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ADAM10 Expression and Promoter Haplotype in Alzheimer's Disease

L.M. Bekris^{1,2}, F. Lutz^{1,2}, G. Li⁴, D.R. Galasko⁵, M.R. Farlow⁶, J.F. Quinn^{8,9}, J.A. Kaye^{8,9}, J.B. Leverenz^{3,4,10,11}, D.W. Tsuang^{1,4}, T.J. Montine¹², E.R. Peskind^{3,4}, and C.E. Yu^{1,2}

¹Geriatric Research, Education, and Clinical Center (GRECC), VA Puget Sound Health Care System, Seattle, WA ²Department of Medicine, University of Washington, Seattle, WA ³Northwest Network VISN-20 Mental Illness Research, Education and Clinical Center (MIRECC), VA Puget Sound Health Care System, Seattle, WA ⁴Psychiatry and Behavioral Sciences, University of Washington School of Medicine ⁵Department of Neurosciences, University of California at San Diego and VA Medical Center San Diego ⁶Department of Neurology, Indiana University School of Medicine, Indianapolis ⁷Department of Neurology, Institute on Aging, University of Pennsylvania, Philadelphia ⁸Department of Neurology, Oregon Health and Science University ⁹Portland VA Medical Center, Portland ¹⁰Department of Neurology, University of Washington School of Medicine, Seattle, WA ¹¹Northwest Network VISN-20 Parkinson's Disease Research, Education and Clinical Center (PADRECC) ¹²Department of Pathology, University of Washington, Seattle, WA

Abstract

Alzheimer's disease is confirmed at autopsy according to the accumulation of brain neuritic plaques and neurofibrillary tangles in the brain. Neuritic plaques contain A β and lower levels of A β correspond to an increase in ADAM10 α -secretase activity. ADAM10 α -secretase activity produces a soluble APP alpha (sAPP α) product and negates the pathological production of A β . In this investigation it was hypothesized that genetic variation with the *ADAM10* promoter is associated with ADAM10 expression levels as well as CSF sAPP α levels. Results from this investigation suggest that the *ADAM10* rs514049–rs653765 C-A promoter haplotype is associated with; 1) higher CSF sAPP α levels in cognitively normal controls compared to AD, 2) higher post mortem brain hippocampus, but not cerebellum, ADAM10 protein levels in low plaque score subjects compared to high plaque score subjects and 3) higher promoter activity for promoter only reporter constructs compared to promoter + 3'UTR constructs in the human neuroblastoma SHSY5Y cell line, but not in HepG2 or U118 cell lines. Taken together, these findings suggest that ADAM10 expression is modulated according to a promoter haplotype that is influenced in a brain region and cell type specific manner.

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Corresponding Author: Lynn M. Bekris, Ph.D., University of Washington Department of Medicine, Division of Gerontology and Geriatric Medicine, Box 358280 VAPSHCS GRECC S182, 1660 South Columbian Way, Seattle, WA 98108, Phone: 206-277-1549, Fax: 206-764-2569, lbekris@u.washington.edu.

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Keywords

ADAM10; haplotype; sAPP α ; Alzheimer's Disease; reporter assay; neuritic plaques; brain; hippocampus; cerebrospinal fluid

Introduction

A β , processed from APP by β - and γ -secretases, accumulates in the brain as amyloid β -containing plaques (Selkoe 1998), one form, called neuritic plaques, is most closely associated with Alzheimer's disease. Alternatively, α -secretase cleavage within the β -amyloid domain negates the cleavage step that produces pathogenic A β . (Selkoe 1998) The membrane-bound disintegrin metalloproteinases (ADAMs) are capable of cleaving APP at the α -secretase site, with ADAM10 as the major α -secretase in the brain. (Fahrenholz, et al. 2000, Postina 2012, Postina 2008) An increase in ADAM10 levels in a mouse model corresponds to a decrease in A β plaque levels. (Postina, et al. 2004)

