Identification of Alzheimer Disease Risk Genotype That Predicts Efficiency of SORL1 Expression in the Brain

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Objective: To identify SORL1 risk genotypes that determine receptor protein expression in the human brain.

Design: DNA, RNA, and proteins were extracted from brain autopsies of Alzheimer disease cases and used for SORL1 genotyping, RNA profiling, and SORLA protein quantification, respectively.

Setting: Specimens were provided by the MRC London Brain Bank for Neurodegenerative Diseases and the Netherlands Brain Bank.

Subjects: Brain autopsy material (frontal cortex) from 88 confirmed cases of sporadic Alzheimer disease.

Results: Our studies identified a SORL1 haplotype in the 3’ gene region consisting of single-nucleotide polymorphisms rs1699102 and rs2070045 that is associated with poor receptor expression in the brain of patients with Alzheimer disease. These gene variations alter the SORL1 transcript sequence, resulting in a change from frequent to rare codon usage in the minor risk genotype. Studies in cultured cells confirm less efficient translation of the minor receptor transcripts into protein.

Conclusion: Our findings suggest a functional mechanism that correlates SORL1 genotype with efficiency of receptor expression in the human brain.

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BRAIN AUTOPSY MATERIAL

Brain autopsy specimens from the frontal cortex were obtained from the Netherlands Brain Bank (Netherlands Institute for Neuroscience, Amsterdam) and the MRC London Brain Bank for Neurodegenerative Diseases (Institute of Psychiatry, King’s College London). The ethnicity of samples was white. All material was collected from donors for or from whom a written informed consent for a brain autopsy and the use of the material and clinical information for research purposes had been obtained by the Netherlands Brain Bank or the MRC London Brain Bank for Neurodegenerative Diseases. DNA, RNA, and membrane proteins were extracted from the snap-frozen tissues by standard procedures and used for genotyping, quantitative reverse transcriptase–polymerase chain reaction (PCR), or enzyme-linked immunosorbent assay (ELISA), respectively. Genotyping was performed using the TaqMan SNP Genotyping Assay, according to the manufacturer’s recommendations, using the 7900HT Fast Real-Time PCR System (Applied Biosystems). RNA was extracted from brain specimens using the Trizol method and transcribed into complementary DNA (High Capacity RNA-to-cDNA Kit; Applied Biosystems). TaqMan gene expression assays were applied to quantify SORLA (Hs00983791_m1) messenger RNA (mRNA) levels. Human β2-microglobulin (Hs00984230_m1) transcript levels were determined as the internal control.

ENZYME-LINKED IMMUNOSORBENT ASSAY

For quantification of SORLA, we developed a custom-made ELISA in 96-well plates (Nunc Maxisorb F96; Thermo Fisher Scientific) coated with 1 µg/mL of rabbit anti-SORLA IgG (IgG 3587) in coating buffer (100mM sodium bicarbonate, pH 9.8) at 4°C overnight. After rinsing in wash buffer (150mM sodium chloride, 7.5mM disodium phosphate, 2.8mM monosodium phosphate, and 0.05% Tween, pH 7.4), coated wells were blocked with wash buffer supplemented with 2.5% casein (Sigma-Aldrich) at 4°C overnight. Protein lyases or recombinant SORLA protein standards (purified from stably transfected CHO cells) were loaded on the wells and incubated overnight at 4°C. The next day, the plates were washed and incubated with mouse monoclonal anti-SORLA antibody (IgG 20c11) at a final concentration of 1 µg/mL in wash buffer overnight and followed by rabbit antimonouse polyhorseradish peroxidase IgG for 2 hours at room temperature (1:2500 in wash buffer; Dako). Colorimetric staining was performed by adding 100 µL of peroxide buffer (Thermo Fisher Scientific) with o-phenylenediamine (Pierce) for 30 minutes at room temperature and measured on an ELISA reader at 450 nm.

CELL CULTURE EXPERIMENTS

The major allele variants of rs2070045 and rs1699102 present in the human SORLA complementary DNA were converted into the minor genotypes by PCR-based site-directed mutagenesis using primers 5′-cag gga ggt gtc Gga gag-3′ and 5′-ctt cat cCg acc agt ccc cgg tg-3′ for rs2070045 and 5′-ctg ccc aaa Cgg cac ttg ca-3′ and 5′-tgca ggc tgc cGt ggc gtc-3′ for rs1699102. Both allele variants were introduced into pcDNA3.1zeo vector and transiently transfected into CHO cells using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were harvested for RNA and protein preparation. SORLA transcript and protein levels were quantified by TaqMan and ELISA as described earlier. For determination of transfection efficiency, zeoC2A3 resistance cassette transcripts and 18s ribosomal RNA were amplified by primers 5′-cGC CGC TAG AGG TGA AT TC-3′ and 5′-TGGA CGA AAT GCT TTC GCT GC-3′ (18s ribosomal RNA) and 5′-AGT TGA CCA GTG CCG TTC-3′ and 5′-GAT GAA CAG GAT GCC GAT-3′ (zeoC2A3 resistance cassette transcripts) applying the Power SYBR Green Real-Time PCR System (Applied Biosystems). Differences in expression levels were calculated using the Pfaffl method.17

