Original Paper

Cholesterol 25-Hydroxylase on Chromosome 10q Is a Susceptibility Gene for Sporadic Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is the most common cause of dementia. It is characterized by β-amyloid (Aβ) plaques, neurofibrillary tangles and the degeneration of specifically vulnerable brain neurons. We observed high expression of the cholesterol 25-hydroxylase *(CH25H)* gene in specifically vulnerable brain regions of AD patients. *CH25H* maps to a region within 10q23 that has been previously linked to sporadic AD. Sequencing of the 5' region of *CH25H* revealed three common haplotypes, *CH25Hχ2*, *CH25Hχ3* and *CH25Hχ4*; CSF levels of the cholesterol precursor lathosterol were higher in carriers of the *CH25Hχ4* haplotype. In 1,282 patients with AD and 1,312 healthy control subjects from five independent populations, a common variation in the vicinity of *CH25H* was significantly associated with the risk for sporadic AD (p = 0.006). Quantitative neuropathology of brains from elderly non-demented subjects showed brain Aβ deposits in carriers of *CH25Hχ4* and *CH25Hχ3* haplotypes, whereas no Aβ deposits were present in *CH25Hχ2* carriers. Together, these results are compatible with a role of *CH25Hχ4* as a putative susceptibility factor for sporadic AD; they may explain part of the linkage of chromosome 10 markers with sporadic AD, and they suggest the possibility that *CH25H* polymorphisms are associated with different rates of brain Aβ deposition.

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Introduction

Alzheimer's disease (AD) is the most common cause of dementia. It is characterized by β-amyloid (βA) plaques, neurofibrillary tangles (NFTs) and the degeneration of specifically vulnerable brain neurons. Degeneration of neurons in AD occurs predominantly in such brain regions as the hippocampus, the inferior temporal cortex, the entorhinal cortex and the amygdala. The relative protection from degeneration of neuronal populations in the frontal and the occipital cortex [1] indicates selective vulnerability of specific neurons in brain regions involved in cognitive and memory processes.

We previously demonstrated specific downregulation of seladin-1 *(DHCR24)*, encoding 24-dehydrocholesterol reductase (seladin-1), in the inferior temporal cortex in AD using a differential mRNA display approach [2]; downregulation of *DHCR24* is reportedly associated with pathologic phosphorylation of tau, the major proteinaceous constituent of NFTs in AD [3].

In this study, we targeted a priori *CH25H*, encoding cholesterol 25-hydroxylase. The intronless *CH25H* codes for a polytopic membrane protein of 272 amino acids and plays an important role in lipid metabolism [4]. By synthesizing 25-hydroxycholesterol, a potent co-repressor of SREBP (sterol regulatory element binding protein) processing, *CH25H* is involved in the transcriptional regulation of lipid-related genes. Importantly, *CH25H* maps within a 30-cM-broad region on chromosome 10q, which has been recently linked to late-onset AD [5–7]. In addition, *CH25H* is known to be upregulated in the spinal cord of patients with amyotrophic lateral sclerosis [8], suggesting a potential role in neurodegeneration.

Because recent genetic evidence suggests that cholesterol- and lipid-related genes are associated with the risk for AD [9–14], we examined the differential expression of *CH25H* in AD, its potential association with disease risk, as well as its association with Aβ plaque deposition.

Subjects and Methods

Differential mRNA Expression Studies

Brain tissues from 12 aged individuals with Braak stages ranging from 0 to 6 were dissected and immediately frozen in liquid nitrogen, on average within 5 h postmortem. Brain areas for differential expression analysis were identified and stored at –80°C until RNA extractions were performed. In order to compare RNA populations from carefully selected postmortem brain tissues (hippocampus, and frontal and inferior temporal cortex), qPCR using the LightCycler™ was employed. The ratio of the normalized amount of candidate gene cDNA from the temporal cortex or hippocampus and frontal cortex was determined (relative quantification). The following strategy was used for normalization: Xenopus β-globin mRNA spiked into the mRNA of each brain tissue was used as a qPCR standard to normalize differences in the cDNA synthesis efficiency. Additionally, the qPCR procedure was applied to a set of housekeeping genes, which were selected as a reference standard for quantification. The ratio of the amount of frontal and temporal mRNA of five such housekeeping genes (cyclophilin B, ribosomal protein S9, β-actin, GAPDH and transferrin receptor) was determined, the mean value from the five ratios calculated and used for normalization of candidate gene expression. Primers for qPCR of *CH25H* were 5′-GGT CAA CAT CTG GCT TTC CG-3′ (forward) and 5′-CAC CAG TCT GTG AGT GGA CCA A-3′ (reverse).

