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Genetic Association of *CDC2* with Cerebrospinal Fluid Tau in Alzheimer's Disease

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Key Words
Alzheimer's disease · *CDC2* · Cell cycle · Tau · β-Amyloid

Abstract
We have recently reported that a polymorphism in the cell division cycle *(CDC2)* gene, designated Ex6 + 7I/D, is associated with Alzheimer's disease (AD). The *CDC2* gene is located on chromosome 10q21.1 close to the marker D10S1225 linked to AD. Active cdc2 accumulates in neurons containing neurofibrillary tangles (NFT), a process that can precede the formation of NFT. Therefore, *CDC2* is a promising candidate susceptibility gene for AD. We investigated the possible effects of the *CDC2* polymorphism on cerebrospinal fluid (CSF) biomarkers in AD patients. *CDC2* genotypes were evaluated in relation to CSF protein levels of total tau, phospho-tau and β-amyloid$_{(1–42)}$ in AD patients and control individuals, and in relation to the amount of senile plaques and NFT in the frontal cortex and in the hippocampus in patients with autopsy-proven AD and controls. The *CDC2* Ex6 + 7I allele was associated with a gene dose-dependent increase of CSF total tau levels ($F_{2, 626} = 7.0$, $p = 0.001$) and the homozygous *CDC2* Ex6 + 7II genotype was significantly more frequent among AD patients compared to controls ($p = 0.006$, OR = 1.57, 95% CI 1.13–2.17). Our results provide further evidence for an involvement of cdc2 in the pathogenesis of AD.

Introduction

In 2000, three independent genetic studies reported a linkage of Alzheimer's disease (AD) to two chromosome 10 markers [1–3]. We have recently reported that the cell division cycle *(CDC2)* gene [4], located close to the marker D10S1225 linked to AD [1, 3], may be responsible for the linkage signal detected on chromosome 10 [5]. We described a novel polymorphism in the *CDC2* gene, designated Ex6 + 7I/D, and found the homozygous variant *CDC2* Ex6 + 7II with a single base insert at position +7 after exon 6 to be significantly overrepresented in AD patients compared to controls [5].

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Cdc2 (also designated cdk1 or $p34^{cdc2}$) is a cyclin-dependent kinase involved in cell cycle regulation [6]. To regulate cell cycle events, cdc2 must form a complex with its regulatory subunit cyclin B1 [7] and elevated expression of cyclin B has been found in the hippocampus in AD patients [8]. The cdc2/cyclin B complex forces the cells to pass the G_1/S checkpoint even in the absence of growth factors and DNA replication [9]. This lack of DNA replication leads to arrest of the cell cycle in G_2, through an inhibition of cyclin B [9, 10]. Factors expressed in G_2 regulate hyperphosphorylation of tau and microtubule destabilization, which is responsible for segregation of chromosomes during mitosis [11]. Additionally, cdc2 is known to be one of the kinases that phosphorylates the amyloid precursor protein (APP) at Thr668 in the G_2/M phase of the cell cycle [12], which may influence amyloidogenic processing of APP in AD.

Hyperphosphorylated tau is the main component of neurofibrillary tangles (NFT) found in the AD brain [13]. The main function of tau is to bind to and thereby stabilize microtubule and the binding ability of tau is modulated through phosphorylation [14]. The active cdc2/cyclin B complex is localized in NFT-bearing neurons [15], and intraneuronal accumulation of active cdc2 can precede the deposition of paired helical filament tau, and can be present in the absence of β-amyloid (Aβ) [16].

The predominant late-onset form of AD (LOAD) is a genetically complex disorder probably involving a range of genetic factors combined with environmental influences. In the vast majority of LOAD, there is no recognizable pattern of classical Mendelian inheritance, although epidemiological, family and twin studies suggest a significant proportion of LOAD being attributable to genetic factors [17]. The only established susceptibility gene is the apolipoprotein E gene *(APOE),* which has been shown to modify both the risk and age at onset of LOAD [18, 19]. However, the ε4 allele only accounts for a fraction of the estimated genetic component in sporadic AD pathogenesis and hence, several susceptibility genes remain to be found.