STATISTICAL ANALYSES

Statistical analyses were performed with GraphPad Prism software (GraphPad Software). Nonparametric tests were used to evaluate all data. The Mann-Whitney test was applied to differences between 2 experimental groups. Differences between more than 2 groups were tested using a Kruskal-Wallis 1-way analysis of variance test with a Dunn post hoc test. To account for multiple comparison, we applied a permutation-based method to control the experiment-wise error rate, as described by Churchill and Doerge,18 considering that the tests are highly correlated owing to strong linkage disequilibrium between SNPs. Haplotype distribution in SORL1 was estimated using MaCH.19 Linkage disequilibrium is shown as D′ and significance for deviation from Hardy-Weinberg equilibrium was obtained from a χ2 test (Haplovie; MIT/ Harvard Broad Institute).

RESULTS

We compiled a sample set of brain autopsy material (frontal cortex) from 88 confirmed cases of AD. Donors were of white ancestry. Specimens were provided by the MRC London Brain Bank for Neurodegenerative Diseases (44 individuals) and the Netherlands Brain Bank (44 individuals). Because no discernable differences in sex, age, or clinical scores were evident, both sample sets were combined into 1 study (Table 1). DNA, RNA, and membrane proteins were extracted from snap-frozen tissues of each individual by standard procedures and used for genotyping, RNA profiling, and SORLA protein quantification, respectively.

For genotyping, we tested 12 SNPs in the SORL1 region that have previously been associated with AD in white populations11,15 (Figure 1A). These SNPs cover the 5′ and 3′ regions of the gene that have both been genetically linked to disease.11 Single-nucleotide polymor-
phism genotyping replicated the reported distribution of major and minor allele frequencies in our sample set (Table 2). Also, linkage disequilibrium analysis confirmed the existence of 2 distinct haplotype blocks in the 5’ and 3’ regions of SORL1 (Figure 1B).

In parallel, we determined SORLA protein concentrations in brain specimens of our sample set. To do so, we developed a sensitive sandwich ELISA using 2 different antihuman SORLA antisera for antigen capture and detection, respectively (see the “Methods” section for details). Replicate determination of SORLA levels in brain extracts demonstrated high interassay precision and reproducibility (eFigure 1A, http://www.archneurol.com). Also, SORLA concentrations determined by ELISA correlated well with semiquantitative analysis of receptor levels by Western blotting in selected samples (eFigure 1B and C).

To explore whether SORLA expression may be associated with distinct genotypes, we next correlated the 12 SNPs in SORL1 with protein concentration in the brain. In doing so, we identified 2 SNPs (rs1699102 and rs2070045) that were significantly associated with protein levels in affected individuals ($P = .01$ and $P = .03$, respectively). In line with poor receptor expression being considered disease promoting, the minor (risk) genotype was consistently correlated with lower receptor levels. SORLA levels in heterozygous carriers were intermediary, further supporting a link between genotypes and receptor expression (Figure 2A and B). The strength of correlation between risk genotype and expression levels for both SNPs was further improved when samples were adjusted for age, sex, and APOE4 status using linear regression analysis ($P = .006$ and $P = .03$, respectively) (Figure 2C and D). No statistically significant association of receptor level with genotype was observed for the remaining 10 SNPs (Figure 3). Analyses adjusted for multiple testing yielded similar findings with statistically significant association of SNP rs1699102 with SORLA brain levels ($P = .02$) and a trend for association with rs2070045 ($P = .10$) (see the “Methods” section for details).

Single-nucleotide polymorphisms rs2070045 (SNP 19) and rs1699102 (SNP 22) clustered in a haplotype block (block 2) in the 3’ region of SORL1 in our sample set (Figure 1B). Haplotype analysis replicated association of the major or minor haplotype with higher or lower SORLA levels, respectively, as shown in Table 3. In detail, haplotype distribution of SNPs in the 5’ haplotype block (block 1) did not show association with SORLA levels, in line with our previous results in the individual SNPs in this region (SNPs 8, 9, and 10) (Table 3). In contrast, significant association of the 3’ haplotype block (block 2) with the major variant TCTCAT (haplotype frequency, 58.7%) correlating with high SORLA levels ($P = .049$) and the minor variant GCCTCCC (haplotype frequency, 26.5%) correlating with low levels ($P = .04$) was reproduced. All other haplotypes were estimated with frequencies less than 5%. However, the strength of association of the minor allele variant ($P = .04$) did not improve as compared with the analysis of the individual SNPs rs2070045 ($P = .03$) and rs1699102 ($P = .01$) on a nominal significance level.

Remarkably, rs1699102 (SNP 22) and rs2070045 (SNP 19) were the only 2 SNPs among the 12 sequence variations tested that were exonic. Although both SNPs represent silent mutations, alteration of the primary transcript sequence may potentially affect SORL1 expression. No significant correlation of genotype with SORL1 mRNA levels ($P = .22$) was seen in a selected set of samples of major or minor SNP genotypes, arguing against an influence of these polymorphisms on transcription efficiency or mRNA stability (Figure 4).