Genetic Association Studies

Genetic studies were conducted on five independent populations: a Swiss sample (174 AD patients and 285 controls), a Mediterranean sample from Greece and Italy (272 AD patients and 125 controls), a Russian sample (74 AD patients and 90 controls), a French sample (551 AD patients and 665 controls) and a US sample derived from the NIMH Human Genetics Initiative and from the National Cell Repository for Alzheimer's Disease (NCRAD; grant No. U24 AG21886; 211 AD patients and 147 controls). The diagnosis of AD was performed according to the NINCDS-ADRDA criteria. The control groups comprised cognitively healthy elderly individuals who were either the spouses of AD patients or subjects recruited from the outpatient clinics of the participating institutions.

Neuropathological Studies

Neuropathological examinations were performed in the brains of 71 elderly individuals (mean age of death: 71.6 years, range 50–91 years, 28 females) devoid of significant neuropathological abnormalities and without signs of dementia, as measured by the Clinical Dementia Rating scale [15]. The evolutionary phases (0–4) of β-amyloidosis in the medial temporal lobe of these subjects were determined as described previously [16, 17]. NFT staging (0–6) was performed according to Braak and Braak [18]. For genotype determination, DNA was extracted from fresh-frozen samples of cerebelli following standard protocols.

CSF Studies

CSF was obtained by lumbar puncture in a subset of the participants of the genetic studies in Zurich. Forty-five AD patients (mean age: 70.1 years) and 27 healthy elderly subjects (mean age: 65.4 years) were included. For CSF $A\beta_{42}$ analysis, we used a sandwich ELISA (INNOTEST β-Amyloid 1–42, Innogenetics) with mAb 21F12 – specific for the free C-terminal end of $A\beta_{42}$ (peptide sequence Aβ 33–42) – as capturing antibody and mAb 3D6 – specific for the N-terminal end of $A\beta_{42}$ (peptide sequence Aβ 1–5) – as detector. CSF lathosterol was measured by means of combined gas chromatography/mass spectrometry [19].

SNP Selection and Genotyping

Information on polymorphic sites of 10q23–24 was derived from the database of single nucleotide polymorphisms (dbSNP) established by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/index.html), and from the Celera database (www.celera.com). The following genes were se-

Fig. 1. *CH25H* mRNA overexpression in vulnerable brain regions in late Braak stages. Expression analysis of *CH25H* mRNA was done in the inferior temporal lobe and the frontal lobe from brains of 12 aged individuals with Braak stages ranging from 0 to 6. A value of 0% indicates equal *CH25H* mRNA expression levels in the inferior temporal lobe and the frontal lobe. Bars represent means of expression ratios ± SEM. Normalization for housekeeping genes. p = 0.016 (Spearman's rank correlation). Comparison of *CH25H* mRNA levels between the hippocampus and the frontal lobe from brains of 9 aged individuals showed significant mRNA elevation up to Braak stage 5 (Rs = 0.8, p = 0.031; data not shown).
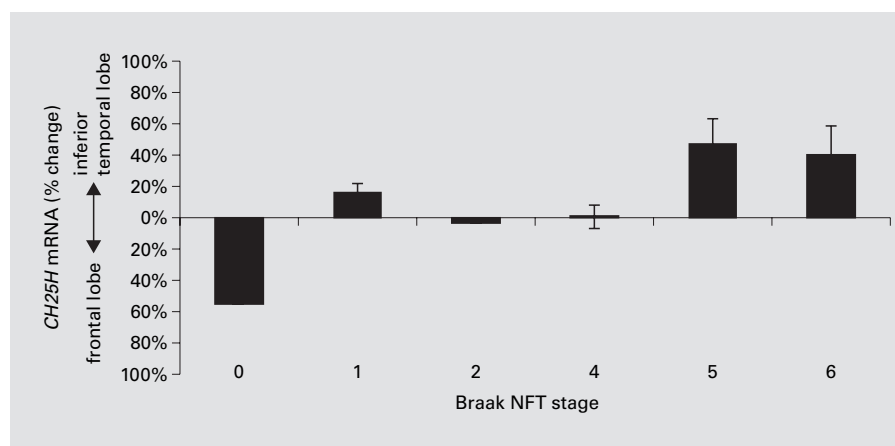

