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Allelic mRNA expression of sortilin-1 (*SORL1*) mRNA in Alzheimer's autopsy brain tissues

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Abstract

Polymorphisms in the gene encoding *SORL1*, involved in cellular trafficking of APP, have been implicated in late-onset Alzheimer's disease, by a mechanism thought to affect mRNA expression. To search for regulatory polymorphisms, we have measured allele-specific mRNA expression of *SORL1* in human autopsy tissues from the prefrontal cortex of 26 Alzheimer's patients, and 51 controls, using two synonymous marker SNPs (rs3824968 in exon 34 (11 heterozygous AD subjects and 16 controls), and rs12364988 in exon 6 (8 heterozygous AD subjects)). Significant allelic expression imbalance (AEI), indicative of the presence of *cis*-acting regulatory factors, was detected in a single control subject, while allelic ratios were near unity for all other subjects. We genotyped 7 SNPs in two haplotype blocks that had previously been implicated in Alzheimer's disease. Since each of these SNPs was heterozygous in several subjects lacking AEI, this study fails to support a regulatory role for *SORL1* polymorphisms in mRNA expression.

Keywords

Alzheimer's disease; *SORL1*; Allelic expression imbalance

1. Introduction

Sortilin-1 (*SORL1*, SorLA/LR11; sortilin-related receptor containing LDLR class A repeats) is a sorting receptor that regulates the intracellular transport and processing of the amyloid precursor protein (APP) in neurons. *SORL1* co-localizes with APP and regulates its trafficking in endocytic compartments [8], resulting in APP sequestration in the Golgi and protection from processing into amyloid- peptide (A β), the main component of senile plaques in Alzheimer's disease (AD) [1].

Previous studies have shown that the expression of *SORL1* protein is reduced in brain tissues from individuals with Alzheimer's disease, which is associated with increased A β production [12]. In contrast, *SORL1* overexpression significantly reduces total cellular APP and

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extracellular A β [8]. In a genetic association study [12], 5 SNPs clustered in two distinct regions of *SORL1* (3 SNPs at the 5' end and 2 SNPs at the 3' end) were found to be significantly associated with AD in several clinical studies in a North European case-control cohort. The G and T alleles of the synonymous SNPs rs2070045 and rs3824968 respectively, enhanced AD risk alleles with odds ratios of 1.79 and 2.16 [12]. Similar associations were found in independent cohorts [3] and in a Chinese cohort [13]. Since the candidate SNPs reside in two clusters of mainly intronic SNPs (Fig. 1), Rogaeva et al. [12] have suggested that these variants affect mRNA expression. Because the *SORL1* candidate variants are frequent, *SORL1* was hailed as the next significant AD biomarker after *APOE4*. However, no single *SORL1* SNP or haplotype was associated with increased risk for AD in all data sets, and the functional polymorphisms remain unknown. Moreover, Liu et al. were unable to confirm linkage of *SORL1* on 11q23.2-q24.2 to AD [6]. A similar negative result was observed in a large Caucasian American case in a more recent study [7].

To assess the role of *SORL1* variants in AD, it is necessary to study the molecular genetic mechanisms. Since nonsynonymous SNPs are rare, we have searched for regulatory polymorphisms affecting transcription and mRNA processing and turnover by measuring allelic mRNA expression in human autopsy brain tissues. Any difference in the mRNA generated from either of the two alleles, termed allelic expression imbalance (AEI), is a strong indicator of the presence of *cis*-acting factors, genetic or epigenetic [2]. AEI ratios offer a quantitative and robust measure of allelic differences in each individual, one allele acting as the control for the other. We used two synonymous marker SNPs in exons representing the 3' and 5' haplotype blocks of *SORL1* (Fig. 1) to measure allelic mRNA expression in AD and in control brain autopsy tissues.

