

Alzheimer's disease: No effect of the CDK5 gene on CSF biomarkers, neuropathology or disease risk

ANNICA SJÖLANDER¹, MALIN E. ANDERSSON¹, HENRIK ZETTERBERG^{1,2}, LENNART MINTHON^{3,4},
NENAD BOGDANOVIC⁵ and KAJ BLENNOW¹

¹Institute of Neuroscience and Physiology, Department of Neurochemistry and Psychiatry,

²Institute of Biomedicine, Department of Clinical Chemistry and Transfusion Medicine, Sahlgrenska University Hospital, Göteborg University, Göteborg; ³Clinical Memory Research Unit, Department of Clinical Sciences Malmö, Lund University;

⁴Neuropsychiatric Clinic, Malmö University Hospital, Malmö; ⁵Neurotec Department, Section of Clinical Geriatrics, Karolinska Institutet, Karolinska University Hospital in Huddinge, Stockholm, Sweden

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Abstract. Cyclin-dependent kinase 5 (cdk5) has been identified as one of the kinases that phosphorylates tau at several Alzheimer's disease (AD)-associated sites. Cdk5 is predominantly expressed in neurons, and has higher activity in AD brains than in non-demented brains. To investigate the effect of the *CDK5* gene on AD, we analyzed an SNP of the *CDK5* gene (rs2069456) in 347 patients with AD and in 157 controls. *CDK5* genetic data was investigated in subgroups in relation to biochemical and neuropathological markers for AD. We found no significant differences in genotype or allele distributions between AD patients and controls. None of the *CDK5* gene variants influenced biomarkers for AD.

Introduction

One of the characteristics of Alzheimer's disease (AD) is the intraneuronal accumulation of neurofibrillary tangles (NFTs). NFTs are composed of a hyperphosphorylated form of the microtubule-associated protein tau (1,2). When tau is in its hyperphosphorylated form, its ability to perform the role of maintaining neuronal morphology by promoting the assembly and stabilization of microtubules is impaired (3). Cyclin-dependent kinase 5 (cdk5) is identified as one of the proline-directed serine/threonine kinases that mediates hyperphosphorylation at several AD-associated phosphorylation sites on tau (4-8). Cdk5 belongs to the cyclin-dependent

kinase (cdk) family. However, in contrast to other cdks, cdk5 is not directly involved in cell cycle events (9). Although cdk5 is expressed in various tissues, its activity predominantly takes place in neurons as its activators, p35 and p39, are restricted to this cell type (10,11). In the brain, cdk5 interacts closely with NFTs (12,13). Significantly higher cdk5 activity is found in AD brains compared to non-demented brains (14). When the cysteine-protease calpain cleaves p35 into p25, a more stable p25-cdk5 complex is formed (15). This is due to the approximately 5-fold longer half-life of p25 compared to p35. Therefore, p25 can give rise to the prolonged activation of cdk5, which in turn leads to the hyperphosphorylation of tau (15). Of note, accumulations of both calpain and p25 are found in human AD brains (16).

Based on these findings, we hypothesized that the *CDK5* gene might be a candidate risk gene for AD. To test this hypothesis, we studied the influence of a single nucleotide polymorphism (SNP) in the *CDK5* gene on the risk of AD and on biochemical and neuropathological markers. No previous study has investigated biochemical or neuropathological markers for AD in relation to *CDK5* genotype data. The rs2069456 SNP (A→C, intron 7, <http://genome.ucsc.edu>) was selected due to its high allele frequency compared to other known SNPs in this gene. The global frequency of the least common variant C allele is reported to be 0.32 (<http://www.ncbi.nlm.nih.gov/sites/entrez>).

Materials and methods

Subjects. The study included a total of 347 patients with AD (120 men and 227 women) and 157 controls (73 men and 84 women) of Caucasian origin. Data on cerebrospinal fluid (CSF) levels of biochemical markers for AD were available for 265 AD patients (75±6.4 years) and for 53 controls (73±7.9 years). Total-tau, phospho-tau₁₈₁ and β -amyloid (A β)₁₋₄₂ levels were determined using established ELISA methods (17,18). Neuropathological post-mortem examinations were performed on 82 AD patients (79±7.9 years) and 100 controls (77±9.4 years). Consequently, data on the amounts of senile plaques (SPs) and NFTs were available.