Fig. 2. LD between SNP *CH25H*1* at –6443 bp and SNP *CH25H*2* at –6627 bp (relative to the start codon of *CH25H*). Haplotypes were reconstructed by including individuals homozygous for one or both SNPs. Subjects heterozygous for both SNPs were excluded.

lected due to possible relevance for AD: *PTEN* (phosphatase and tensin homolog), *ACTA2* (α_2-actin), *TNFRSF6* (tumor necrosis factor receptor superfamily, member 6), *CH25H* (cholesterol 25-hydroxylase), *LIPA* (lipase A), *PPP1R3C* (protein phosphatase 1, regulatory subunit 3C), *CYP2C8* [cytochrome P$_{450}$, subfamily IIC (mephenytoin 4-hydroxylase), polypeptide 8], *TLL2* (tolloid-like 2), *SLIT1* (slit homolog 1), *PGAM1* (phosphoglycerate mutase 1), *SFRP5* (secreted frizzled-related protein 5), *HPA2* (heparanase 2), *GOT1* (glutamic-oxaloacetic transaminase 1), *COX15* (COX15 homolog), *WNT8B* (wingless-type MMTV integration site family), *NEURL* (neuralized-like), *SLK* (Ste20-related serine/threonine kinase), and *GSTTLp28* (glutathione-S-transferase like). In these genes, the following SNPs were analyzed: rs1903858, rs1389, rs1324551, hCV399212, rs13500, rs1131706, rs1556478, rs1051339, rs1051338, rs1044563, rs2068888, rs1058932, rs730179, rs902471, rs716838, rs1126878, rs2075430, rs1556971, rs2236278, rs2970, rs1047425, rs805657, and rs4925. SNP identification in the ORF, the 5′ and the 3′ region of *CH25H*, was done by double-stranded sequencing of 40 chromosomes on an ABI PRISM® 310 Genetic Analyzer. The Masscode™ system was used for SNP genotyping [20].

Statistics

Genotype and allelic frequencies between AD patients and controls were compared by Fisher's exact tests. Forward and backward unconditional logistic regression analyses were done for the simultaneous assessment of the influence of age, gender, *APOE* and *CH25H* genotypes on the risk for developing AD. The estimate haplotype frequencies program was used to test for linkage disequilibrium (LD) between SNPs and for significance of haplotype distribution between AD cases and controls [21]. The FBAT and SDT algorithms were used for family-based association analysis [22]. Phases of β-amyloidosis between groups were compared with the U test by Wilcoxon, and Mann and Whitney. The significance of correlation between *CH25H* mRNA expression levels and NFT stages was assessed by Spearman's rank correlation coefficient. For the comparison of CSF Aβ$_{42}$ and lathosterol levels, t tests were used.

Results

First-Stage Analysis

Using comprehensive differential display [23, 24] and real-time quantitative PCR analyses, we observed high *CH25H* (encoding cholesterol 25-hydroxylase) expression in such vulnerable brain regions as the inferior temporal cortex and the hippocampus in AD patients (fig. 1).