To further investigate the relationship between the potential susceptibility gene *CDC2* and AD, we analyzed 988 individuals for the previously described *CDC2* Ex6 + 7I/D polymorphism [5]. The individuals included in this study can be divided into a clinical and a neuropathological postmortem subgroup. To explore possible neurobiological effects of the *CDC2* polymorphism, we measured cerebrospinal fluid (CSF) protein levels of total tau, phospho-tau and $A\beta_{42}$ in the clinical subgroup. Autopsies from brain tissue were obtained from the neuropathological subgroup, and consequently the amount of senile plaques (SP) and NFT could be measured. The mean count of SP and NFT for each brain region could then be investigated in relation to the *CDC2* polymorphism. Since we wanted to have as high power as possible for each comparison, we performed an analysis including all individuals available for each trait. We chose to include all available AD patients and controls in our study since we had the opportunity to perform an association study on the *CDC2* gene in a much larger population compared with the one used in our previous paper [5].

Materials and Methods

Subjects

Included in the study were 788 patients with AD (306 men and 482 women, mean age 75 ± 7.8 years) and 200 controls (82 men and 118 women, mean age 73 ± 9.1 years). They can be divided into a clinical (695 AD patients, 98 controls) and a neuropathological postmortem subgroup (93 AD patients, 102 controls). In the clinical subgroup, CSF samples were available for 560 (total tau), 541 (phospho-tau) and 498 ($A\beta_{42}$) AD patients and for 75 (total tau), 44 (phospho-tau) and 75 ($A\beta_{42}$) control individuals. Neuropathological examinations of brain tissue were available for the postmortem subgroup.

Clinically diagnosed patients underwent a thorough clinical investigation, which 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 diagnosis of probable AD was made according to the NINCDS-ADRDA criteria [20]. None of the AD patients had a family history of dementia suggestive of autosomal dominant AD. Clinical controls were individuals without a history, symptoms or signs of psychiatric or neurological disease, malignant disease, or systemic disorders (e.g. rheumatoid arthritis, infectious disease). Individuals with Mini Mental State Examination (MMSE) scores [21] below 28 were excluded as controls. The neuropathological AD patients fulfilled the clinical NINCDS criteria for probable AD [20] and met the neuropathological CERAD criteria for definitive AD [22]. The postmortem examination revealed no infarcts or other changes that could account for the dementia. Two areas of the right hemisphere, the frontal lobe and the anterior part of the hippocampal formation were fixed in 10% buffered neutral formalin for 4–6 weeks, and thereafter embedded in paraffin blocks. Six-micrometer-thick sections were stained with Bielschowsky staining. The absolute number of SP and NFT was counted in five randomly selected fields at a magnification of ×125, and a mean count of SP and NFT was obtained for each brain region and rated on a four-step scale (0 = no, 1 = mild, 2 = moderate, and 3 = severe) [23]. All AD brains had a histopathological score above 5 (mean ± SD 7.5 ± 2.0). Neuropathological controls were individuals who had died from cardiac disease or malignant disease. Their medical records revealed no history of dementia, or psychiatric or neurological diseases. The postmortem examination revealed no macroscopic infarcts, and all brains had a histopathological score [23] of 4 or lower (mean ± SD 1.3 ± 1.4).

