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Genetics of Clusterin Isoform Expression and Alzheimer’s Disease Risk

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Abstract

The minor allele of rs11136000 within CLU is strongly associated with reduced Alzheimer’s disease (AD) risk. The mechanism underlying this association is unclear. Here, we report that CLU1 and CLU2 are the two primary CLU isoforms in human brain; CLU1 and CLU2 share exons 2–9 but differ in exon 1 and proximal promoters. The expression of both CLU1 and CLU2 was increased in individuals with significant AD neuropathology. However, only CLU1 was associated with the rs11136000 genotype, with the minor “protective” rs11136000T allele being associated with increased CLU1 expression. Since CLU1 and CLU2 are predicted to encode intracellular and secreted proteins, respectively, we compared their expression; for both CLU1 and CLU2 transfected cells, clusterin is present in the secretory pathway, accumulates in the extracellular media, and is similar in size to clusterin in human brain. Overall, we interpret these results as indicating that the AD-protective minor rs11136000T allele is associated with increased CLU1 expression. Since CLU1 and CLU2 appear to produce similar proteins and are increased in AD, the AD-protection afforded by the rs11136000T allele may reflect increased soluble clusterin throughout life.

Introduction

Clusterin (CLU, APOJ) has been implicated in diseases ranging from cancer to Alzheimer’s disease (AD) (reviewed in [1,2,3,4]). Although the primary role of clusterin in AD is unclear, CLU is implicated in AD by several lines of evidence, including (i) CLU mRNA and clusterin protein is increased in AD [5,6], (ii) clusterin is a component of plaques [4,5,7], (iii) clusterin modulates AD-related pathways such as inflammation and apoptosis [1,8,9] and (iv) clusterin acts as an amyloid-beta (Aβ) chaperone to alter Aβ aggregation and/or clearance ([10,11], reviewed in [4,12,13,14]). The physiologic relevance of CLU to AD was confirmed recently when CLU single nucleotide polymorphisms (SNPs) were associated with AD risk [15,16,17,18,19]. Overall, CLU genetic variation is essentially unequivocally associated with AD given the robust statistical power of the initial genome-wide association studies and subsequent replication studies [15,16,17,18,19]. How CLU SNPs modulate clusterin to alter AD risk is unknown.

Two CLU isoforms, CLU1 and CLU2, have been reported that consist of nine exons and differ only in their first exons and associated proximal promoters; CLU1 is predicted to encode a nuclear protein and CLU2 a secreted protein [reviewed in [20]]. Additional reported isoforms include a CLU isoform that lacks exon 5 and a CLU isoform that lacks exon two, which encodes the leader sequence, resulting in another nuclear clusterin [21,22]. Here, we investigated the hypothesis that CLU isoforms are differentially modulated by AD status and AD-associated SNPs. We identified CLU1 and CLU2 as the major CLU isoforms in human brain. Quantitative expression studies show that both CLU1 and CLU2 are increased in AD but only CLU1 is associated with rs11136000. Lastly, although CLU1 and CLU2 are predicted to produce intracellular and secreted proteins, respectively, immunofluorescence and Western blot studies indicate that CLU1 and CLU2 both produce secreted proteins that are similar to those detected in the human brain. Overall, we interpret our results as suggesting that SNP-mediated increases in secreted, soluble clusterin expression may act to reduce AD risk.

Methods

Ethics Statement

The work described here was performed with approval from the University of Kentucky Institutional Review Board.

Cell Culture

SH-SY5Y (human neuroblastoma) and HepG2 (human hepatocellular carcinoma) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin at 37°C in a humidified 5% CO2 - 95% air atmosphere.

CLU Expression Plasmid

Expression plasmids encoding CLU1 and CLU2 were generated from SH-SY5Y cellular mRNA that was reverse transcribed by using the primer 5’-TAGGGTGCAAAAGCAACAT-3’ which corresponds to sequence just after the CLU stop codon. CLU1 and CLU2 cDNAs were then amplified by PCR with forward

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primes 5’-TGAGTCATGCAGTTTGAGCAG-3’ (CLU1) and 5’-ATGATGAGACAGTCCGCTGCTG-3’ (CLU2) used in combination with the common reverse primer 5’-CTCCCTGCCTGCTTGGT-3’. PCR fragments were ligated into pEcoN3.1/V5-His-TOPO T/A cloning vector (Invitrogen, Carlsbad, CA). Clones encoding CLU1 and CLU2 were detected by PCR screening and clone integrity confirmed by sequencing.