2. Materials and methods

Human brain tissues- 26 human autopsy brain tissues were obtained from the polar region of the frontal lobe of patients with histologically confirmed AD and 51 normal prefrontal cortex brain samples from various tissues banks (Ohio State University Neurodegenerative Disease Brain Tissue Repository (Buckeye Brain Bank), Tissue Banks at the University of Maryland). The AD patients were all Caucasians; the average estimated age at onset of AD was 69.9 ± 8.8 years, and the average age at death was 80.3 ± 9.0 . Seven out of the 26 AD patients had early onset AD (estimated age at onset less than 65). The controls (subjects that had not been diagnosed as having AD) were from different ethnic origins (31 Caucasians, 7 African Americans, 8 Hispanics, 2 African Americans/Hispanics, and 3 Caucasians/Hispanics) and had died of unknown reasons at average age of 43.4 ± 15.6 years. Genotyping was performed of de-identified specimen, under a local IRB approval.

Genomic DNA and RNA preparation

Preparation of genomic DNA, RNA, and cDNA were performed as described in previous papers [10;14;18].

Genotyping of candidate SNPs in *SORL1* using multiplexed SNaPshot and an allele-specific PCR melting curve assay (GC clamp allele-specific assay)

Four SNPs (rs3824968, rs12364988, rs689021, rs2070045) were analyzed by SNaPshot (Applied Biosciences, Foster City, CA) using primers listed in Supplemental table 1. The detailed method has been published previously [10]. The extended primers, labeled with different fluorescent dyes, were run on an ABI 3730 capillary electrophoresis instrument (Applied Biosciences, Foster City, CA), then analyzed with Gene Mapper 3.0 software [18]. The extension primers were sufficiently separated by capillary electrophoresis to permit simultaneous analysis of rs12364988, rs689021, rs2070045.

Three additional candidate SNPs in *SORL1* (rs1699102, rs2282649, rs1010159) were genotyped using a GC clamp allele-specific assay [9], with three primers for each SNP (Supplemental table 1). The fluorescence melting curves of the amplicons were analyzed on an ABI7000 cyclor.

mRNA Analysis Using Real-time PCR

Real-time PCR was performed with the same PCR amplification primers used for SNaPshot analysis of rs3824968, with 10 µl PCR SYBR master mixture (Applied Biosciences, Foster City, CA). The mixtures were run on an ABI 7000 thermocycler, and cycle thresholds (Ct's) were measured. As an internal control, β -actin was amplified for the same samples. Expression levels were determined by normalizing the measured cyclic threshold to β -actin. The mRNA level was expressed as the difference $Ct_{SORL1} - Ct_{\beta\text{-actin}}$ [4] [18].

Quantitative analysis of allelic ratio in mRNA and genomic DNA

Two exonic (synonymous) marker SNPs rs3824968 and rs12364988 were chosen for the measurement of AEI in AD samples; only one marker SNP rs3824968 was used for measurement of AEI in control samples. PCR amplification of DNA and cDNA (see above) was followed by a primer extension reaction using primers listed in Supplemental table 1, and fluorescently labeled dideoxynucleotides. The fluorescent products were run on an ABI 3730 capillary electrophoresis instrument (Applied Biosciences, Foster City, CA) and analyzed with Gene Mapper 3.0 software [18]. Peak area ratios were calculated to measure the relative amount of DNA and mRNA (as cDNA) of the two alleles.

Assuming that the two alleles were present in equal amounts in genomic DNA, measured DNA and cDNA ratios were normalized to the average of genomic DNA ratios using the equation: normalized ratio = measured DNA (or cDNA) ratio/mean of genomic DNA ratio. For genomic DNA, none of the samples significantly deviated from the mean, representing a 1/1 allele ratio.