Genetic Analysis

Genomic DNA was extracted from whole blood samples and brain tissue using the GenoPrep™ DNA Blood kit and DNA MagAttract kit (Qiagen, Germany) together with the GenoM™-48 Robotic Workstation (GenoVision, Norway). Approximately 10 mg of brain tissue or 100 μl of whole blood was used for each extraction. The isolated genomic DNA was stored at –20°C until it was used for gene analysis. The primer sequences for the PCR amplification of the *CDC2* gene were designed using the Lasergene 4.0 software (DNAstar Inc., Madison, USA) according to the *CDC2* genomic DNA sequence deposited in the NCBI database (accession No. 14739455 and D32223). PCR amplification was performed with the forward primer 5′-GCACCATATTTGCTGAACTAG-3′ and the reverse primer 5′-GTCCTGTAAAGATTCCACTTC-3′ (Invitrogene, Life Technologies) in a final volume of 50 μl, containing approximately 20 ng of template DNA, 1.5 m*M* $MgCl_2$, 200 μ*M* dNTPs, and 1.25 unit of Taq DNA polymerase in 1 × PCR buffer and 0.4 pmol/μl of each primer with the following PCR program: 95°C for 4 min, 35 cycles of 95°C for 1 min, 57°C for 45 s and 72°C for 1 min. A final extension step of 72°C for 10 min completed the reaction. DNA sequencing was performed using cycle sequencing with fluorescent dNTPs (ABI PRISM, Applied Biosystems, UK), separation by capillary electrophoresis and detection by laser-induced fluorescence in an ABI 3100 genetic analyzer (Perkin-Elmer, USA). The sequencing reactions were conducted in both the forward and reverse directions using the primers previously described.

CSF Collection

In both AD patients and controls, CSF samples were obtained by lumbar puncture in the L_3/L_4 or L_4/L_5 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. All CSF samples were collected according to standard conditions and were then frozen and stored at –80°C pending biochemical analyses without being refrozen. Further details of CSF collection have been described earlier [24].

CSF Analysis

Levels of CSF total tau were determined using a sandwich ELISA, a solid-phase enzyme immunoassay constructed to measure both normal and hyperphosphorylated tau (Innotest™ hTAU-Ag, Innogenetics, Ghent, Belgium) [25]. The wells are coated with an antihuman tau monoclonal antibody, AT120. CSF samples are incubated in these wells together with a pair of biotinylated tau-specific monoclonal antibodies, HT7 and BT2.

Quantification of CSF phospho-tau was done using another sandwich ELISA (Innotest phospho-tau_{181P}, Innogenetics), which specifically measures phospho-tau phosphorylated at Thr181 [26]. The detection limit for this ELISA for CSF samples is 20 pg/ml. To capture the protein, the monoclonal antibody HT7 was used. CSF samples and thereafter the biotinylated antibody AT270 were added, as described previously in detail [26].

CSF $A\beta_{42}$ was determined using a sandwich ELISA (Innotest $A\beta_{1-42}$, Innogenetics), specifically measuring the 42 amino acid fragment of $A\beta_{42}$ [24, 27]. The monoclonal antibody 21F12, which is highly specific to the C-terminus of the Aβ peptide, was used as capturing antibody, and the biotinylated monoclonal antibody 3D6, specific to the N-terminus, was used as detector. The sensitivity for the ELISA for CSF samples was 50 pg/ml.

All analyses were run on the same batch of antibodies and ELISA plates. Day-to-day imprecision (coefficient of variation) for all biochemical analyses was less than 10%.

Statistics

Genotype and allele distributions for the *CDC2* Ex6 + 7I/D polymorphism were analyzed using the Pearson χ^2 test. Comparisons involving three or more groups were performed using analysis of variance (ANOVA) and since the CSF traits are slightly skewed, we also performed the nonparametric Kruskal-Wallis analysis of ranks. Odds ratios and 95% confidence intervals were calculated according to Altman [28]. Comparing observed and expected genotype frequencies using a χ^2 test assessed deviations from the Hardy-Weinberg equilibrium. All analyses were performed using the SYSTAT program version 10.2 (SPSS Inc., Chicago, Ill., USA).

Ethics

All clinical evaluations were made without knowledge of the results from the genetic analyses. The local Ethics Committees approved the study. All patients and controls (or their closest relatives) gave their informed consent to participate in the study, which was conducted in accordance with the provisions of the Helsinki Declaration.