Human Autopsy Tissue
De-identified human brain specimens were provided by the University of Kentucky AD Center Neuropathology Core [25,26]. AD and non-AD designations followed NIARI neuropathology guidelines, which include indices of neuritic senile plaques and neurofibrillary tangles, and provide a likelihood staging of AD neuropathological diagnosis [25,26]. Individuals with “low” AD neuropathology were cognitively intact prior to death and had no or low likelihood of AD by NIARI criteria; their average age at death was 81.8 ± 10.2 (mean ± SD, n = 17). Individuals with “high” AD neuropathology represented a combination of demented individuals with high likelihood of AD by NIARI criteria (n = 27) and cognitively intact individuals that were found to have moderate or high AD neuropathology at death (n = 7); their average age at death was 81.9 ± 6.2 (mean ± SD, n = 34). The average post-mortem interval (PMI) for low AD neuropathology individuals was 3.0 ± 0.8 hours (mean ± SD, n = 17) while the PMI for high AD neuropathology individuals was similar (3.2 ± 0.8 hours (n = 34)). Choroid plexus samples were from six individuals with an average age at death of 80.0 ± 3.3 years and PMI of 2.9 ± 1.1 hours. Fetal tissue RNA samples were obtained commercially (Stratagene, Santa Clara, CA) and have been described previously [27].

PCR Amplification
Total RNA was extracted from human brain specimens and converted to cDNA in 1 μg aliquots with random hexamers and reverse transcriptase (SuperScript III, Invitrogen), essentially as we described previously [24,28,29,30]. PCR primers were designed such that the splicing of each internal CLU exon as well as CLU1 and CLU2 were evaluated (Table 1). In initial screening, cDNA pooled from five high AD neuropathology and five low AD neuropathology samples were subjected to PCR-amplification (Platinum Taq, Invitrogen) by using each primer pair and a PCR profile consisting of initial denaturation for 5 minutes at 95°C, followed by 27–32 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and final extension at 72°C for 7 min (Perkin Elmer 9600). PCR products were separated by polyacrylamide gel electrophoresis, stained with SYBR Gold and visualized by using a fluorescence imager (Fuji FLA-2000). The identity of the PCR products was confirmed by direct sequencing (Davis Sequencing, Davis, CA).

Real-time PCR
The expression level of CLU1 and CLU2 was quantified by real-time PCR. Each isoform was specifically amplified by using a sense primer corresponding to sequence within their respective exon 1, i.e., 5’-GGGAGGAGACAGGGCTGCTAAAT-3’ for CLU1 and 5’-AGATTTGATCCGGTGAGGA-3’ for CLU2, and an anti-sense primer corresponding to sequence at the exon 2–3 boundary, i.e., 5’-GAGATTTGATCCGGTGAGGA-3’. Note that the CLU1 sense primer contains the TATA sequence suggested by Schepeler et al to be present only in a longer “CLU3/4” isoform [31]. The 20 μl real-time PCR mixture contained approximately 20 ng of brain cDNA, 1 μM of each primer and 1 × PerfeCTaq PCRSupermix (Quanta Biosciences, Gaithersburg, MD) was subjected to real-time PCR (Bio-Rad, Hercules, CA). PCR profiles consisted of pre-incubation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 20 s. Specificity of the reactions was evaluated by showing a single PCR product by gel electrophoresis and by performing a melting curve analysis after PCR amplification. The PCR product copy number in each sample was determined relative to standard curves that were amplified in parallel and were based upon previously purified and quantified PCR products. The copy numbers were then normalized to the geometric mean of the copy numbers of hypoxanthine-guanine phosphoribosyltransferase 1 and ribosomal protein L13A as described (Vandesompele et al., 2002; Zhang et al., 2005). All real-time PCR assays were repeated twice.

Genotyping
DNA samples were genotyped for rs11136000 by using unlabeled PCR primers and TaqMan FAM and VIC dye-labeled MGB probes (Pre-designed SNP Genotyping Assay, Applied Biosystems, Foster City, CA) on a real-time PCR machine (Bio-Rad, Hercules, CA).

