GENETIC RISK FACTORS IN ALZHEIMER'S DISEASE

Ph.D. Thesis

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Abbreviations

5HT: serotonin

5HT2A: serotonin receptor type 2A

5HTT: serotonin transporter

5HTTLPR: serotonin transporter gene-linked polymorphic region

α7 nAChR: alpha-7 nicotinic acetylcholine receptor

ACE: Addenbrooke's Cognitive Examination

AD: Alzheimer's disease

APOE: apolipoprotein E

APP: amyloid precursor protein

Aβ: amyloid-β

BACE1: β-site APP cleaving enzyme-1

BDNF: brain-derived neurotrophic factor

CALHM1: calcium homeostasis modulator 1

CHRFAM7A: fusion of the CHRNA7 exons 5-10 and FAM7A exons A-E

CHRNA7: α7 nAChR subunit

DHCR24: 24-dehydrocholesterol reductase

FAM7A: family with sequence similarity 7A

IFNG: Interferon-γ

MMSE: Mini-Mental State Exam

NINCDS/ADRDA: National Institute of Neurological and Communicative Disorders and Stroke / Alzheimer's Disease and Related Disorders Associations

OR: odds ratio

PCR: polymerase chain reaction

PLAG4A: cytosolic phospholipase A_2, group IVA

PLAU: urokinase-type plasminogen activator

PTGS2: prostaglandin endoperoxide synthase 2

Seladin1: Selective AD Indicator

SLC6A4: solute carrier family 6 member 4 gene encoding serotonin transporter

SNP: single nucleotide polymorphism

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder representing the most common cause of dementia in the elderly population (Ritchie and Lovestone, 2002). The clinical manifestation of AD is characterized by progressive memory impairment and cognitive deficits. Typically, AD begins with subtle and poorly recognized failure of memory worsening inevitably and finally, incapacitating the patient. AD pathology is characterized by the presence of extracellular senile plaques composed of amyloid-β (Aβ) peptid and intraneuronal neurofibrillary tangles containing hyperphosphorylated tau protein. The amyloid and the tau hypotheses consider these proteins as inducers and key players of the disease (Selkoe et al., 2001).

Genes have a varied influence on developing AD, ranging from the autosomal-dominant inheritance in the familial forms (1-5% of cases) to the polygenic background in late-onset (>65 years of age) sporadic AD (≥95% of cases). The complex genetic model of sporadic AD suggests that several heterogeneous susceptibility sets of genes may converge on the pathological processes that underlie the disease. However, so far only the apolipoprotein E (APOE) gene has been definitively associated with the risk for AD (Brouwers et al., 2008).

APOE is involved in lipid transport and metabolism. Furthermore, it plays a specific role in the central nervous system, including neuronal development, regeneration and certain neurodegenerative processes. The polymorphism of the APOE gene determines three isoforms of APOE protein (ε2, ε3, ε4) with different conformation and lipid binding properties. The APOE ε4 isoform prefers very low density lipoprotein and it is less effective in cholesterol transport as compared to the other APOE isoforms (Cedazo-Mínguez and Cowburn, 2001).

The genetic epidemiology of sporadic AD remains a very active area of research, since a large part of the genetic aetiology is still poorly understood and remains unresolved. The aim of our work was to contribute to this field investigating gene polymorphisms presumably involved in AD pathogenesis. The candidate gene polymorphisms in this study were selected and grouped on the basis of the following in AD supposedly leading processes: Aβ metabolism, cholesterol metabolism, neuroinflammation and neuronal dysfunction.

I. Amyloid-β metabolism and AD

The amyloid precursor protein (APP) follows two distinct cleavage pathways competing α- and β-secretases, and both pathways are active in normal metabolism (Selkoe et al., 2001). The predominant cleavage of APP is mediated by the α-secretase generating non-

amyloidogenic products. The cleavage leading to Aβ generation is mediated by β- and γ-secretases. The β-site APP cleaving enzyme-1 (BACE1) gene at locus 11q23.3 encodes β-secretase, the key and rate-limiting enzyme in the cascade of Aβ formation. Genetic variations of BACE1 may act on the Aβ generation and thereby influence neurodegenerative processes leading to AD. The rs638405 polymorphism of BACE1 is a nucleotide change in exon 5 (C786G) with no alteration at the amino acid level (Val262) (Murphy et al., 2001).

The PLAU gene encodes urokinase-type plasminogen activator (uPA). The uPA serine protease converts the inactive plasminogen to the active plasmin form, and it is also capable to degrade Aβ directly. Plasmin promotes α-cleavage of APP, degrades secreted and aggregated Aβ thereby blocks Aβ neurotoxicity (Finckh et al., 2003). The frequent single nucleotide polymorphism (SNP) of PLAU (rs2227564, C1788T) is located in exon 6, and results in an amino acid change at codon 141 (Pro141Leu).

An increasing body of evidence supports the major contribution of the dysregulation of calcium homeostasis in accelerating pathological changes in AD, i.e. Aβ accumulation (Bezprozvanny and Mattson, 2008). The calcium homeostasis modulator 1 (CALHM1) gene has been recently identified and it appears to modulate the intracellular calcium levels. An SNP (rs2986017) at nucleotide 257 (C/T) producing amino acid substitution at codon 86 (Pro86Leu) has been identified. The Leu allele has been reported to be associated with AD, and in vitro demonstrated to result in impaired CALHM1 function leading to decreased calcium permeability and reduced cytoplasmic calcium levels (Dreses-Werringloer et al., 2008). Intracellular calcium levels were reported to affect the metabolism of APP and thereby the levels of Aβ (Green et al., 2008; Tanzi et al., 2008).