Correspondence to: Dr Annica Sjölander, Department of Clinical Chemistry and Transfusion Medicine, Molecular Biology, The Sahlgrenska Academy at Göteborg University, Sahlgrenska University Hospital, SE-413 45 Göteborg, Sweden
E-mail: annica.sjoland@neuro.gu.se

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Individuals included in the study were recruited at the Clinical Memory Research Unit, Department of Clinical Sciences Malmö, Lund University, Sweden, and at the Neurotec Department, Section of Clinical Geriatrics, Karolinska Institutet, Karolinska University Hospital in Huddinge, Stockholm, Sweden. The study was approved by the local ethics committees in Sweden. The patients (or their closest relatives) and the control subjects gave their informed consent to participate in the study, which was conducted in accordance with the provisions of the Helsinki Declaration.

Patients in the clinical group were clinically diagnosed with probable late-onset AD according to the NINCDS-ADRDA criteria (19). Clinical diagnoses included a medical history, physical, neurological and psychiatric examination, screening laboratory tests, ECG, X-ray of the chest, EEG, and computerized tomography of the brain. The Mini-Mental State Examination (MMSE) was performed according to standard procedures (20). No patient had a family history of autosomal dominant dementia. In the clinical control group, only individuals without history, symptoms or signs of psychiatric or neurological disease, malignant disease, or systemic disorders were included. Individuals with MMSE scores below 28 were not recruited to the control group. In the post-mortem group, AD patients were clinically diagnosed with probable late-onset AD according to the NINCDS-ADRDA criteria (19), and were confirmed as definitive AD if the neuropathological diagnoses fulfilled the neuropathological CERAD criteria (21). All AD brains had a histopathological score of >5 and revealed no infarcts or other changes that could account for the dementia. Included in the neuropathological control group were individuals who had succumbed to cardiac or malignant disease. They had no history of dementia, psychiatric or neurological disease, and all brains had a histopathological score (22) of ≤ 4 . Clinical and post-mortem diagnoses were made without knowledge of the results of the biochemical and/or genetic analyses, and *vice versa*.

Genetic analysis. Gene designations follow the recommendations of the HUGO Gene Nomenclature Committee (23).

Genomic DNA was extracted from whole blood samples or brain tissue using the GenoPrep™ DNA Blood kit or DNA MagAttract kit (Qiagen, Hilden, Germany) together with the GenoM-48 Robotic Workstation (GenoVision, Oslo, Norway). Approximately 100 μ l of whole blood or 10 mg of brain tissue was used for each extraction. The isolated genomic DNA was stored at -20°C until it was used for gene analysis.

Primers surrounding the *CDK5* rs2069456 SNP were designed using the human *CDK5* genomic sequence information deposited in the UCSC database (<http://genome.ucsc.edu>). To diminish the risk of hairpin structures in the following Dynamic Allele Specific Hybridization (DASH) procedure, the PCR product was analysed with a DNA folding analysis program (MFOLD Zucker software). DASH probes matching the A and C variants of the PCR product were used. PCR was carried out using AmpliTaq Gold® (Applied Biosystems, Foster City, USA) in a final volume of 25 μ l containing 5–20 ng of template DNA. The reaction was optimized by examining the size and specificity of the PCR product on a 2.5% agarose gel (SeaPlaque® GTG® Agarose, FMC BioProducts, Philadelphia, USA). Optimal conditions were determined as:

4.0 mM MgCl_2 , 200 μM dNTPs, 0.5 U of Taq, 0.36 pmol/ μ l of the forward biotinylated primer (5'-Biotin-TCCGCT-GTTACTCAGCTGAGG-3') and 0.44 pmol/ μ l of the reverse primer (5'-GACTCCCTCCCCCAATCCC-3'), (Invitrogen, Carlsbad, USA) in 1X PCR buffer. The thermal cycling profile was designed as follows: 10 min initial denaturation at 95°C followed by 40 cycles of 30 sec at 95°C , 45 sec at 59°C , 1 min at 72°C and a final extension step of 10 min at 72°C . Genotyping of the SNP was performed using the G-containing probe 5'-ATCCCATCGTCTAGCTC-Rox-3' (MWG Biotech, Ebersberg, Germany), as it gave the best separation of melting temperature. DASH procedure followed a previous description by Prince *et al* (24).