Statistical Analysis
Variation in CLU isoform expression were analyzed as a function of rs11136000 genotype, AD neuropathology, sex and age by using a general linear model (SPSS v.18 (IBM, Somers, NY)). A dominant mode of inheritance was assumed to maximize statistical power.

Immunofluorescence
CLU expression plasmids were transfected into SH-SY5Y cells by using FuGene HD Transfection Reagent as directed by the manufacturer (Roche Applied Sciences, Indianapolis, IN). Briefly, 3.75 × 10^4 cells/well were maintained in a poly-L-lysine treated 8-well chamber coverglass (Lab-Tek, Nunc, Rochester, NY) and transfected the next day with CLU1 or CLU2 expression plasmid. Twenty-four hours after transfection, cells were washed with phosphate buffered saline (PBS) and fixed with ice-cold methanol for 5 minutes. Non-specific antibody binding sites were blocked by incubating the cells with 5% goat serum in PBS with 0.1% Tween-20 (PBST) for 1 hour and the cells were then incubated with mouse anti-V5 antibody (1:1000 dilution, ab27671, Abcam, Cambridge, MA) and either rabbit anti-calnexin antibody (1:200 dilution, SPA-860, Stressgen, Victoria, BC, Canada) or rabbit

<table>
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<th>Table 1. PCR Primers for evaluating splice variation.</th>
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<td><strong>Exons Amplified</strong></td>
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<td>1a-5 (For CLU1)</td>
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<tr>
<td>1b-5 (For CLU2)</td>
</tr>
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<td>4-6</td>
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<td>5-9</td>
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doi:10.1371/journal.pone.0033923.t001

April 2012 | Volume 7 | Issue 4 | e33923
anti-TGN46 antibody (1:1000 dilution, ab16052, Abcam) in 5% goat serum at 4°C overnight. The next day, cells were washed with PBST three times and incubated with a mixture containing Alexa-488 goat anti-mouse IgG antibody (1:200 dilution, Molecular Probes, Carlsbad, CA) and Alexa-560 goat anti-rabbit IgG antibody (1:200 dilution, Molecular Probes) in 5% goat serum at room temperature for an hour. After washing with PBST, cells were stained with 10 μg/ml of Hoechst 33258 (Molecular Probes) for 30 minutes, washed with PBS, and subjected to fluorescence microscopy.

Western Blot
SH-SY5Y cells or HepG2 cells were grown in a 100 mm dish (2.6 × 10⁶ cells/dish) for 24 hours and then transfected with CLU or CLU2 expression plasmid by using FuGene HD Transfection Reagent as directed by the manufacturer (Roche Applied Sciences). Twenty-four hours after transfection, cell medium was replaced with Opti-MEM (Invitrogen) and cells maintained for another 24 hours. The conditioned medium was collected and the cells washed with 5 ml of room-temperature PBS. Cells were then lysed in 0.5 ml of RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) containing 1× protease inhibitor cocktail (Roche Applied Sciences) for 30 minutes on ice with occasional rocking. Cell lysates were centrifuged at 10,000×g for 10 minutes at 4°C and the supernatant collected. Conditioned medium was centrifuged at 25,000×g for 10 minutes and 0.5 ml of the supernatant was collected and supplemented with 1× protease inhibitor cocktail (Roche Applied Science). Human brain anterior cingulate tissue samples were prepared by homogenizing ~0.5 mg tissue in RIPA buffer supplemented with 1× protease inhibitor cocktail, centrifuging at 14,000×g for 10 minutes at 4°C, and then collecting the supernatant. After the protein concentration of each sample was determined (Micro BCA Protein Assay Reagent Kit, Pierce, Rockford, IL), 15 or 20 μg protein were mixed with SDS sample loading buffer containing β-mercaptoethanol, boiled for 5 minutes and subjected to polyacrylamide gel electrophoresis on a 7.5% polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Bio-Rad). The blots were then incubated with 5% nonfat dry milk for 1 hour at room temperature and probed overnight with mouse anti-V5 antibody (1:5000 dilution, Abcam) or mouse anti-clusterin antibody (1:200, B-5 Santa-Cruz) at 4°C. After washing with PBST four times for 5 minutes each, the blots were incubated with peroxidase-conjugated sheep anti-mouse antibody (1:1,000 dilution, Jackson ImmunoResearch) for 1 hour at room temperature. Bound peroxidase was visualized by using a SuperSignal West Pico kit (Pierce) and a molecular imager (Chemidoc XRS System, Bio-Rad). For studies involving PNGase F digestion, each protein sample was treated with PNGase F as directed by the manufacturer (New England Biolabs, Ipswich, MA). Briefly, 15 μg protein of each sample was denatured at 100°C for 10 minutes and incubated with 1,500 units of PNGase F at 37°C for 2 hours. Samples were then analyzed by Western blot as described above.