To explore alternative effects of SORL1 sequence variations on receptor expression, we generated SORLA complementary DNA constructs encoding the major or minor alleles of rs1699102 and rs2070045 (eFigure 2A). SORLA was efficiently expressed from both constructs following transient transfection of Chinese hamster ovary (CHO) cells (eFigure 2B). To accurately quantify SORLA expression, we transiently transfected replicate CHO cell layers with both constructs and determined SORLA mRNA and protein levels using quantitative reverse transcriptase–PCR and ELISA, respectively. As the internal control, we quantified the levels of zeozin resistance cassette transcripts encoded by the vector backbone (eFigure 2A). Levels of zeozin resistance cassette transcripts relative to 18s ribosomal RNA were identical in major and minor haplotype transfectants, demonstrating equal transfection efficiency (Figure 5A). Also, no difference in SORLA transcript levels relative to zeozin resistance cassette transcripts was observed, indicating identical transcription efficiency of both SORLA minigene variants (Figure 5B).
However, a significant 30% decrease in receptor protein levels was detected in transfectants expressing the minor compared with the major haplotype in multiple replicate experiments (Figure 5C). Because the sequence variations encoded by rs2070045 (SNP 19) switch serine codon usage from frequent to rare, our findings argue that this SORL1 haplotype affects brain SORLA expression by altering translation efficiency.

COMMENT

In recent years, major efforts have been dedicated to unraveling the mechanisms that may predispose carriers of SORL1 risk alleles to poor receptor expression in the brain. Yet, the results have been confusing. Herein, we present the first study, to our knowledge, that correlates SORL1 genotypes with protein levels in the brain of patients with AD. Our data uncovered a 2-SNP risk genotype in SORL1 that predicts receptor expression in patients likely by affecting the efficiency of translation of the receptor transcript.

SORL1 is one of the most extensively characterized AD risk genes, with a total of 50 SNPs studied previously. Although not all SNPs have been replicated in all cohorts, most reports agree on the existence of 2 haplotype blocks in the 5'/H11032 and 3'/H11032 gene regions that are associated with risk of sporadic AD. In particular, a 3' haplotype block consisting of 5 SNPs (SNPs 19-23) (Figure 1A) has been associated with AD in several European populations. Our analysis replicates the existence of this haplotype block structure in our sample set (Figure 1B).

Because gene expression profiling in lymphoblasts had uncovered 2.5-fold lower levels of SORL1 transcripts in patients with AD compared with controls, an effect of
SORL1 sequence variations on gene transcription had been proposed. In line with this hypothesis, Grear and colleagues identified association of SORL1 mRNA levels with rs661057 (SNP 4) in the 5’ intronic gene region in AD cases. Similarly, McCarthy et al reported association of SORL1 transcripts with SNPs rs7945931 and rs2298723 in the 5’ haplotype block in a cohort of healthy controls. However, in the latter study, association was only seen in the temporal but not the frontal cortex, with the minor allele variants being associated with a 2-fold increase in SORL1 mRNA. Finally, Rogaev and colleagues identified a haplotype in the 3’ SORL1 region (SNPs 22-24) associated with a 50% reduction in mRNA in lymphoblasts. Interestingly, regression analysis also suggested that this genotype accounts for only 14% of the variance, implicating additional genetic or non-genetic factors in modulation of SORLA expression.

In our studies, we have tested association of SORL1 risk alleles with SORLA protein levels in the brain, more directly correlating genotype with neuronal receptor expression. We focused our analyses on the frontal cortex, the brain region in which reduction in receptor protein has been shown by Western blot and immunohistological analysis. In our studies, we identified a novel haplotype composed of 2 SNPs wherein the minor (risk) variant significantly correlates with low receptor expression (Figure 2). Intentionally, we performed our studies in samples from patients with AD because we reasoned that genotype effects might be promoted by a sensitized background of the diseased brain, a situation seen for many multifactorial diseases. Obviously, neurodegenerative processes may affect neuronal protein expression secondarily. However, such impact on SORLA expression has been ruled out in previous studies in individuals with familial forms of AD and in aged AD mouse models.

As documented in Figure 2, the 2 proximal SNPs (rs2070045 and rs1699102) in the 3’ haplotype block are significantly associated with SORLA protein levels. A similar trend of association of the minor allele variant with...
experiments were performed and measured in triplicate. Changes in codon usage of rs2070045 are shown for both genotypes. A total of 6 transcripts relative to zeozin resistance cassette transcripts (B), and zeozin transcripts relative to 18s ribosomal RNA (A), levels of transiently transfected with constructs encoding minor or major receptor expression. Replicate layers of Chinese hamster ovary cells were transiently transfected with constructs encoding minor or major receptor expression. In conclusion, our study identified correlation of SORL1 risk genotypes with SORLA expression in the brain, further supporting the genetic basis of insufficient receptor activity in the brain as an important risk factor in AD.

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