The genomic structure and function of the *ADAM10* promoter has been described. (Prinzen, et al. 2005) The 5'-UTR of *ADAM10* has been reported to repress the rate of *ADAM10* translation (Lammich, et al. 2010) and retinoic acid and acitretin stimulate promoter activity and increase ADAM10 levels in human cell lines and a mouse model. (Endres, et al. 2005, Tippmann, et al. 2009) Genetic variation within the *ADAM10* promoter has recently been associated with cerebrospinal fluid (CSF) sAPP α levels in AD compared to cognitively normal control subjects, implicating an important ADAM10 role in AD. (Bekris, et al. 2011) *ADAM10* mRNA has been reported to be high in AD (Gatta, et al. 2002) while ADAM10 protein levels have been reported to be low in AD CSF and platelets. (Colciaghi, et al. 2002, Colciaghi, et al. 2004, Tang, et al. 2006) ADAM10 immunostaining has been reported to be reduced in AD brain. (Bernstein, et al. 2009) Given the likely influence of ADAM10 expression levels on sAPP α levels in AD it is important to further characterize the influence of *ADAM10* promoter genetic variation on both transcription and translation (mRNA and protein) of ADAM10 in AD compared to cognitively normal controls.

Since, to our knowledge human brain *ADAM10* mRNA and protein levels have not been previously measured in parallel in the same sample, and since an association between *ADAM10* promoter genetic variation, ADAM10 expression, and neuritic plaque load have not been described, the aim of this investigation was to determine whether *ADAM10* promoter genetic variation is associated with differential ADAM10 brain expression in subjects with high neuritic plaque score compared to subjects with low neuritic plaque score as well as CSF sAPP α levels.

Methods

Population Description

Two regions of human post-mortem brain (PMB) (cerebellum; CB, hippocampus; HP) from each subject were obtained from the Neuropathology Core of the Alzheimer's Disease Research Center (ADRC) at the University of Washington. Use of human tissue was approved by the University of Washington Institutional Review Board. All tissue was obtained following informed consent, flash frozen at time of autopsy, and stored at -80°C . Patients with late-onset AD (n=21) volunteers in the UW ADRC, where they were diagnosed during life with probable AD per National Institute of Neurological and Communicative Disease – Alzheimer's disease and Related Disorders (McKhann, et al. 1984) and confirmed by post-mortem neuropathologic examination to have AD (Braak, et al. 1991, Mirra, et al. 1991) (Table 1). Control individuals (n=22) were volunteers in the

UW ADRC, were never diagnosed with a CNS disorder and post-mortem examination showed age-related changes only; including sparse to moderate neuritic plaques. (Braak, et al. 1991, Mirra, et al. 1991) Sample size was constrained by limits on post-mortem brain interval (PMI), Braak stage and neuritic plaque score. Sample criteria for inclusion in this investigation were: (1) a low PMI less than or equal to 8 hours to limit mRNA and protein degradation in brain samples and (2) diagnosis in life of AD as well as AD confirmed at autopsy.

Cerebrospinal fluid (CSF) sAPP α levels were obtained from 104 healthy cognitively normal control subjects (Table 1). Use of human CSF and DNA was approved by the University of Washington Institutional Review Board. Following informed consent, all subjects underwent extensive evaluation that consisted of medical history, family history, physical and neurologic examinations, laboratory tests, and neuropsychological assessment; information was obtained from subjects and from informants for all patients. Mini-Mental State Examination (MMSE) scores of all cognitively normal subjects' were between 26–30 and Clinical Dementia Rating Scale (CDR) scores were 0 as previously described. (Peskind, et al. 2006). All AD patients were participants in research clinical cores at respective institutions. Clinical diagnoses of AD were made according to well-established consensus criteria. (McKhann, et al. 1984, Petersen, et al. 1999)

Cerebrospinal Fluid

All CSF samples were obtained as previously described. (Peskind, et al. 2006, Peskind, et al. 2005) Samples were separated into aliquots at the bedside and frozen immediately on dry ice and stored at -80°C until assayed. Concentrations of sAPP α in the 10th ml of collected CSF were measured by immunoassays as previously described. (Johnson-Wood, et al. 1997)