3. Results

Genotyping Results

26 AD human brain tissues were genotyped for the two marker SNPs used subsequently in allelic expression analysis, rs3824968 (a potential risk factor SNP) at the 3' end (exon34) and rs12364988, located in a 5' haplotype block of the transcribed gene (exon6) (Fig. 1). We also genotyped 5 additional SNPs; rs1699102, rs2282649, rs1010159, rs689021 and rs2070045 all implicated as potential risk factors in AD [12] (Supplemental Table 2). For each SNP, we determined minor allele frequency (MAF) and tested for Hardy Weinberg Equilibrium (HWE) (Table 1). All SNPs were in HWE, except for rs12364988 possibly a result of the rather small number of subjects for this analysis. For each SNP pair, we also calculated linkage disequilibrium (LD) (Table 2), showing substantial LD of SNPs rs1699102, rs2282649, rs1010159 and rs2070045 with the marker SNP rs3824968 (3' haplotype block) and LD of rs689021 with the marker SNP rs12364988 (5' haplotype block). Moreover, allele frequencies of marker SNPs rs3824968 and rs12364988 were similar to those of the additional 5 candidate SNPs. Therefore, the 2 marker SNPs are suitable for assessing effects of all proposed *SORL1* candidate SNPs on mRNA expression. For the control brains, 19 of 51 subjects were heterozygous for the marker SNP rs3824968 and therefore eligible for allelic mRNA expression analysis.

mRNA analysis using real-time PCR

SORL1 mRNA expression was successfully measured in 26 AD human brain tissues and 16 control human brain tissues using quantitative RT-PCR, with β -actin as internal standard. The

expression of *SORL1* mRNA was significantly more variable than that of β -actin, showing a normalized Ct range (relative to β -actin) of 3.9–8.5. Shown in Supplemental Figure 1, *SORL1* mRNA levels varied over a 20-fold range. The mean cycle threshold for *SORL1* mRNA (25.9 ± 1.2) indicated a sufficiently robust expression in most tissues analyzed for accurate analysis of allelic mRNA expression ratios. Comparing AD subjects and controls, after normalization to the Ct's of β -actin, the normalized *SORL1* Cts in the controls ranged between 4.88 and 6.33, not significantly different from the AD tissues.

There was no significant correlation between *SORL1* mRNA expression and genotype for any of the seven SNPs included in this study in AD subjects. If any effect would have occurred, a much larger sample number is needed, as total mRNA levels are subject not just to *cis*-acting factors but also, and more prominently, to numerous *trans*-effects.

Allelic expression of *SORL1* in human autopsy brain tissues of AD patients

The marker SNPs rs3824968 and rs12364988 yielded 11 and 8 heterozygous samples, respectively. The measured peak area ratios (SNaPshot) representing allelic ratios in genomic DNA were 1.71 ± 0.08 (S.D.) and 0.74 ± 0.04 , respectively. The ratios did not deviate significantly from the mean in any given sample ($<3SD$), indicating an absence of copy number variation (although loss of one allele (hemizygosity) would not be detectable by this method). Therefore, all genomic DNA ratios for *SORL1* were normalized to 1, as reference standard for mRNA ratios. After normalizing the allelic mRNA ratios to the mean genomic DNA ratio, the A/T allelic mRNA ratios of marker rs3824968 (exon 34) did not deviate significantly from unity (Fig. 2). These results indicate the absence of *cis*-acting factors on allelic mRNA expression, measured in the region of exon 34.

Use of SNP rs12364988 as a marker SNP, located at some distance in exon 6 (Fig. 1), similarly failed to reveal any expression imbalance, confirming and extending the absence of allelic expression imbalance in heterozygous samples for this SNP, using a 5' upstream region of the mRNA (Fig. 2). Combined, the absence of AEI in two distinct regions of *SORL1* mRNA suggest that alternative splicing or truncation events are unlikely to have occurred as a result of *cis*-acting factors.

Because rs3824968 and rs12364988 reside in different haplotype blocks and are not in linkage disequilibrium, it is possible that we could have missed AEI in some portion of the mRNA - for example as a result of differential processing - in subjects heterozygous for only one of the two marker SNPs. However, three subjects were compound heterozygotes, and yet each failed to show AEI at both marker SNP sites.

