similar in both samples. 

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that is predominantly expressed in brain and possesses structural and functional homologies to LDL receptor–related protein, a receptor etiologically linked to AD. On the basis of these considerations, we selected LR11 for further examination in control and AD brains.

**LR11 PROTEIN EXPRESSION IN HUMAN BRAIN**

To establish the potential biological relevance of changes in LR11 gene expression for AD, we examined LR11 in control and AD brains at the level of protein expression. Immunohistochemistry of 13 AD brains and 7 controls showed a remarkable reduction in LR11 expression in AD (Figure 2A and B). In control brains, pyramidal neurons in the frontal cortex showed strongly labeled small cytoplasmic puncta throughout the cell body and the proximal dendrites (Figure 2C). In striking contrast, there was dramatic loss of LR11 staining in pyramidal neurons in AD frontal cortex (Figure 2D). The difference between control and AD brains was remarkably consistent, and marked loss of LR11 staining in pyramidal neurons was found in each of the AD cases examined. In addition to neurons, punctate LR11 staining was also found in glial cells. However, unlike pyramidal neurons, glial staining in frontal cortex was preserved in AD brains (Figure 2E and F). Hematoxylin-counterstained hippocampal dentate granule neurons showed strong LR11 immunoreactivity in controls (G), but very little staining in AD brain (H). Scale: in A and B, bars indicate 100 µm; in C-H, 10 µm.

**Table 2. Identities of Consistently Altered Transcripts in Microarray Screens**

<table>
<thead>
<tr>
<th>Fold Change*</th>
<th>Gene Name Function Accession No.</th>
<th>Function</th>
<th>Accession No.</th>
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<tr>
<td>-1.8</td>
<td>LDL receptor relative with 11 repeats (LR11)</td>
<td>LDL receptor</td>
<td>Y08110</td>
</tr>
<tr>
<td>-2.4</td>
<td>Interferon-γ receptor 1</td>
<td>Interferon receptor</td>
<td>J03143</td>
</tr>
<tr>
<td>-2.1</td>
<td>Stimulated trans-acting factor (Staf-50)</td>
<td>Transcription regulation</td>
<td>AA853455</td>
</tr>
<tr>
<td>-1.8</td>
<td>Pleckstrin</td>
<td>PKC substrate</td>
<td>X07743</td>
</tr>
<tr>
<td>-2.3</td>
<td>Amylo-(1,4-1,6)-transglycosylase 1</td>
<td>Glycogen branching enzyme</td>
<td>L07956</td>
</tr>
<tr>
<td>+1.9</td>
<td>Homo sapiens SNC73 mRNA</td>
<td>Immunoglobulin heavy chain</td>
<td>AF067420</td>
</tr>
</tbody>
</table>

Abbreviations: AD, Alzheimer disease; LDL, low-density lipoprotein; mRNA, messenger RNA; PKC, protein kinase C substrate.

*Change in transcript abundance compared with reference control mRNA.
in neuronal LR11 staining by immunocytochemistry. Western blotting indicated relatively modest reduction in LR11 band intensity in total cortical homogenates. This difference may reflect the contribution of glial LR11, which is retained in AD cortex (Figure 2E and F).

Our findings suggest a novel link between LR11 and AD; to our knowledge, this report is the first to identify a candidate disease-associated gene in an unbiased microarray screen of blood from patients with AD. Peripheral cells express genes associated with AD, and model some processes involved in pathological changes of AD brains. The structure and function of LR11 as a mosaic ApoE receptor lends biological plausibility to the microarray results, and examination of LR11 in brain strongly supports the hypothesis that it plays a role in AD. In agreement with previous studies, we detected LR11 in widespread populations of neurons in neocortex, limbic cortex, and cerebellum. In AD brains, LR11 immunoreactivity was lost with remarkable consistency. Moreover, LR11 staining was decreased specifically in neurons, but staining was preserved in glia. The loss of immunoreactivity was not simply due to cell loss, as hematoxylin counterstaining showed otherwise healthy-appearing neurons (Figure 2). Most of the AD brains in this study were from patients with late-stage disease. Additional studies of patients with mild AD and mild cognitive impairment will be helpful in determining whether LR11 plays a role in early stages of disease development.

The unique multidomain structure of LR11 suggests potential roles as a cell-surface lipoprotein receptor and as an intracellular sorting receptor. There is a cluster of extracellular binding repeats and a cytoplasmic internalization sequence that are present in all endocytosis competent lipoprotein receptors. In addition, LR11 contains a VPS10 homology domain near the amino terminus and a Golgi-localized, gamma-ear-homology domain, adenosine diphosphate–ribosylation (ARF)–binding protein (GGA) binding domain in the cytoplasmic tail. The VPS10 domains are involved in trafficking from the Golgi to the vacuole in yeast, and GGAs have been shown to mediate trafficking between Golgi and the endosomal–lysosomal system. Given its structural features, LR11 seems to be ideally positioned to interact with AD-associated proteins, and additional studies suggest that LR11 expression may influence levels of Aβ (K.O., unpublished data, 2003).

This exploratory study suggests that gene expression analysis of widely available lymphoblasts derived from patients with AD holds promise as a primary screen for candidate genes associated with AD. Our current studies, using this approach, identified the brain ApoE receptor, LR11, as an intriguing candidate molecule for sporadic AD. Further studies using larger sample sizes and refined microarray and bioinformatics procedures coupled with mechanistic validation of candidates are warranted.

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REFERENCES