Post-Mortem Brain DNA, RNA, and Protein Extraction and Quantification

DNA, RNA, and protein were extracted from between 1 and 3 mg of post-mortem brain tissue using the Qiagen Allprep DNA/RNA MiniKit (Qiagen, Valencia, CA). Protein was isolated from the column flow through (after the ethanol step) using an acetone precipitation step according to Qiagen protocol (Qiagen) which precipitates soluble and insoluble protein. The protein sample was resuspended in immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCL (pH 6.8), 0.5% NP-40, 0.5% Na-deoxycholate, 5 mM EDTA, 50 mg/ml pepstatin, 50 mg/ml leupeptin, 10 mg/ml aprotinin, 0.25 mM PMSF). *ADAM10* mRNA was measured in triplicate by quantitative real time RT-PCR (QRT-PCR) (Applied Biosystems, Foster City, CA). Beta-actin was used as a loading control. Minus RT samples were included and any samples that had a positive minus RT were repeated. All QRT-PCR results are presented as a ratio of ADAM10/Actin. ADAM10 protein levels were measured in triplicate by Western blot using the primary antibody; rabbit polyclonal anti-human ADAM10 antibody AB19026 (Millipore, California) and the secondary antibody; ECLTM anti-rabbit IgG Horseradish Peroxidase (Sigma, St. Louis, MO). Western blot band intensity was quantified using ImageJ software (U. S. National Institutes of Health, <http://rsb.info.nih.gov/ij/>, 1997–2007). Results above the 97th percentile or below the 3rd percentile were considered as outliers and removed from the database (n=4). Data are presented as ADAM10/Actin (mRNA) or ADAM10 band intensity minus background (protein). Low plaque subjects were categorized using a neuritic plaque score of absent, sparse or moderate (AD n=8; Controls n=22). High plaque subjects were categorized using a neuritic plaque score of frequent or frequent diffuse (AD n=13).

SNPs and Genotyping

Two ADAM10 promoter region SNPs (rs514049 and rs653765) were chosen according to the following criteria: (1) minor allele frequency greater than 5% and (2) available through

Applied Biosystems. Genotypes were determined as previously described. (Bekris, et al. 2008) Briefly, TaqMan allelic discrimination detection using 96-well plates was utilized (Applied Biosystems). PCR reactions were carried out using a 9700 Gene Amp PCR System (Applied Biosystems). Plates were subjected to end-point read in a 7500 Real-Time PCR System (Applied Biosystems). All SNPs were in Hardy–Weinberg equilibrium. No significant association with AD compared to cognitively normal controls in the CSF or PMB sample was found for either ADAM10 promoter region SNP (rs514049 p-values: 0.683 and 0.215, respectively or rs653765 p-values: 0.572 and 0.543, respectively, data not shown). ADAM10 promoter region SNP frequencies are described in Figures 1 and 2. ADAM10 promoter region SNPs (rs514049 and rs653765) are highly correlated ($p < 0.0001$; R^2 : 0.61) suggesting promoter region linkage disequilibrium. Haploview prediction shows *ADAM10* haplotype blocks (Barrett, et al. 2005) and ENCODE marks (Rosenbloom, et al. 2012) located at both the promoter and the 3'UTR regions of the *ADAM10* gene (data not shown). Genetic variation in the 3'UTR is rare (rare allele frequency of 3.5%: rs77874096), was not available through Applied Biosystems and was not identified through sequencing of reporter constructs. Thus, ADAM10 3'UTR genetic variation was not investigated here.

Generation of Regulatory Haplotype Reporters

DNA regions of interest were PCR amplified and DNA sequenced from the post-mortem brain genomic DNA. Primer sequences are shown in Figure 4 Panel A. ADAM10 promoter haplotypes for each regulatory genetic region of interest were PCR amplified from genomic DNA identified by TaqMan allelic discrimination detection (see above) and were validated according to sequenced SNP content once inserted into the reporter constructs. ADAM10 promoter region haplotypes were inserted 5' to the luciferase gene of the pGL4.10[luc2] vector (Promega) to produce promoter only constructs (Figure 4, Panel A). To produce promoter-3'UTR constructs, haplotype variants of the ADAM10 3'UTR were inserted into the promoter constructs 3' to the luciferase gene (Figure 4, Panel A). The In-Fusion® PCR Cloning System (Clontech) was used for all the cloning procedures. After propagating the recombinant DNA in *E. coli* host cell, the reporter constructs were isolated and purified by ion-exchange column (Qiagen). Inserts of all constructs were fully DNA-sequenced to validate the correct genetic content.