We then sequenced the ORF and 6.8 kb of the 5′ genomic region of *CH25H* and identified two synonymous SNPs, four 3′ SNPs, and six 5′ SNPs. Haplotype analysis revealed three common haplotypes, designated *CH25Hχ2*, *CH25Hχ3* and *CH25Hχ4*, composed of SNP *CH25H*1* at –6443 bp and SNP *CH25H*2* at –6627 bp (fig. 2).
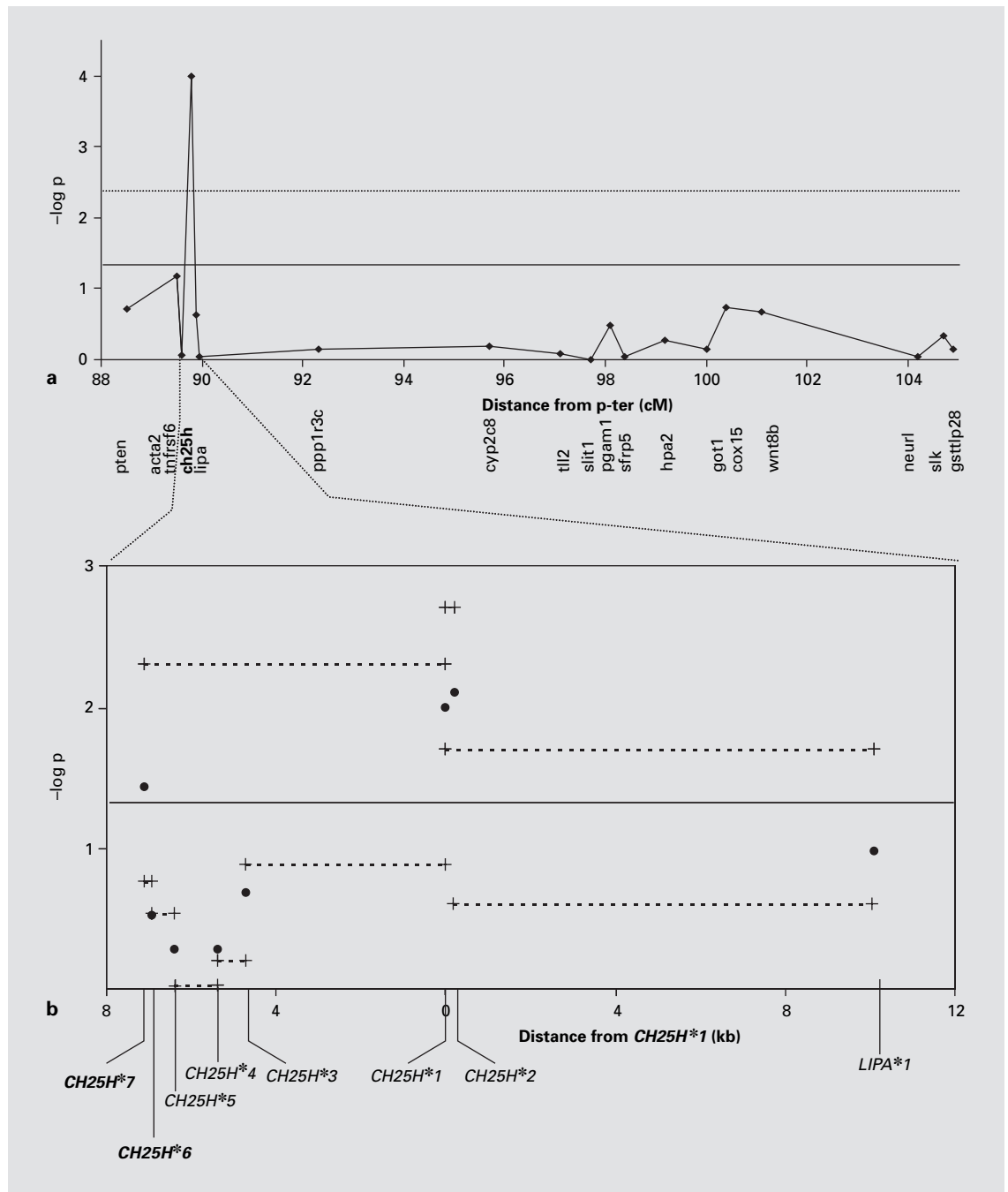
Fig. 3. SNPs in the 5′ UTR of CH25H were significantly associated with AD. **a** Allelic association of SNPs on 10q with AD. Values on the y-axis represent the negative logarithm of the significance p (χ^2 test for allelic association). The horizontal continuous line represents the significance level of 0.05, the dotted line represents the significance level after Bonferroni correction for all SNPs analyzed. Distance from p-ter is given in the x-axis in cM according to the NCBI map. **b** Fine mapping of the *CH25H* locus at 90 cM. *CH25H*1*: [T-6443C], corresponds to SNP rs13500; *CH25H*2*: [A-6627T], corresponds to SNP rs1131706; *CH25H*3*: [C-1710T]; *CH25H*4*: [A-054G]; *CH25H*5*: [A-44G]; *CH25H*6*: [T503C]; *CH25H*7*: [A656G]; *LIPA*1*: corresponds to SNP rs1556478. SNP positions were calculated in relation to the start codon of *CH25H*.
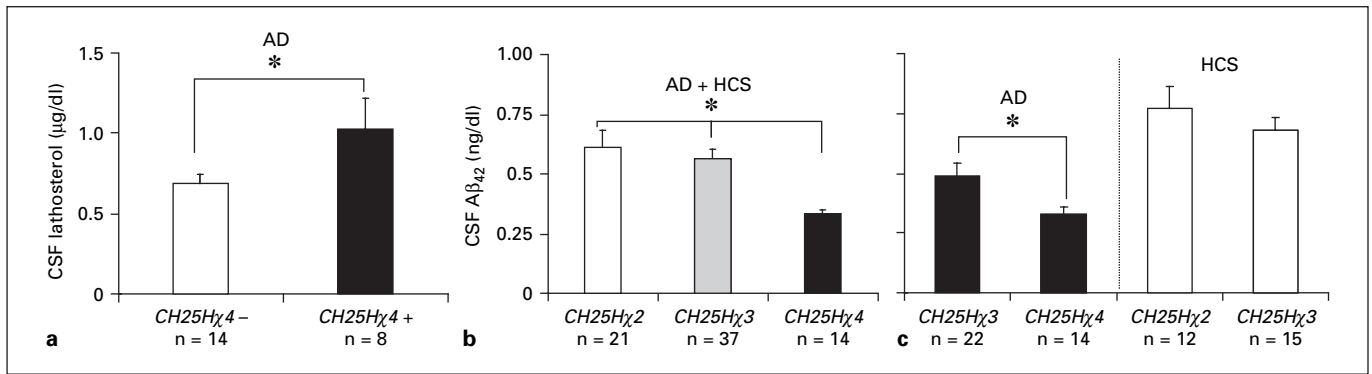
Fig. 4. a Higher CSF concentrations of lathosterol in diseased carriers of the *CH25Hχ4* haplotype as compared to diseased non-carriers (* p = 0.05, Student's t test). **b** Low CSF concentrations of soluble $A\beta_{42}$ in *CH25Hχ4* carriers, intermediate concentrations in *CH25Hχ3* carriers, and high concentrations in *CH25Hχ2* carriers (* p = 0.002, ANOVA). **c** Significantly lower CSF concentrations of soluble $A\beta_{42}$ in diseased *CH25Hχ4* carriers as compared to diseased *CH25Hχ3* carriers (* p = 0.014, Student's t test). The difference between healthy *CH25Hχ3* and *CH25Hχ2* carriers was not significant. Bars represent means ± SEM. AD = AD patients; HCS = healthy control subjects.