Allelic expression of *SORL1* in control human autopsy brain tissue

The marker SNP rs3824968 yielded 19 heterozygous samples, of which 16 yielded AEI measurements. The measured peak area ratios (SNaPshot) representing allelic ratios in genomic DNA and cDNA were 1.70 ± 0.18 (S.D.) and 1.68 ± 0.18 (S.D.), respectively. Similar to AD brain tissue results, the A/T allelic mRNA ratios normalized to the mean genomic DNA ratio of marker rs3824968 (exon 34) did not deviate significantly from unity in 15 tissues (Fig. 2). However, one sample showed a large cDNA allelic ratio of 4.9 ± 0.9 (after normalization to gDNA), demonstrating the presence of a robust *cis*-acting factor (this sample was treated as an outlier and not used for calculating the mean cDNA allelic ratio of the 15 subjects).

4. Discussion

Clinical association studies implicate *SORL1* variants as an AD risk factor [12]. This potentially important finding must be validated by identifying the polymorphisms responsible for altered

SORL1 functions. Because the implicated polymorphisms are frequent but do not include nonsynonymous SNPs that could have affected protein functions, and since low *SORL1* expression was associated with AD, we suspected that any genetic variants would affect transcription and mRNA processing, including maturation, splicing, and turnover.

We have applied allelic mRNA expression analysis to *SORL1* in prefrontal cortex tissues from AD patients and controls. This brain region is typically involved in late stage AD, and therefore relevant for assessing *cis*-acting regulatory polymorphisms that may vary from one tissue to another. In a series of previous studies we have demonstrated that allelic mRNA ratios can be measured accurately in human autopsy brain and yield important information on the presence or absence of *cis*-acting regulatory polymorphisms [11;15;18]. The results of the present study failed to detect any substantial allelic expression imbalance (less than 20% deviation from unity), except in one subject not previously diagnosed with AD. As we had measured AEI in 32 subjects total (70 chromosomes), the frequency of a possible allele causing this substantial expression difference could be as high as 2–3%, but probably much less in this population. Nevertheless, given the potential importance of *SORL1* in the etiology of AD, a larger study is needed to establish allele frequency, underlying mechanism (gain or loss of function) and possible clinical relevance.

The absence of AEI in all other subjects strongly argues against a role of the frequent candidate SNPs in mRNA expression. Were any of these SNPs functionally relevant, we would have expected detectable AEI, resulting from several possible mechanisms, for example gene regulation, mRNA processing, and mRNA turnover. However, we cannot exclude the possibility that these events can occur in other brain regions.

While the present study was relatively small, the group of 26 AD subjects, and 16 with AEI measurements, it was sufficient to address possible functionality of the frequent *SORL1* candidate SNPs. For each of the 7 candidate SNP analyzed, there were several subject heterozygous for one or more of them (Tables 1, Supplemental table 2), yet no AEI was present. Measuring allelic mRNA expression ratios obviates the influence of *trans*-acting factors and mitigates problems with post-mortem degradation, assuming both alleles degrade at similar rates. In several previous studies where we had detected AEI, we were able to identify regulatory polymorphisms consistent with these results [5;18] [17]. The results therefore argue strongly against any of the tested SNP to affect transcription, mRNA processing and turnover in the tissues studied.

Measuring total mRNA levels (relative to β -actin), we did not find any association between mRNA levels and genotype, as expected because mRNA levels are subject to multiple *trans*-acting factors, and post-mortem degradation. The lower *SORL1* levels in AD subjects relative to controls were offset by lower β -actin levels that we used for normalization. Possibly, mRNA levels were generally lower in these AD tissues.