Cell Culture

Human neuroblastoma SHSY5Y cells (ATCC) were grown in 44.5% Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) with 44.5% F12 (Gibco), 10% fetal bovine serum (Gibco), and 1% penicillin/streptomycin (100 μ U/ml penicillin, and 100 μ g/ml streptomycin) at 37°C in a 5% CO₂ atmosphere. Human hepatocytoma HepG2 cells (ATCC) and human U118 astrocytoma cells (ATCC) were grown in 89% DMEM (Gibco), 10% fetal bovine serum (Gibco), and 1% penicillin/streptomycin (100 μ U/ml penicillin, and 100 μ g/ml streptomycin) at 37°C in a 5% CO₂ atmosphere. Cells were passaged at 5×10^4 per well into 96-well tissue culture plates 48 hours prior to transfection.

Luciferase Reporter Construct Transfection and Assay

SHSY5Y, HepG2 and U118 cells were transiently transfected for 48 hours with firefly luciferase pGL4.10[luc2] haplotype reporter constructs (as the experimental construct) and a renilla luciferase pGL4.75[hRluc/CMV] construct (as an internal loading control) within the same well using Lipofectamine 2000 (Invitrogen), according to manufacturer instructions. Firefly reporter constructs contained either the ADAM10 promoter region haplotype or the ADAM10 promoter region haplotype and the ADAM10 3'UTR (Figure 4, Panel A). All transfection assays included duplicate transfections within the same assay and assays were performed in triplicate. Failed transfection reactions were duplicated, leading to 6 – 8 replicate transfections for each haplotype reporter construct. SHSY5Y, HepG2 and U118

transiently transfected cells were harvested after 48 hours using the Dual Glo™ Luciferase Assay System (Promega) which allows for high throughput analysis of firefly luciferase (pGL4.10[luc2] constructs in the first step. In the second step, after quenching the firefly luminescence and activating the renilla luciferase (pGL4.75[hRluc/CMV]), the internal loading control was analyzed. Luciferase luminescent counts per second (CPS) were measured using a Wallac Victor2 1420 Multilabel Counter. Luminescent counts for both firefly luciferase and renilla luciferase are presented as a ratio (Firefly / Renilla) that represents the reporter construct activity relative to the loading control.

Statistical Analysis

CSF sAPP α levels, PMB ADAM10 expression levels and the reporter activity were analyzed using SPSS (Version 13). ANOVA and linear regression analyses were utilized where CSF sAPP α level, PMB ADAM10 expression level or reporter activity level was the dependent variable and genotype or haplotype was the independent variable and either disease status (AD or control), neuritic plaque score (low plaque or high plaque) or reporter construct (promoter only or promoter – 3'UTR) were the selection variable. Covariates; *APOE* ϵ 4 status and age, were also taken into account (used as additional independent variables) in separate models. All p-values are Bonferroni corrected for multiple comparisons.

Results

ADAM10 genetic variation and CSF sAPP α levels

ADAM10 promoter SNPs rs514049 and rs653765 are located within the previously described *ADAM10* promoter. (Prinzen, et al. 2005) In controls, CSF sAPP α levels were significantly higher for rs514049 CC genotype carriers compared to CA, AA carriers (p-value, 0.004) and compared to AD CC carriers (p-value, 0.001) (Figure 1, Panel A). In AD, CSF sAPP α levels were higher for rs514049 CA, AA carriers compared to CC carriers (Figure 1, Panel A). In controls, CSF sAPP α levels were significantly higher for rs653765 AA genotype carriers compared to AG, GG carriers (p-value, 0.009) and compared to AD AA carriers (p-value, 0.010) (Figure 1, Panel B). Significance remained after taking into account covariates; *APOE* ϵ 4 status and age (data not shown).

ADAM10 mRNA and protein expression stratified by plaque score and promoter SNPs

ADAM10 mRNA expression was higher in high plaque score subjects compared to low plaque score subjects in cerebellum (CB) (p-value, 0.037, data not shown) but not hippocampus (HP), and was higher in AD compared to controls in CB (p-value, 0.018; data not shown) but not HP.

CB (data not shown) or HP *ADAM10* mRNA expression was not significantly different upon stratification by *ADAM10* promoter rs514049 genotype or neuritic plaque category (Figure 2, Panel A).