DNA sequencing was carried out for seven samples to confirm DASH genotyping. Amplification was performed using Taq DNA polymerase (Roche, Basel, Switzerland). Optimal conditions were 1.0 mM MgCl_2 , 0.4 pmol/ μ l primers (forward: 5'-TTCAGAATGGGGAGCTGAAATTG-3'; reverse: 5'-ACTTAGAGGACTGGGGAGGAG-3' (Invitrogen), and 55°C hybridization temperature. In each reaction, 5–20 ng of template DNA was used. PCR products were purified using MicroSpin™ S-300 HR Columns (Amersham Biosciences, Piscataway, USA) and sequenced in both forward and reverse directions using cycle sequencing with fluorescent dNTPs (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit, Applied Biosystems), separation by capillary electrophoresis and detection by laser-induced fluorescence in an ABI PRISM 3100 Genetic Analyzer (Perkin-Elmer, Waltham, USA).

Apolipoprotein E (*APOE*) genotyping was performed using minisequencing as previously described (25). Two SNPs, rs7412 and rs429358, were used to determine the presence of the $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$ alleles.

Cerebrospinal fluid collection. In the AD patients and controls, CSF samples were obtained by lumbar puncture in the L3/L4 or L4/L5 interspaces under standardized conditions. A CSF volume of 12 ml was collected and gently mixed to avoid gradient effects, followed by centrifugation to remove cell debris. CSF samples were collected according to standard conditions, then frozen and stored at -80°C pending biochemical analysis without being refrozen. Details regarding CSF collection have previously been described (26).

Cerebrospinal fluid analysis. Levels of CSF total-tau (Innotest™ hTAU-Ag), phospho-tau [Innotest phospho-tau(181P)] and $\text{A}\beta_{1-42}$ [Innotest β -amyloid(1-42)], all from Innogenetics (Gent, Belgium), were determined using a solid-phase enzyme immunoassay as previously described (17,18,26,27). The detection limit for these assays on CSF samples is 20–50 pg/ml. The analyses were run on the same batch of antibodies and ELISA plates. Day-to-day imprecision (CV) for all biochemical analyses was $<10\%$.

Statistical analysis. *CDK5* genotypes and allele frequencies comparing AD patients and control subjects were statistically analysed using Pearson's χ^2 test. Testing for associations between genotypes and CSF biomarkers (total-tau, phospho-tau, $\text{A}\beta_{1-42}$) and histopathological scoring (SPs and NFTs) was performed using the analysis of variance (ANOVA). Since the

Table I. *CDK5* and *APOE* genotype and allele frequencies in Alzheimer's patients and controls.

	Genotype frequencies							Allele frequencies				
	<i>CDK5</i>			<i>APOE</i>			No.	<i>CDK5</i>		<i>APOE</i>		
	No.	AA (%)	AC (%)	CC (%)	No ϵ 4 (%)	One ϵ 4 (%)	Two ϵ 4 (%)	A (%)	C (%)	ϵ 2/ ϵ 3 (%)	ϵ 4 (%)	
Patients	347	170 (49.0)	151 (43.5)	26 (7.5)	96 (27.7)	179 (51.6)	72 (20.7) ^a	694	491 (70.7)	203 (29.3)	371 (53.5)	323 (46.5) ^a
Controls	157	82 (52.2)	61 (38.9)	14 (8.9)	112 (71.3)	41 (26.1)	4 (2.6)	314	225 (71.7)	89 (28.3)	265 (84.4)	49 (15.6)

CDK5, cyclin-dependent kinase 5; *APOE*, apolipoprotein E; No., number of individuals. ^ap<0.001 compared to controls, Pearson's χ^2 test.

Table II. Cerebrospinal fluid protein levels in Alzheimer's disease patients and controls.

CSF protein	Patients (n=265)	Controls (n=53)	P-value
Total-tau	670.4±21.2	335.9±18.0	P<0.001
Phospho-tau	84.3±2.1	61.2±3.1	P<0.001
β -amyloid ₁₋₄₂	479.0±6.8	738.0±23.7	P<0.001

CSF, cerebrospinal fluid. Data are the mean values (pg/ml) \pm SEM; CSF protein levels between Alzheimer's disease patients and controls were compared using Kruskal-Wallis one-way analysis of variance.