Results
To begin to evaluate CLU expression, we screened human brain cDNA to identify CLU isoforms present in brain. Previously reported isoforms include CLU1 and CLU2, which are identical in exons 2–9 but differ in exon 1, as well as isoforms lacking exons 2 or 5 ([21,22], reviewed in [20]). RT-PCR analyses of pooled human brain cDNA samples detected only CLU1 and CLU2 (Figure 1A–1B). CLU2 translation is predicted to result in a secreted protein, beginning at the initial ATG in exon 2 that is common to both CLU2 and CLU1 (Figure 1C). In contrast, CLU1 translation is predicted to initiate at an ATG within its exon 1, which is in-frame with the ATG site in the common exon 2. Hence, CLU1 is predicted to encode a protein identical to that encoded by CLU2 except that the CLU1 protein would contain 52 additional amino acids at its amino terminus (Figure 1C). This change is predicted by PSORTII to result in an intracellular and likely nuclear, form of clusterin (reviewed in [20]). In summary, CLU1 and CLU2 are the primary CLU isoforms in brain and are predicted to encode intracellular and secreted proteins, respectively.

To evaluate whether rs11136000, the primary AD-related CLU SNP ([15,16,17,18,19]), is associated with CLU expression, we quantified CLU1 and CLU2 expression in a series of brain samples. The CLU isoforms were quantified in separate real-time PCR assays that used forward primers corresponding to their unique exon 1 and a common reverse primer that targeted the exon 2–3 boundary. Copy numbers were determined relative to a standard curve for each isoform and normalized to the geometric mean of housekeeping genes [23,32]. The expression of CLU1 and CLU2 were modestly coordinately regulated (Figure 2A), with CLU2 being consistently greater than CLU1; the overall CLU2:CLU1 ratio was 3.35±1.84 (mean ± SD, n = 51). To gain further insights into CLU isoform expression, we quantified CLU1 and CLU2 in a series of single human fetal tissue samples as well as a set of six adult choroid plexus samples (Figure 2B). Among the fetal tissues, the CLU2:CLU1 ratio varied widely from 0.3 (skin) to 6.2 (kidney) (Figure 2B). The CLU2:CLU1 ratio in choroid plexus, which produces cerebrospinal fluid, was 5.72±0.65 (mean ± SD, n = 6).

We next evaluated CLU isoform expression as a function of AD neuropathology, rs11136000 status, sex and age. We found that CLU1 expression was significantly increased with high AD neuropathology and the minor rs11136000T allele (Figure 2C, Table 2). In contrast, CLU2 expression was significantly increased with AD neuropathology but not rs11136000 genotype, and decreased with age (Figure 2D–E, Table 3). Overall, both CLU1 and CLU2 expression was increased with AD, confirming prior results. However, only CLU1 expression was associated with rs11136000. Since the minor rs11136000T allele was associated with both increased CLU1 expression and reduced AD risk [15,16,17,18,19], we interpret these results as suggesting that increased CLU1 expression is associated with reduced AD risk.

CLU1 and CLU2 are predicted to encode intracellular and secreted proteins, respectively (Figure 1). Therefore, we evaluated the proteins produced by CLU1 and CLU2 transfected cells to discern whether these corresponded to the intracellular and secreted forms of clusterin that have been reported [4,33]. When each isoform was transiently transfected into neural SH-SY5Y cells, immunofluorescence analyses showed a similar subcellular localization pattern (Figure 3). The proteins produced by CLU1 and CLU2 tended to concentrate within the Golgi apparatus, as established by double-labeling with an antibody against trans-Golgi network protein 46 (TGN46) (Figure 3A). Relatively modest amounts of clusterin were also detected within the endoplasmic reticulum (ER), as established by double labeling with calnexin (Figure 3B). Nuclear, cytosolic, or mitochondrial-associated clusterin was not observed. Hence, the clusterin produced by CLU1 and CLU2 manifests an intracellular localization that includes the ER and Golgi, consistent with a possible secretory pathway for both proteins.