PMB ADAM10 protein levels were not significantly different in AD compared to controls or in low plaque subjects compared to high neuritic plaque subjects for either CB or HP (data not shown). Upon stratification by ADAM10 promoter rs514049 genotype, HP ADAM10 mature (60kDa) protein levels were significantly higher for low plaque CC carriers compared to high plaque CC carriers (p-value, 0.009) (Figure 2, Panel B). CB showed no significant differences in ADAM10 protein levels upon stratification by ADAM10 promoter rs514049 genotype (data not shown). HP ADAM10 mature (60kDa) protein levels were higher for high plaque rs514049 CA, AA compared to CC carriers (p-value, 0.003) and compared to low plaque CA, AA carriers (p-value, 0.026) (Figure 2, Panel

B). HP ADAM10 precursor (85kDa) protein levels were lower for high plaque rs514049 CC carriers compared to low plaque CC carriers (p-value, 0.015) and high plaque CA, AA carriers (p-value, 0.001) (Figure 2, Panel C). HP ADAM10 mRNA/ADAM10 precursor (85kDa) protein expression was higher in high plaque rs514049 CC carriers compared to CA, AA carriers (p-value, 0.002) (Figure 2, Panel D).

CB (data not shown) and HP *ADAM10* mRNA expression was not significantly different according to *ADAM10* promoter rs653765 genotype or plaque category (Figure 2, Panel E). HP ADAM10 mature (60kDa) protein levels were higher for high plaque rs653765 AG, GG carriers compared to AA carriers (p-value, 0.022) and compared to low plaque AG, GG carriers (p-value, 0.031) (Figure 2, Panel F). HP ADAM10 precursor (85kDa) protein levels were lower for high plaque rs653765 AA carriers compared to AG, GG carriers (p-value, 0.001) (Figure 2, Panel G). HP ADAM10 mRNA/ADAM10 precursor (85kDa) protein expression was not significantly different according to *ADAM10* promoter rs653765 genotype or plaque category (Figure 2, Panel H). Significance remained after taking into account covariates; *APOE* ϵ 4 status and age (data not shown).

ADAM10 promoter haplotype effect on CSF sAPP α and PMB ADAM10 levels

A total of four rs514049 – rs653765 ADAM10 promoter haplotypes were analyzed and only 2 haplotypes showed a significant association with expression levels (Figure 3). Control *ADAM10* C-A haplotype carriers had significantly higher CSF sAPP α levels compared to A-G carriers (p-value, 0.004) or AD ADAM10 C-A haplotype carriers (p-value, 0.001) (Figure 3, Panel A). Significance remained after taking into account covariates; *APOE* ϵ 4 status and age (data not shown).

Low neuritic plaque *ADAM10* C-A haplotype carriers had significantly higher ADAM10 protein (60kDa) levels than high amyloid plaque *ADAM10* C-A haplotype carriers (p-value, 0.009) in the HP but not the CB (data not shown). In contrast, high amyloid plaque *ADAM10* C-A haplotype carriers had significantly lower ADAM10 protein (60kDa) levels than *ADAM10* A-G haplotype carriers (p-value, 0.041) in the HP but not the CB (data not shown) (Figure 3, Panel B). C-A haplotype carriers had significantly discrepant HP *ADAM10* mRNA compared to protein (*ADAM10* mRNA relative to ADAM10 protein (85 kDa) expression) when comparing low plaque subjects to high plaque subjects (p-value, 0.009; data not shown). Significance remained after taking into account covariates; *APOE* ϵ 4 status and age in most cases (not where p-values were greater than 0.04, data not shown).

ADAM10 reporter construct structure and ADAM10 haplotype reporter activity

Reporter constructs were designed to test whether an *ADAM10* promoter haplotype influences expression with or without an additional regulatory mechanism, such as post-transcriptional 3' region regulation (3' untranslated region: 3'UTR) (Figure 4). *ADAM10* promoter and promoter – 3'UTR luciferase reporter constructs were designed as either promoter only constructs containing one of four rs514049 – rs653765 haplotypes or promoter and 3'UTR constructs containing one of four rs514049 – rs653765 haplotypes. Significant differences were found between two haplotypes and are shown in Figure 4.

ADAM10 promoter C-A haplotype reporter activity in HepG2 cells was significantly higher than the A-G haplotype (p-value, 0.048) (Figure 4, Panel B). *ADAM10* promoter C-A haplotype activity in SHSY5Y cells was significantly higher than the *ADAM10* promoter – 3'UTR C-A haplotype (p-value, 0.036) (Figure 4, Panel B). In contrast, *ADAM10* promoter – 3'UTR C-A haplotype activity in SHSY5Y cells was significantly lower than the *ADAM10* promoter – 3'UTR A-G haplotype (p-value, 0.037) (Figure 4, Panel B). No

significant difference in *ADAM10* reporter haplotype was seen in U118 cells (Figure 4, Panel B).