Genetic data in Caucasian populations (www.celera.com) indicate that both *CH25H*1* and *CH25H*2* are located within an extended haplotype covering *CH25H* and *LIPA* (encoding lipase A, also known as cholesterol ester hydrolase). *LIPA* was not found to be differentially expressed in our analysis. Because the reported linkage peak on 10q may result from the combined effect of multiple susceptibility genes, we assessed the association between AD and 18 possibly relevant genes within a 20-cM-broad region on 10q23–24. Both SNPs and extended haplotypes were analyzed in 446 AD patients and 410 unrelated control subjects from Switzerland and the Mediterranean region (Greece and Italy). SNP *CH25H*1* showed significant allelic association with AD (p = 0.0002, fig. 3a). Fine mapping of an 18-kb region around *CH25H*1* and subsequent estimated haplotype analysis revealed strong LD between SNPs *CH25H*1* and *CH25H*2* in the Swiss population (p < 0.001) and weak LD in the Mediterranean population (p = 0.02). Association mapping of two synonymous SNPs in *CH25H*, five SNPs in the 5′ region of the gene and three SNPs in the adjacent *LIPA* gene in the Swiss population revealed significant allelic association of *CH25H*1*, *CH25H*2*, and *CH25H*7* with AD (fig. 3b). Haplotype *CH25Hχ* reached the highest significance of association with AD. Significant allelic and haplotypic association of *CH25H*1* and *CH25Hχ* with AD was also observed in the combined sample (p = 0.0002, p = 0.00003, respectively; table 1). In the Mediterranean sample, significant association was observed for *CH25H*1* and *CH25Hχ* (p < 0.05), but not for *CH25H*2* (p > 0.1). An