CLU1 is predicted to encode a 501 amino acid protein with a molecular weight of 57.8 kDa while CLU2 is predicted to encode 449 amino acids, totaling 52.5 kDa. During maturation of secreted...
Figure 1. **CLU1** and **CLU2** are the primary **CLU** isoforms expressed in human brain. The exon layout for the **CLU1** and **CLU2** isoforms is shown (A). Coding and non-coding exonic regions are represented by black and white boxes, respectively, while introns are shown as thin black lines. The first ATG site in each isoform is shown as well. Evaluation of **CLU** splicing by RT-PCR identified only **CLU1** and **CLU2** in human brain cDNA (B), i.e., single PCR products are observed upon amplification from exon 1a - exon 5 (lane 1), exon 1b - exon 5 (lane 2), exon 4 - exon 6 (lane 3) and exon 5 - exon 9 (lane 4). Molecular weight markers are shown at the left. The predicted protein sequences encoded by **CLU1** and **CLU2**, beginning with the initial ATG sites shown in A, are shown in panel C. The sequence in gray font is unique to **CLU1** while sequence in black font is common to **CLU1** and **CLU2**. The boxed amino acid sequence represents signal sequence that is removed from clusterin-2 upon its translocation to the ER. Two nuclear localization sequences, as predicted by PSORT II (http://psort.ims.u-tokyo.ac.jp/form2.html), are underlined. The asterisk shows the clusterin cleavage site that separates the α- and β- subunits.

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Figure 2. **Expression of **CLU** isoforms in human tissues.** **CLU1** and **CLU2** isoforms were quantified by isoform-specific RT-PCR in a series of adult human cingulate and choroid plexus samples (A–B) as well as a human fetal tissues (B). Within A, each marker reflects the expression of **CLU1** and **CLU2**, normalized to housekeeping genes, in a single brain sample. Within B, the value in parentheses following the tissue name is the **CLU1:****CLU2** ratio. **CLU1** expression shows a positive association with AD neuropathology and rs11136000T allele (C). **CLU2** expression was increased in individuals with AD pathology and decreased with age (D–E).

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clusterin, the protein is N-glycosylated and proteolyzed to generate a heterodimer that is maintained by disulfide bonds [20]. To compare the size of the proteins produced by CLU1 and CLU2, we performed Western blot analyses of transfected SH-SY5Y cells; ectopic clusterin was detected via a V5 epitope tag (Figure 4 A–B). Cell lysates contained both intact and cleaved clusterin while conditioned medium contained only cleaved clusterin; the clusterin protein produced by CLU1 and CLU2 appeared equal in size (Figure 4A). To discern whether glycosylation differences may obscure differences in the clusterin molecular weight derived from their primary sequence, the protein mixtures were digested with PNGase F to remove N-linked sugars prior to electrophoresis; the size of the proteins produced by CLU1 and CLU2 transfected cells were both decreased similarly by PNGase treatment (Figure 4B). We considered the possibility that CLU translation may show cell-type specificity, and therefore repeated this study in an unrelated cell line by transfected SH-SY5Y cells, CLU1 and CLU2 in human brain. Since CLU is increased in AD and CLU1 is increased with the minor rs11136000T allele, which is also associated with reduced AD risk, we interpret our results as suggesting that increased CLU expression throughout life may reduce AD risk. Overall, these results identify a possible means underlying the association of rs11136000 with AD risk.