Discussion

Given the emerging knowledge of ADAM10s critical role in the attenuation of A β production, (Kojro, et al. 2001, Postina 2012, Postina 2008, Postina, et al. 2004, Selkoe 1998) it is important to characterize the impact of *ADAM10* genetic variation on ADAM10 expression in the brain. In this investigation, CSF sAPP α levels were found to be significantly higher in cognitively normal controls with either the *ADAM10* rs514049 CC genotype or the rs653765 AA genotype (Figure 1). Both of these *ADAM10* promoter genotypes showed an opposite lower level effect in AD carriers of either the rs514049 CC or the rs653765 AA genotypes. These results confirm previous work (Bekris, et al. 2011) and implicate an ADAM10 regulatory mechanism that is specific to the microenvironment of the AD brain and *ADAM10* promoter genetic content.

ADAM10 mRNA expression was found to be significantly higher in CB from subjects with high neuritic plaque score compared to subjects with low neuritic plaques, but not in the HP, and *ADAM10* mRNA expression was higher in AD compared to controls in CB, but not in HP (data not shown). Similar results have been reported by others where higher *ADAM10* mRNA expression in AD HP and CB combined were found. (Gatta, et al. 2002)

Interestingly, PMB ADAM10 protein levels were not significantly different in AD compared to controls or in low neuritic plaque subjects compared to high neuritic plaque subjects (data not shown). Others have reported that CSF and platelet ADAM10, as well as ADAM10 brain immunostaining, are lower in AD compared to controls. (Bernstein, et al. 2009, Colciaghi, et al. 2002) Thus, taken together, these results suggest that ADAM10 mRNA and protein levels are not correlated implicating a post-transcriptional regulatory influence on ADAM10 expression in the brain.

Upon stratification by *ADAM10* promoter genetic variation, high plaque subjects that carried either the *ADAM10* rs514049 CC genotype or the rs653765 AA genotype, showed significantly lower mature *ADAM10* (60 kDa) and precursor (85kDa) protein expression in brain HP, compared to the rs514049 CA, AA or the rs653765 AG, GG collapsed genotypes (Figure 2). Interestingly, this brain ADAM10 protein expression pattern according to genotype shows a similar pattern as CSF sAPP α levels (Figure 1). In contrast, brain *ADAM10* mRNA levels were not associated with promoter genetic variation and did not correspond to the pattern seen for protein levels (Figure 2). These discrepant *ADAM10* mRNA and protein levels were further demonstrated by a significant difference between *ADAM10* mRNA relative to ADAM10 protein for carriers of the rs514049 CC genotype in high plaque subject HP (Figure 2). These discrepant ADAM10 mRNA and protein levels suggest a post-transcriptional regulatory mechanism specific to both the AD brain microenvironment and ADAM10 promoter genetic content where specifically the rs514049 C allele in combination with the rs653765 A allele may confer a differential impact on ADAM10 expression.

To determine whether a combination of these two ADAM10 SNPs in the form of an *ADAM10* rs514049 – rs653765 C-A promoter haplotype differentially impacts expression levels, *ADAM10* haplotype specific CSF sAPP α levels and PMB ADAM10 protein levels were analyzed by comparing a C-A promoter haplotype with other promoter haplotypes. Control *ADAM10* C-A haplotype carriers were found to have significantly higher CSF sAPP α levels compared to A-G carriers or AD C-A haplotype carriers (Figure 3). Similarly, low neuritic plaque *ADAM10* C-A haplotype carriers were found to have significantly

higher HP ADAM10 protein (60kDa) levels than high plaque *ADAM10* C-A haplotype carriers. In contrast, high neuritic plaque *ADAM10* C-A haplotype carriers have significantly lower HP ADAM10 protein (60kDa) levels than *ADAM10* A-G haplotype carriers in the HP (Figure 3). These results support the concept that an *ADAM10* promoter haplotype plays a role in ADAM10 expression in the brain where the *ADAM10* C-A promoter haplotype showed an opposite lower level effect in AD and high neuritic plaque compared to controls and low neuritic plaque C-A haplotype carriers.