additive interaction between *APOE4* and *CH25Hχ4* was observed in the combined sample. Compared with individuals lacking the *APOE4* allele and the *CH25Hχ4* haplotype, the odds ratio for carriers of both *APOE4* and *CH25Hχ4* was 5.5 (95% confidence interval: 2.8–10.9). The odds ratios and 95% confidence intervals were 3.1 (2.1–4.5) for *APOE4* carriers and 2.7 (1.5–4.8) for *CH25Hχ4* carriers. In addition to *CH25Hχ*, two additional haplotypes containing *CH25H*1* showed significant, yet less pronounced association with AD (fig. 3b). Seventeen SNPs within the examined region on 10q failed to show significant allelic association with AD in the study populations.

SNP *CH25H*2* is located within the core sequence (CTTG) of the functional binding site for the steroidogenic factor 1 (SF-1) [25, 26], which is involved in the transcriptional regulation of steroid hydroxylases [27]

Table 1. Significantly different distribution of the *CH25Hχ4* haplotype and the *CH25H*1 T* allele between AD patients and healthy control subjects (HCS) in the combined sample of the first-stage analysis

	HCS, % (n = 410)	AD, % (n = 446)	p value
CH25Hχ4 haplotype	11.4	22.2	0.00003
*CH25H*1 T* allele	13.9	23.8	0.0002
*CH25H*2 A* allele	23.9	20.8	0.28

Table 2. Brain βA load in the medial temporal lobe differs between *CH25H* haplotypes and alleles

	n	βA load	p value
CH25Hχ haplotype			
χ4	10	1.5 ± 0.9	
χ3	44	1.0 ± 0.6	0.002
χ2	17	0.0 ± 0.0	
*CH25H*1 T* allele			
T+	10	1.5 ± 0.9	0.127
T−	61	0.0 ± 0.3	
*CH25H*2 A* allele			
A+	19	0.0 ± 0.0	<0.001
A−	52	1.0 ± 0.6	

Brain βA load: values represent blindly scored phases of β − A load and are given as medians ± SEM. Statistical comparisons: H test (*CH25Hχ* haplotypes) and U test (*CH25H*1* and *CH25H*2* alleles).