The association between CLU expression and rs11136000 was discerned because the CLU isoforms were analyzed separately. For example, when we analyzed total CLU expression in the same fashion as reported here, total CLU was not associated with rs11136000 (p = 0.51) although a robust association with AD pathology was observed (p = 0.005, Ling et al., unpublished observations). The lack of a significant association between rs11136000 and total CLU is somewhat disconcerting since CLU1 and CLU2 seem to produce a similar protein. We speculate that (i) the threshold for clusterin biological significance may be less than the threshold for overall CLU statistical significance and/or (ii) CLU1 may be enriched in a cell type different than CLU2, which may affect its functionality. We note that the lack of an association between total CLU and rs11136000 was similar to prior reports that total CLU mRNA or clusterin protein were not associated with rs11136000 [17,34]. As the clusterin produced by CLU1 and CLU2 appears similar, discerning an association between rs11136000 and clusterin would be especially challenging in tissues with a large proportion of CLU2. In this regard, CLU2 was the major isoform in choroid plexus, suggesting that an association between cerebrospinal fluid clusterin and rs11136000 would be difficult to detect. The differential regulation of CLU1 and CLU2 was not unexpected because CLU1 and CLU2 have separate proximal promoter regions. Consistent with this observation, Cochrane et al found that androgen-treatment in a prostate cancer cell line decreased CLU1 expression while enhancing CLU2 expression [35]. Although the identification of the functional SNPs and transcription factors that modulate

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**Table 2. Analysis of CLU1 Expression.**

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<th>P value</th>
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<td>0.01675</td>
<td>0.00551</td>
<td>0.004</td>
</tr>
<tr>
<td>Rs11136000 (dominant model)</td>
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<td>0.00798</td>
<td>0.020</td>
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<tr>
<td>Sex</td>
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<td>0.01503</td>
<td>0.640</td>
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<tr>
<td>Age</td>
<td>-0.000112</td>
<td>0.000104</td>
<td>0.286</td>
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CLU1 expression was analyzed as a function of rs1136000, AD neuropathology, sex and age by using a general linear model. The estimated marginal means for CLU1 in rs1136000 CC versus CT/TT individuals were 0.112±0.013 and 0.151±0.010 (mean ± SE), respectively. The estimated marginal means for low and high AD neuropathology were 0.106±0.014 and 0.157±0.009 (mean ± SE), respectively. These values were modeled with age equal to 82.3 years.

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**Table 3. Analysis of CLU2 Expression.**

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CLU2 expression was analyzed as a function of rs1136000, AD neuropathology, sex and age by using a general linear model. A dominant model of inheritance was assumed for rs11136000. The estimated marginal means for low and high AD neuropathology were 0.332±0.062 and 0.526±0.041 (mean ±SE), respectively. These values were modeled with age equal to 82.3 years.

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**Discussion**

The main findings of this report are several. First, CLU1 and CLU2 are the primary CLU isoforms in human brain and differ in their first exon and proximal promoter. Second, the expression of only CLU1 was associated with rs11136000 while both CLU1 and CLU2 were increased with robust AD neuropathology. Third, CLU1 and CLU2 produce secreted clusterin proteins that are similar to each other and to clusterin in human brain. Since CLU is increased in AD and CLU1 is increased with the minor rs11136000T allele, which is also associated with reduced AD risk, we interpret our results as suggesting that increased CLU expression throughout life may reduce AD risk. Overall, these results identify a possible means underlying the association of rs11136000 with AD risk.
CLU expression is beyond the scope of this report, we note that rs11136000 resides within intron 3 and is common to both CLU1 and CLU2. Hence, rs11136000 is likely not functional but rather is in linkage with a functional SNP more proximal to the CLU1 promoter. In summary, the association between rs11136000 and CLU1 was dependent upon analyzing the two CLU isoforms separately.

Since earlier predictions were that CLU2 encodes a secreted protein while the additional 52 amino-terminal residues encoded by CLU1 would result in its intracellular localization [20], we expected clusterin in CLU1 and CLU2 transfected cells to differ in cellular locale and size. In support of this prediction, the ATG translation initiation sites in the CLU1-specific exon 1a as well as the common exon 2 both satisfy Kozak consensus sequence requirements (http://bioinfo.iitk.ac.in/AUGPred/). However, clusterin in CLU1 and CLU2 transfected cells was localized to the secretory pathway and was equivalent in size under conditions that would easily discern their predicted five kDa size difference, especially after PNGase F treatment to remove sugar residues. Hence, CLU1 and CLU2 generally produce soluble clusterin protein.

The most parsimonious interpretation of these data is that CLU1 translation is generally initiated at the common exon 2 ATG. The possibility exists that a portion of CLU1 translation is initiated at the exon 1a ATG at levels too low to be readily detected or that produce an unstable protein which is rapidly degraded. Distinguishing among these possibilities is a future direction for this work. Overall, we summarize our current results by noting that the proteins produced from CLU1 and CLU2 in vitro are similar to each other, and similar in size to those present in human brain, leading us to interpret our data as supporting the possibility that CLU1 and CLU2 generally produce secreted clusterin protein in vitro and in vivo.