Results

We studied the *CDC2* Ex6 + 7I/D gene variant in relation to quantitative measurements of total tau, phospho-tau and $A\beta_{42}$ proteins in CSF. All analyses were conducted in both AD patients and controls. We sought to systematically test for the potential impact of the *CDC2* Ex6 + 7I/D gene variant upon these AD-related quantitative traits, as well as MMSE scores, age at onset, and a neuropathological index of plaque and tangle scores in autopsy-confirmed AD patients. Comparisons of these CSF traits between AD patients and controls have previously been performed demonstrating highly significant differences, whereby higher total tau and phospho-tau levels and lower $A\beta_{42}$ levels are evident in AD patients compared to controls [29], which was also true for the present sample (table 1). This represents the full extent of traits that are at present available for these subgroups. To model these effects, we tested each trait in turn using ANOVA to explore significant differences in genotypic means. For CSF traits, this initial screen was performed in combined patients and controls. For MMSE and SP-NFT scores, this was only done in AD patients.

ANOVA omnibus tests were significant for CSF levels of total tau ($F_{2, 632} = 8.1$; $p = 0.0003$), phospho-tau ($F_{2, 582} = 3.8$; $p = 0.023$) and $A\beta_{42}$ ($F_{2, 570} = 3.9$; $p = 0.021$). There was no evidence of an effect upon MMSE scores, age at onset, or SP-NFT scores. Individuals homozygous

Table 1. CSF protein levels (pg/ml) in AD patients and controls

CSF protein	AD	Controls	p values
Total tau	670.9 ± 13.9 (n = 560)	344.2 ± 19.4 (n = 75)	<0.0001
Phospho-tau	83.7 ± 1.5 (n = 541)	60.0 ± 3.6 (n = 44)	<0.0001
$A\beta_{42}$	478.4 ± 6.5 (n = 498)	716.0 ± 23.1 (n = 75)	<0.0001

Results are mean values ± SEM. CSF protein levels between AD patients and controls have been compared using the Kruskal Wallis one-way ANOVA.