The SNPs analyzed in this study have shown haplotypic association with AD in the Northern European data sets [12]. Four SNPs at the 3' end of *SORL1* showed overlapping haplotypes of CTT at rs1699102, rs3824968, rs2282649 and TTC at rs3824968, rs2282649, rs1010159 associated with Alzheimer's disease in the Caucasian data set. These haplotypes were also present in our study, indicating that AEI could also not have arisen from a combination of SNPs as suggested by the haplotype associations. Since two clinical studies failed to reveal an association of *SORL1* with AD [16] [7], the question remains open as to whether and to what extent *SORL1* variants contribute to disease risk.

Our analysis argues against regulatory mechanisms but leaves open a number of possible molecular genetic mechanisms that still need to be explored. First, we cannot exclude that alternative splicing has been affected by a *cis*-acting polymorphism, leading to mRNA variants

that have equal turnover, thereby failing to result in detectable AEI. However, inspection of EST databases did not reveal the presence of substantial splice variants. Neither of the two exonic SNPs is associated with exonic splicing enhancer sites (using the online RESCUE-ESE tool). Second, any polymorphism, including synonymous and nonsynonymous SNPs, and SNPs in the 3' and 5' untranslated regions in mRNA, can affect the rate of translation and thus protein levels, by several mechanisms including altered codon usage or other regulatory effects. For example, the two selected marker SNPs rs3824968 and rs12364988, while being synonymous, are present in the mature mRNA and could alter translation. Measuring *SORL1* protein levels in association with genotype will require a larger sample cohort and precisely specified brain regions because protein levels are also subject to *trans*-acting factors, generating more variability farther downstream of genetic variants in *SORL1*. Nevertheless, the previous suggestion that SNPs occurring in at least two different clusters of intronic SNPs regulate expression of *SORL1* mRNA is not supported by the present study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

SORL1	sortilin-related receptor
AD	Alzheimer's Disease
AEI	allelic expression imbalance

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Figure 1.

Genomic map of *SORL1* showing the location of SNPs genotyped in this study. The SNPs indicated by the shaded boxes are the two marker SNPs

Figure 2.

SORL1 allelic mRNA expression ratios of prefrontal cortex. a: Control subjects heterozygous for marker rs3824968 (A allele/T allele). b: AD subjects heterozygous for marker rs3824968 (A allele/T allele). c: AD subjects heterozygous for marker rs12364988 (G allele/A allele). Allelic mRNA ratios were normalized to the mean gDNA ratios, set at unity. (a: n=2 two independent assays; b,c: n=4 two independent assays in duplicate)(c: only one measurement was used for sample B71).

Table 1

Minor allele frequencies of *SORL1* in the AD brains tested

SNP	rs12364988	rs689021	rs2070045	rs1699102	rs3824968	rs2282649	rs1010159
Allele	G/A	G/A	G/T	C/T	A/T	C/T	G/A
Minor allele	A	A	T	C	A	T	G
MAF	0.46	0.46	0.29	0.31	0.29	0.32	0.42
HWE R	0.38	0.16	0.16	0.13	0.03	0.08	0.21
HWE P value	0.05	0.43	0.42	0.53	0.88	0.69	0.28

Table 2

Linkage disequilibrium between the two marker SNPs, rs12364988 and rs3824968, and 5 additional SNPs of SORL. Linkage disequilibrium (LD) was calculated from genotyping results in 26 AD subjects, using HelixTree. The results show the expected two haplotype blocks, 5' represented by rs12364988 and rs689021, 3' by the other 5 SNPs.

Marker1	Marker2	LD Correlation R	D Prime
rs12364988	rs689021	0.83	0.83
rs12364988	rs2070045	0.18	0.27
rs12364988	rs1699102	0.05	0.08
rs12364988	rs3824968	0.07	0.10
rs12364988	rs2282649	0.07	0.10
rs12364988	rs1010159	0.02	0.02
rs3824968	rs689021	0.17	0.29
rs3824968	rs2070045	0.70	0.70
rs3824968	rs1699102	0.91	1.00
rs3824968	rs2282649	0.91	1.00
rs3824968	rs1010159	0.74	1.00