A logical extension of the finding that AD risk is reduced with a genetic variant that correlates with increased CLU expression is that other factors that increase CLU expression may also reduce AD risk. Furthermore, since CLU expression is increased in AD without reversing the disease, we speculate that enhanced CLU expression reduces AD risk only if CLU expression is increased well before AD onset, mimicking the likely SNP effects. One possible modulator of interest is age-dependent changes in sex hormones since CLU isoforms are differentially regulated by androgens [35]. Stress has also been shown to upregulate CLU in many instances [36] and may cause the AD-associated increase in CLU observed here. In terms of possible therapeutic agents, multiple histone deacetylase inhibitors increase CLU expression robustly in vitro [37]. Interestingly, one member of this class, valproate, increases CLU expression in human astrocytes [37] and reduces amyloid accumulation as well as behavioral deficits in mouse amyloid models [38,39]. While valproate may also act via other mechanisms [39], the actions of valproate on CLU expression may be relevant to its neuroprotection and merit further exploration to reduce AD risk.

Limitations of this study include that rs11136000 and AD neuropathological status capture only ~20% of the variance in CLU expression. Identifying the unknown factors that account for the additional variance, which could include epigenetic factors, will facilitate understanding of CLU expression. Moreover, variability in these factors could facilitate or obscure the SNP association with CLU1 expression described here, suggesting that replication of these data in additional samples is necessary for their

Figure 3. **CLU1 and CLU2 encoded proteins are localized to the ER and Golgi.** SH-SY5Y cells were transfected with vectors encoding CLU1 and CLU2; the resultant clusterin expression was localized by a vector-derived V5 epitope tag. The subcellular localization for both proteins overlapped with the Golgi, as shown by co-labeling with antibody against TGN46 (A) and with the ER, as shown by co-labeling with an antibody against calnexin (B).

**A**

<table>
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<th>Hoechst/Nuclei</th>
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<th>V5/CLU</th>
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**B**

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<th>Calnexin/ER</th>
<th>V5/CLU</th>
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acceptance by the research community. Additionally, since the nuclear form of clusterin arising from an isoform lacking exon 2 has been associated with apoptosis [21,22], this isoform may be transiently expressed and hence below our limits of detection, especially in comparison with CLU1 and CLU2.

In conclusion, the AD-protective allele of rs11136000 was associated with increased expression of CLU1 but not CLU2 in our dataset. Both CLU1 and CLU2 produce a soluble, secreted clusterin protein that is similar to that observed in human brain. Discerning the association between rs11136000 and CLU1 depended upon analyzing these two CLU isoforms separately.

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Author Contributions
Conceived and designed the experiments: IL SE. Performed the experiments: IF JB JS. Analyzed the data: IL JB JF DF SE. Wrote the paper: IF SE.

References

Figure 4. CLU1 and CLU2 produce clusterin proteins that are similar to each other and to clusterin in human brain. Transfection of CLU1 and CLU2 resulted in proteins of similar size, appearing as both intact and cleaved clusterin in cell lysates and conditioned media; the proteins resulting from CLU transfection were detected via their V5 epitope tag (A–B). PNGase F treatment prior to electrophoresis reduced the size of the proteins produced by CLU1 and CLU2 similarly (B). The apparent doublet in the protein encoded by CLU1 at 37 kDa was variable between experiments and likely reflects incomplete glycosylation as the doublet collapsed to a single band after PNGase F digestion. Results similar to those depicted in B were obtained when CLU1 and CLU2 were transfected into HepG2 cells (C). When transfected and endogenous clusterin proteins were detected with an antibody raised against a carboxyl-terminal clusterin epitope (D–E), intact clusterin from transfection (T-labeled arrow) was slightly larger than endogenous clusterin (E-labeled arrow), reflecting the ~5.1 kDa V5 epitope and His tags in the ectopic clusterin (D). The bands labeled “Fragments” are clusterin alpha subunits derived from variably glycosylated endogenous and transfected clusterin. Overall, the cellular clusterin proteins are similar in size to the human brain clusterin, as discerned for the intact and cleaved clusterin under reducing conditions (D) or as the heterodimeric clusterin under non-reducing conditions (E).

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