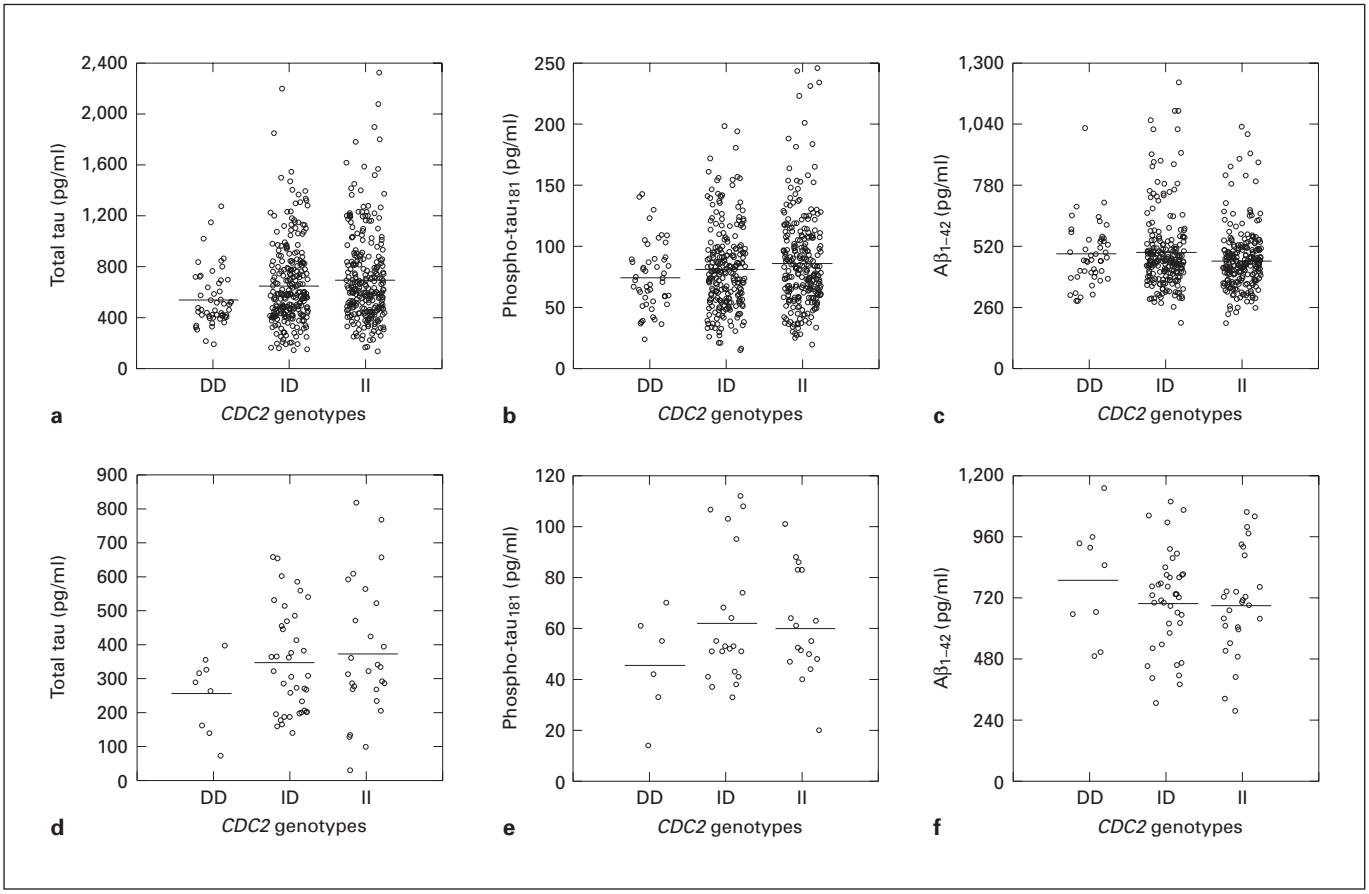

Fig. 1. Circles represent individual values of protein levels of total tau, phospho-tau and $A\beta_{42}$ in CSF. **a–c** AD patients. **d–f** Controls. Horizontal lines indicate the mean value in the respective group. **a** Total tau mean values (in pg/ml ± SEM) for II: 704.5 ± 22.0; ID: 651.9 ± 20.0; DD: 552.3 ± 31.0. **b** Phospho-tau levels for II: 87.2 ± 2.5; ID: 81.7 ± 2.2; DD: 76.7 ± 3.9. **c** $A\beta_{42}$ levels for II: 461.0 ± 8.4; ID: 495.5 ± 11.4; DD: 481.5 ± 19.9. **d** Total tau levels for II: 370.3 ± 38.3; ID: 346.1 ± 24.5; DD: 257.8 ± 36.4. **e** Phospho-tau levels for II: 61.0 ± 5.1; ID: 63.3 ± 5.7; DD: 45.8 ± 8.4. **f** $A\beta_{42}$ levels for II: 700.8 ± 39.6; ID: 709.0 ± 30.8; DD: 791.9 ± 74.8.

for the insertion allele had elevated total tau and phospho-tau levels and reduced $A\beta_{42}$ levels in CSF, consistent with what is expected from studies of average trait levels in patients and controls for a pathogenic allele (fig. 1). The potential pathogenic effect of this allele is also supported by findings showing that it is elevated in AD patients compared to controls. Since the CSF traits are slightly skewed, we also considered it important to confirm the significance of effects with a nonparametric test. Significances using the Kruskal-Wallis analysis of ranks were