Table 3. Genetic findings of SNP *CH25H*1* in ethnically independent populations

*CH25H*1* genotypes	Control subjects	AD patients
Swiss sample		
Total	285	174
C/C	246 (86.3%)	133 (76.4%)
C/T or *T/T*	39 (13.7%)	41 (23.6%)
Statistics	$\chi^2 = 7.3$, d.f. = 1, p = 0.007	
Mediterranean sample		
Total	125	272
C/C	107 (85.6%)	207 (76.1%)
C/T or *T/T*	18 (14.4%)	65 (23.9%)
Statistics	$\chi^2 = 4.7$, d.f. = 1, p = 0.031	
Russian sample		
Total	90	74
C/C	78 (86.7%)	55 (74.3%)
C/T or *T/T*	12 (13.3%)	19 (25.7%)
Statistics	$\chi^2 = 4.0$, d.f. = 1, p = 0.045	
French sample		
Total	665	551
C/C	530 (79.7%)	452 (82.0%)
C/T or *T/T*	135 (20.3%)	99 (18.0%)
Statistics	$\chi^2 = 1.1$, d.f. = 1, p = 0.304	
NIMH/NIA sample		
Total	147	211
C/C	129 (87.8%)	164 (77.7%)
C/T or *T/T*	18 (12.2%)	47 (22.3%)
Statistics	$\chi^2 = 5.9$, d.f. = 1, p = 0.015	
Combined sample		
Total	1,312	1,282
C/C	1,090 (83.1%)	1,011 (78.9%)
C/T or *T/T*	222 (16.9%)	271 (21.1%)
Statistics	$\chi^2 = 7.5$, d.f. = 1, p = 0.006	

and lipoprotein receptors [28]. Because allele *A* of *CH25H*2* eliminates the SF-1 binding site, which results in impaired activity of SF-1-dependent regulatory regions [25], and because *CH25H* is a potent regulator of cholesterol synthesis [4], we examined whether the *CH25H*2*-containing haplotype *CH25Hχ* is associated with the levels of metabolic precursors of cholesterol. We found that the concentration of the cholesterol precursor lathosterol in CSF of *CH25Hχ4* carriers was significantly higher than in non-carriers (fig. 4a).

To investigate whether *CH25Hχ* exerts effects relevant to the pathophysiology of AD, we examined whether *CH25H* haplotypes differentially affected Aβ plaque pathology. Brain samples from the medial temporal lobes of AD patients are ill suited for the study of genetic effects on amyloid load because they invariably show highest levels of Aβ plaque pathology. Therefore, we prevented such ceiling effects and examined the brains of 71 elderly non-demented subjects (age at death ≥ 50 years). We observed that both *CH25Hχ4* and *CH25Hχ3* were associated with high scores of brain Aβ deposition, whereas no Aβ deposits were present in *CH25Hχ2* carriers (p = 0.002, table 2). In contrast, Braak's NFT staging [18] was similar among haplotype groups (p = 0.7). The *CH25Hχ*-related differences in brain Aβ deposition were paralleled by low CSF levels of $A\beta_{42}$ in *CH25Hχ4* carriers, intermediate levels in *CH25Hχ3* carriers, and high levels in *CH25Hχ2* carriers (fig. 4b, c).

Second-Stage Analysis

The significant association of SNP *CH25H*1* with the risk for AD prompted us to validate this finding in ethnically independent case-control series from Russia (n = 164) and France (n = 1,216). We also genotyped a large sample of siblings who were part of the NIMH Human Genetics Initiative and the NCRAD (grant No. U24 AG21886) and applied family-based and case-control analytical methods.

Consistent with the findings in the Swiss and Mediterranean populations, the *T* allele of *CH25H*1* was significantly overrepresented in Russian AD patients as compared to control subjects (p = 0.031, table 3). This was not the case in the French sample (p = 0.3). Family-based analysis (using both the FBAT and the SDT algorithms)

in the NIMH sample also failed to yield significant results (p > 0.1). Discrepancies between family-based and case-control approaches are common and may be related to the differential proportions of positive family history between samples [29]. Therefore, we selected from the sib-pair sample a group of AD patients and unrelated control subjects to compare the family-based and case-control results in the same population. We selected the controls (n = 147) from sibships with at least one control subject. Where two or more control subjects were present, the oldest one was chosen. Unrelated AD patients (n = 211) were selected from the remaining sibships. Where possible, autopsy-verified cases and controls were selected. Association analysis in that sample confirmed the significant results obtained in the Swiss, Mediterranean and Russian populations: the *T* allele of *CH25H*1* was significantly overrepresented in the US AD patients as compared to the control subjects (p = 0.015, table 3). Importantly, the genotype frequencies were nearly identical across populations. In addition, a compound analysis in the pooled case-control sample (1,282 AD patients and 1,312 control subjects) revealed a significant effect of the *CH25H*1* SNP on AD risk (p = 0.006).