Since *ADAM10* promoter haplotype was found to be associated with HP ADAM10 expression, next we sought to demonstrate a functional impact by *ADAM10* promoter haplotypes on promoter activity by designing reporter constructs that contained the *ADAM10* promoter haplotypes of interest. In addition, since ADAM10 mRNA and protein expression was discrepant, it was hypothesized that a post-transcriptional regulatory mechanism was involved in ADAM10 expression. Since the 3' region of a gene is often a key player in post-transcriptional regulation, (Siegel, et al. 2011) reporter constructs were designed to contain the *ADAM10* promoter region and the *ADAM10* 3' region (3' UTR). The *ADAM10* promoter C-A haplotype reporter activity was significantly higher than the A-G haplotype, in HepG2 cells, and this effect was abolished by adding the 3' UTR to the reporter construct (Figure 4). *ADAM10* promoter C-A haplotype activity in SHSY5Y cells was significantly higher than the *ADAM10* promoter – 3' UTR C-A haplotype (Figure 4). In contrast, *ADAM10* promoter – 3' UTR C-A haplotype activity in SHSY5Y cells was significantly lower than the *ADAM10* promoter – 3' UTR A-G haplotype suggesting that *ADAM10* regulatory structure is influenced by a promoter haplotype in a cell type specific manner.

Since the pattern of *ADAM10* haplotype specific reporter activity in human neuroblastoma SHSY5Y cells is similar to *ADAM10* haplotype associated expression in high plaque HP, compared to low plaque HP, as well as *ADAM10* haplotype associated AD CSF sAPP α expression in controls compared to AD, it may be speculated that the neuronal AD microenvironment activates an *ADAM10* 3' UTR regulatory mechanism, such as microRNA related post-transcriptional inhibition. For example, specifically, a trans-acting factor complex up-regulated in AD HP neurons may efficiently interact with both the ADAM10 C-A promoter and the 3' UTR to inhibit ADAM10 expression whereas inhibition may be disrupted in the presence of the A-G promoter haplotype. In contrast, under “normal” neuronal conditions, (i.e. in cognitively normal controls, low plaque subjects or reporter constructs without the ADAM10 3' UTR) an ADAM10 3' UTR regulatory mechanism is not active thus the C-A promoter and A-G haplotypes produce comparable levels of protein. The 3' UTR contains only one reported SNP (rs77874096) with a low rare allele frequency further suggesting that this region plays an important role in gene regulation.

In addition, a post-translational mechanism may contribute ADAM10 protein expression. Evidence of post-translational effects on ADAM10 expression are seen in Figure 2 Panel B where the A-G promoter haplotype mature 60 kDa protein is decreased, while the precursor 85 kDa protein is not, in low plaque subjects. Furthermore, in HepG2 cells the A-G haplotype promoter only constructs show lower reporter activity compared to the C-A haplotype.

Interestingly, the association between *ADAM10* promoter genetic variation and HP expression levels according to plaque score was not seen in the CB (data not shown) suggesting brain region specific expression. Thus, it is important to note that AD CSF is collected at an earlier disease stage compared to PMB. End stage AD (PMB), compared to early stage AD (CSF), has increased HP atrophy as well as neurodegeneration and astroglial activation. (Jack, et al. 2011, Steele, et al. 2012) This might suggest that the *ADAM10*

promoter haplotype correlation with expression levels represents primarily astrocyte expression. However, the human U118 astrocytoma cell line did not show a significant correlation with *ADAM10* promoter haplotype while the human SHSY5Y neuroblastoma cell line showed a significant difference between *ADAM10* promoter haplotypes suggesting that neurons, not astrocytes, may play a more important role in *ADAM10* expression. Since only a few cell lines were used in this investigation further investigation of *ADAM10* promoter haplotype activity in other cell types, including multiple astrocytic and neuronal cell lines, may help elucidate cell type specific expression.

Contrasting AD genetic association studies of *ADAM10* (Cai, et al. 2012, Kim, et al. 2009, Laws, et al. 2011) as well as a lack of association between AD risk and *ADAM10* promoter SNPs in the present investigation (data not shown), indicate that it remains unclear whether *ADAM10* genetic variation is associated with AD risk. The results of this exploratory investigation implicate both brain region and cell type specific *ADAM10* promoter haplotype association with AD related phenotypes, such as neuritic plaque score. This suggests that it is important for future studies to use brain amyloid imaging or brain *ADAM10* immunochemistry to increase the power to detect a moderate genetic risk factor such as *ADAM10* in AD.