Table 2. *CDC2* and *APOE* genotype and allele frequencies

a Genotype frequencies

CDC2	II	ID	DD
AD (n = 788)	376 (47.7)**	336 (42.6)*	76 (9.6)
Controls (n = 200)	73 (36.5)	102 (51.0)	25 (12.5)
APOE	No *APOE* ε4	One *APOE* ε4	Two *APOE* ε4
AD (n = 787)	247 (31.4)	410 (52.1)	130 (16.5)***
Controls (n = 198)	136 (68.7)	56 (28.3)	6 (3.0)

b Allele frequencies

CDC2	I	D
AD (n = 1,576)	1,088 (69.0)**	488 (31.0)
Controls (n = 400)	248 (62.0)	152 (38.0)
APOE	*APOE* ε4 negative	*APOE* ε4 positive
AD (n = 787)	247 (31.4)	540 (68.6)***
Controls (n = 198)	136 (68.7)	62 (31.3)

Figures in parentheses are percentages. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$. Genotype and allele frequencies have been compared using Pearson's χ^2 test.

$p = 0.0002$ for total tau, $p = 0.065$ for $A\beta_{42}$ and $p = 0.058$ for phospho-tau levels.

Given the potential for confounding that was introduced by including both AD patients and controls in preliminary analyses, we tested for a possible interaction using a second-order factorial ANOVA model, focusing on the total tau trait (on the basis of the weaker effects for $A\beta_{42}$ and phospho-tau levels). This was also done to explore the potential interaction of age, gender, or *APOE* genotype (specifically ε4 carrier or noncarrier status). None of the interaction terms for any of these covariates was significant. With special regard to the patients and controls, the same trends for *CDC2* Ex6 + 7I/D can be seen in both groups (fig. 1). A final adjusted model, including all of these covariates, was evaluated for the effect of the *CDC2* Ex6 + 7I/D marker on CSF total tau levels. This was highly significant, but slightly attenuated in comparison to the unadjusted model ($F_{2, 626} = 7.0$; $p = 0.001$). We note that this final p value survives formal Bonferroni correction for multiple testing given 6 omnibus tests for all quantitative traits, 3 nonparametric tests, 4 interaction tests, and a final adjusted test ($p_{corrected} = 0.014$).

Genotype distributions for the *CDC2* Ex6 + 7I/D conformed to the Hardy Weinberg equilibrium in both AD patients and controls. Genotype and allele frequencies for the Ex6 + 7I/D marker are shown in table 2. The *CDC2* Ex6 + 7I/D genotype distribution differed significantly between AD patients and controls ($p = 0.017$). Specifically, the homozygous *CDC2* Ex6 + 7II genotype ($p = 0.006$) and the *CDC2* Ex6 + 7I allele frequencies ($p = 0.009$) were significantly increased in AD (table 2). The odds ratio for having AD was 1.57 (95% CI 1.13–2.17) for the *CDC2* Ex6 + 7II genotype, and 1.35 (95% CI 1.07–1.7) for the *CDC2* Ex6 + 7I allele. The *APOE* ε4 genotype and allele frequencies were significantly increased in AD ($p < 0.0001$; table 2). Stratification for age, gender, or *APOE* ε4 carrier status did not affect the results. There were no significant differences in genotype or allele frequencies between the subgroups and the whole group. Additionally, there were no significant differences in mean age and sex distribution between the clinical subgroup, used for comparisons between gene variants and biochemical parameters, and the whole group. We can therefore conclude that these subgroups are representative for the whole material in response to these parameters.

Discussion

To explore possible effects of the *CDC2* Ex6 + 7I/D polymorphism on CSF biomarkers we examined AD patients in whom we measured CSF levels of total tau, phospho-tau and $A\beta_{42}$. We found the *CDC2* polymorphism to be associated with elevated CSF total tau protein levels in AD patients. The *CDC2* I allele influenced CSF total tau protein levels in a dose-dependent manner, suggesting a biochemical effect and an involvement of cdc2 in the pathogenesis of AD. Interestingly, the same tendency could be seen in controls. This was, however, not significant, probably due to less power since the sample size was smaller.