Discussion

Since the first report of genetic linkage of sporadic AD to chromosome 10q [6], subsequent studies narrowed down the genetic region of interest [5, 30, 31]. By combining the information of genome scans with a novel candidate gene approach that is based upon specific information on gene expression levels in the brain, we found evidence that *CH25H* is expressed differentially in brains of patients with AD, and that genetic variants in the vicinity of this gene are associated with the risk for sporadic AD. We obtained significant associations in four ethnically independent populations, whereas no significant association was observed in a sample of French patients and control subjects. Analysis in the pooled sample of 1,282 AD patients and 1,312 control subjects confirmed the significant association of *CH25H*1* with AD. It is therefore unlikely that the findings reported here are due to random effects or multiple testing. They rather underscore the importance of ethnicity as an important confounding factor in genetic association analyses.

In addition to the genetic findings in the case-control populations, we found evidence that *CH25H* is related to the pathophysiology of AD, because the risk haplotype *CH25Hχ4* was associated with high brain Aβ load and low levels of soluble $A\beta_{42}$ in the CSF. The association of *CH25Hχ4* with high Aβ load in the medial temporal lobe suggests that this genetic variant enhances pathologic amyloidogenesis in vulnerable areas of the human brain. At the same time, *CH25Hχ4* was found to be related to *low* levels of soluble $A\beta_{42}$ in the CSF. Aβ is secreted as a soluble protein to the extracellular space, including CSF, as part of the normal constitutive metabolism of amyloid precursor protein [32]. Since the CSF is in direct contact with the extracellular space of the brain, biochemical changes in the brain may be reflected in the CSF. However, the relationship between brain and CSF Aβ is only poorly understood. Nevertheless, a decrease in CSF $A\beta_{42}$ in AD might reflect the deposition of the protein in senile plaques with low levels of soluble peptide remaining to diffuse to the CSF [33]. Indeed, low levels of CSF $A\beta_{42}$ are robustly observed in AD, even in the very early stages [34]. Alternatively, the reduction in $A\beta_{42}$ levels in the CSF of patients with AD may be secondary to a disturbance in the metabolism of amyloid precursor protein and Aβ in already dysfunctional neurons. Either way, our results show an aggravation of this AD-related phenotype (i.e. low $A\beta_{42}$ levels in the CSF) in carriers of the *CH25Hχ4* haplotype and, thereby, are in favor of the hypothesized relationship between *CH25H* and amyloid metabolism. In addition, carriers of *CH25Hχ4* had increased CSF levels of the cholesterol precursor lathosterol, supporting possible physiological differences of the haplotypes in the transcriptional regulation of *CH25H*.

We conclude that *CH25H* is genetically associated with the risk for sporadic AD, with the severity of Aβ deposition in the brain and with the levels of soluble $A\beta_{42}$ in the CSF. By modulating the fluidity and structural integrity of the cell membrane, cholesterol influences the membrane-bound proteolytic processing of amyloid precursor protein. We therefore hypothesize that the association of *CH25H* with AD is linked to the effects of *CH25H* on cellular cholesterol biosynthesis. In support of this hypothesis, the examined *CH25H* haplotype in the 5′ flanking region of the gene was related to the CSF levels of metabolic precursors of cholesterol.

CH25H is located within an AD-linked chromosomal region on 10q. The available genetic data suggest the existence of more than one susceptibility gene in this region, possibly proximal to *CH25H*. Further studies are needed for the identification of these genes, which in sum may contribute to a genetic risk profile for sporadic late-onset AD.

Acknowledgment

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