In summary, the *ADAM10*C-A promoter haplotype is associated with; 1) higher CSF sAPP α levels in controls compared to AD, 2) higher HP PMB *ADAM10* protein levels in subjects with a low neuritic plaque score compared to those with a high neuritic plaque score, and 3) higher reporter activity for *ADAM10* promoter only constructs compared to promoter – 3'UTR constructs in a human neuronal cell line. Taken together, these findings suggest that *ADAM10* expression, as well as sAPP α levels, and thus possibly A β accumulation, may be influenced by an *ADAM10* regulatory structure that is impacted by both promoter genetic content and AD microenvironment. Thus, AD therapeutic strategies aimed at modulating *ADAM10* expression, in the hopes of affecting sAPP α and A β levels, may need to take into account the entire *ADAM10* regulatory region structure as well as promoter genetic content.

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Figure 1. ADAM10 promoter SNPs and CSF APPα levels

CSF APPα levels in control subjects and AD patients according to rs514049 collapsed genotype (Panel A). CSF APPα levels in control subjects and AD patients according to rs653765 collapsed genotype (Panel B). (Bonferroni corrected for multiple comparisons p-values: * $p < 0.05$, ** $p < 0.01$)

Figure 2.

Hippocampus (HP) *ADAM10* mRNA and protein levels stratified by low and high plaque score and *ADAM10* promoter SNPs. *ADAM10* mRNA (Panel A), *ADAM10* mature (60kDa) protein (Panel B), *ADAM10* precursor (85kDa) protein (Panel C) and *ADAM10* mRNA / *ADAM10* precursor (85kDa) protein expression according to rs514049 genotype (Panel D). *ADAM10* mRNA (Panel E), *ADAM10* mature (60kDa) protein (Panel F), *ADAM10* precursor (85kDa) protein (Panel G) and *ADAM10* mRNA / *ADAM10* precursor (85kDa) protein expression according to rs653765 genotype (Panel H). (Bonferroni corrected for multiple comparisons p-values : * p < 0.05, ** p < 0.01).

Figure 3. ADAM10 promoter haplotype influence on CSF APPα and PMB HP ADAM10
 CSF APPα levels in control subjects and AD patients according to *ADAM10* promoter rs514049 – rs653765 haplotypes C-A and A-G (Panel A). PMB HP mature ADAM10 (60kDa) protein levels in control subjects and AD patients according to *ADAM10* promoter rs514049 – rs653765 haplotypes C-A and A-G (Panel B). (Bonferroni corrected for multiple comparisons p-values: * $p < 0.05$, ** $p < 0.01$).

Figure 4. ADAM10 promoter haplotype luciferase reporter activity. ADAM10 promoter luciferase reporter construct structure (Panel A). Reporter construct activity is presented as firefly luciferase CPS relative to the internal loading control renilla luciferase CPS (Firefly / Renilla). Results are stratified by; 1) cell type (HepG2; human hepatocytoma, SHSY5Y; human neuroblastoma; U118; human astrocytoma), 2) rs514049 – rs653765 haplotypes C-A and A-G and 3) the presence or absence of the *ADAM10* 3'UTR region (*ADAM10* promoter only reporter constructs or *ADAM10* promoter – 3'UTR reporter constructs) (Panel B) (Bonferroni corrected for multiple comparisons p-values : * p < 0.05).

Table 1**Population sample description**

Cerebrospinal fluid sample used to measure APP α levels (Panel A). Post-mortem brain (PMB) sample used to measure ADAM10 expression (Panel B). Two sided Fisher Exact p-values are shown for the comparison between cognitively normal control subjects and AD patients.

Cerebrospinal fluid sample: APPα levels.				
A		Controls	AD	p-value
	n =	104	39	
	Mean Age (Range)	67 (52–88)	74 (56–87)	<0.0001
	Mean Age-At-Onset (Range)		69 (51–82)	
	% Male	50	56	0.574
	% <i>APOE</i> ϵ 4+	38	72	<0.0001
Post-mortem brain sample: ADAM10 levels.				
B		Controls	AD	p-value
	n =	22	21	
	Mean Age (range)	87 (77–95)	81 (60–93)	0.005
	Mean Age-at-onset (Range)		74 (55–89)	
	% Male	50	48	0.833
	% <i>APOE</i> ϵ 4+	18	67	0.002