CSF protein levels were slightly skewed, we also performed the non-parametric Kruskal-Wallis analysis of rank. Comparing observed and expected genotype frequencies using the χ^2 test assessed deviations from Hardy-Weinberg equilibrium. All analyses were performed using the SYSTAT program version 10.2 (SYSTAT Software GmbH, Erkrath, Germany). P-values <0.05 was considered statistically significant.

Results

This study was well powered (>95%) to significantly detect a minor allele resulting in a relative risk >2. Genotype frequencies conformed to Hardy-Weinberg equilibrium in both AD patients and control subjects. We verified the well-established association between the *APOE* genotype and AD (Table I). The *APOE* ϵ 4 allele was significantly more frequent in AD patients compared to control subjects (Table I).

Frequencies of the *CDK5* genotype (p=0.54) or allele (p=0.68) did not differ between AD patients and control subjects (Table I). *CDK5* genotype and allele frequencies were almost identical in the clinical (p=0.17, p=0.50) and the post-mortem (p=0.57, p=0.42) populations (data not shown).

Comparisons of CSF biomarkers in AD patients and control subjects have previously revealed highly significant differences (28). As expected, we found that the AD patients had increased levels of total-tau and phospho-tau and decreased levels of $A\beta_{1-42}$ in CSF compared to the control group (Table II). When analyzing the results for a possible association between *CDK5* gene variants and levels of CSF biomarkers, no such association was found (Table III). Neither total-tau (p=0.62), phospho-tau (p=0.66) nor $A\beta_{1-42}$ (p=0.34) CSF levels were influenced by the *CDK5* SNP (Table III).

Table III. Biochemical markers in cerebrospinal fluid and *CDK5* genotypes in Alzheimer's disease patients and controls.

CSF protein	AA	AC	CC
Patients (no.)	132	111	22
Total-tau	682.2±32.1	632.8± 28.7	789.3± 79.6
Phospho-tau	86.1± 3.3	80.5±2.9	92.4±6.2)
β -amyloid ₁₋₄₂	468.5± 9.6	492.2±10.5 ^a	475.9±23.2
Controls (no.)	27	17	9
Total-tau	332.8± 25.6	345.6± 30.9	326.7±49.0
Phospho-tau	58.9±4.6	64.7±5.0	61.3±8.3
β -amyloid ₁₋₄₂	746.4±33.6	708.9±44.0	767.4±53.8

CSF, cerebrospinal fluid. Data are the mean values (pg/ml) \pm SEM. ^aEvaluated in 110 patients only.

As expected, the histopathological scores of SPs and NFTs were significantly higher in AD patients compared to control subjects (p<0.001). However, no correlation was found between SP-NFT scores and *CDK5* genotypes (p=0.18, data not shown).

We also explored whether the results were influenced by *APOE* status. *CDK5* genotype and allele frequencies did not differ in AD patients with different numbers of *APOE* ϵ 4 alleles (data not shown).

Discussion

Several genetic association studies have previously been performed for AD (29). To date, the ϵ 4 variant of the *APOE* gene is the best established risk gene for AD (30,31).

In the present study, we investigated a *CDK5* SNP (rs2069456) with a relatively high population frequency (<http://genome.ucsc.edu>) and examined whether it was associated with an increased risk of AD. Since we had access to CSF samples and brain tissue from subsets of the participants, *CDK5* gene data was examined in relation to biochemical and neuropathological markers for AD.

We considered *CDK5* to be a good candidate gene for study, since accumulating evidence indicates that the cdk5 protein participates in several AD-associated events. Cdk5 has been found to be involved in AD-specific tau hyperphosphorylation, and interacts closely with the NFTs (8,12,13). Cdk5 is predomi-

nantly expressed in brain tissue, and elevated Cdk5 activity is evident in brain tissue from AD patients (10,14). Interestingly, the evidence suggests that cdk5 is a key player linking A β toxicity to tau pathology and neurodegeneration. A β treatment has been found to transform cdk5 into a hyperactive kinase through its activators, p25 or p29 (32). Accumulation of p25 is found in human AD brains (33), and cdk5 closely interacts with NFTs (12).

Despite the high power of the present study, the *CDK5* SNP did not influence AD risk, neither in the whole population, nor in subsets of samples. The results were not affected following *APOE* ϵ 4 stratification of AD patients. *CDK5* gene variants had no effect on CSF biomarkers or neuropathological scores for AD. In conclusion, we found no evidence indicating that the *CDK5* gene significantly contributes to the pathogenesis of AD.

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