The results from the *CDC2* genotyping of the AD patients and controls were in line with our previous results [5] where the homozygous *CDC2* Ex6 + II genotype was significantly overrepresented in AD patients compared to controls. We note that *APOE* ε4, the strongest known genetic risk factor for AD, was more frequent in AD patients compared to controls in our study group (table 2). When we explored potential interactions of age, gender, or *APOE* genotype, none of these covariates affected the results significantly. This suggests that the relationship between the *CDC2* gene and AD is independent of *APOE* ε4 status.

Although mutations in the APP are causative for familial AD, the role of Aβ in sporadic AD is unclear. The amyloid cascade hypothesis suggests that deposition of Aβ into SP is the event causative for the formation of NFT and neurodegeneration [30]. Tau pathology has been observed in the absence of SP in other neurodegenerative disorders, such as progressive supranuclear palsy, Pick's disease and corticobasal degeneration. Alternatively, cell cycle events may precede the formation of SP and NFT found in AD. The general assumption is that adult neurons in the brain are postmitotic and unable to re-enter mitosis and thereby lack the ability to divide; conversely, accumulating evidence shows that postmitotic neurons in AD are able to re-enter the cell cycle. Specific cell cycle components have been found to be activated in the AD brain [8, 15, 31] and altered regulation of cdks and cyclins point towards cell cycle activation in postmitotic neurons in AD [31, 32]. The active cdc2/cyclin B complex is localized in NFT-bearing neurons [15], and this accumulation of active Cdc2 can occur before the formation of paired helical filaments, and without involvement of Aβ [16]. Cdc2 has been suggested to be one of the kinases involved in AD pathology since it is able to phosphorylate tau protein at sites known to be phosphorylated in AD [16, 33, 34]. In the present study, we detected a nonsignificant trend towards increased phospho-tau levels in patients with the *CDC2* II and ID genotypes (fig. 1b). In our ELISA method, we measured tau protein phosphorylated at residue 181, which is a relatively isolated phosphorylation site in the proline-rich region [25] and is preferentially phosphorylated by proline-directed kinases [35]. Even though it is evident that cdc2 activity is increased in AD, it needs to be determined whether cdc2 alters tau phosphorylation in a direct or indirect manner.

Additionally, cell cycle events have been linked to neuronal cell death [36]. Several studies support the hypothesis that cell cycle proteins are expressed in postmitotic neurons, thus force them to re-enter the cell cycle and by this means eventually cause neuronal death [36]. Critical check points of the cell cycle regulate the destiny of the cell, towards further progression through the cell cycle, growth arrest or induction of an apoptotic pathway. In terminally differentiated cells, like neurons, an apoptotic response might prevent an improper activation of the cell cycle [37]. Cdc2 activity is elevated in apoptotic cells and many of the sites on tau that are hyperphosphorylated during apoptosis are also found in the AD brain [34, 38, 39]. Cdc2 can induce phosphorylation of the Bcl-2 associated death agonist (Bad) at Ser128, which in turn results in activation of Bad-mediated apoptosis [40, 41], and previous results have found elevated levels of Bad protein in the AD brains [42]. Moreover, APP can be phosphorylated by cdc2, which results in altered Aβ production [12]. In turn, Aβ can stimulate proliferating, drive cultured neurons into the cell cycle and thereby maintain the proliferating signal [43, 44].

In summary, the present study further supports the involvement of cdc2 in AD and is in line with our previous study [5]. The *CDC2* Ex6 + 7I allele influences CSF total tau protein levels in a dose-dependent manner where individuals with II and ID genotypes have elevated protein levels of CSF total tau compared to DD individuals. Biological studies of the cdc2 protein are needed to investigate how activity and expression are affected by the *CDC2* polymorphism.

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