II. Cholesterol metabolism and AD

Cholesterol is a necessary structural component and cell fluidity modulator of the cell membrane. Most of the CNS cholesterol is produced via local de novo synthesis. The role of cholesterol in AD is a controversial topic, but it seems that an optimal amount of cell cholesterol may be critical for brain homeostasis (Peri and Serio, 2008). The amount of cell cholesterol has a major impact on Aβ generation and cell resistance against Aβ toxicity.

The 24-dehydrocholesterol reductase (DHCR24) gene at locus 1p33-p31.1 encodes Seladin1 (Selective AD Indicator), an enzyme that is involved is cholesterol biosynthetic pathway. Seladin1 catalyses the conversion of desmosterol to cholesterol and confers resistance against Aβ and oxidative stress induced apoptosis by effective inhibition of caspase-3 activity, and prevention of p53 degradation (Peri and Serio, 2008). Seladin1 also

affects the Aβ generation via the modulation of membrane cholesterol content. SNPs of the DHCR24 gene were identified: the rs638944 (intron 2 G/T) and the rs600491 (intron 5 C/T).

III. Neuroinflammation and AD

An increasing body of evidence supports the major contribution of inflammatory processes in accelerating pathological changes in AD (Lukiw and Bazan, 2000). Interferon-γ (IFNG) plays an important role in the induction of the immune-mediated inflammatory response (Blasko, 2001). In human neuroblastoma cells pre-treatment with IFNG increased the expression of cytosolic phospholipase A_2, group IVA (PLAG4A) resulting in an elevated release of arachidonic acid and a subsequently elevated level of prostaglandins (Bate et al., 2006). The T allele of the IFNG T874A polymorphism correlates with an increased level of IFNG (Pravica et al., 2000).

Phospholipase A_2 is a superfamily of enzymes that include key modulators of cerebral phospholipid metabolism. The PLA2G4A catalyzes the release of arachidonic acid from membrane phospholipids. A polymorphic site for Ban*I* restriction enzyme in PLA2G4A gene is an A to G base change (Wei and Hemmings, 2004). Prostaglandin endoperoxide synthase 2 (PTGS2) is a key enzyme in prostaglandin biosynthesis converting arachidonic acid to prostaglandin E2. A functional polymorphism (G-765C) in the promoter region of the PTGS2 gene has been identified and significantly lower promoter activity has been reported for the C allele (Papafili et al., 2002).

IV. Neuronal dysfunction and AD

IV.1. Cholinergic dysfunction

AD is associated with a progressive loss of cholinergic neurons and a consequent acetylcholine deficit, particularly in the temporal and parietal neocortex and hippocampus. The extensive degeneration of the cholinergic neurons in the basal nucleus of Meynert and in the medial septal nucleus is responsible for the loss of up to 95% of the cholinergic innervation to the cortex. It is also presumable that altered activity of acetylcholine receptors determined by genetic variations can influence the cholinergic transmission.

Alpha-7 nicotinic acetylcholine receptors (α7 nAChRs) are homopentamer, ligand-gated cationic channels. They are widely expressed in the central nervous system with high levels in the regions relevant to memory functions and involved in processing of sensory information, such as hippocampus (Weiland et al., 2000). The α7 nAChR subunit gene (CHRNA7) at region 15q13.1 is duplicated from exon 5 to 10 (Gault et al., 1998; Riley et al., 2002). The partially duplicated CHRNA7 and four other exons originated from Family with

sequence similarity 7A (FAM7A) gene form a hybrid gene (CHRFAM7A). A -2bp deletion polymorphism at position 497-498 in exon 6 was identified, which is specific to CHRFAM7A and does not occur in CHRNA7 (Gault et al., 1998). The -2bp deletion causes a frameshift, introducing a stop codon within exon 6 and therefore a truncation in a putative gene product. Since CHRFAM7A is reported to be expressed as mRNA, possible regulatory effects should also be considered.

IV.2. Serotonergic dysfunction

Serotonergic involvement in AD is supported by findings including cerebrospinal fluid alteration of serotonin (5HT) and loss of synthesizing neurons and 5HT receptors in AD. The solute carrier family 6 member 4 (SLC6A4) gene at locus 17q11.1-q12 encodes 5HT transporter (5HTT). The promoter region of the SLC6A4 gene shows a 22 bp tandem repeat polymorphism, which is designated as 5HTT gene-linked polymorphic region (5HTTLPR). The two major alleles have 14 and 16 repeats, thus differing in 44 bp, and were denoted as short (S) and long (L) alleles. The 5HTTLPR polymorphism determines dose-dependent 5HT reuptake from the synaptic cleft, the S allele is less effective.

The gene HTR2A, which codes for the 5HT receptor type 2A (5HT2A) is located at 13q14–q21. The rs6313 polymorphism of HTR2A is a nucleotide change in exon 1 (T102C), and does not alter the serine at position 34 (silent mutation). The activity of the C allele has been shown to be significantly decreased as compared to the T allele.

IV.3. Brain-derived neurotrophic factor

The brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors, produced by cortical neurons. Besides its general role in neurodevelopment, BDNF has important functions in the adult brain such as promoting the survival and maintaining the structural integrity of neuronal cells (Murer et al., 2001). The activity-dependent expression of BDNF plays a role in modulating synaptic changes associated with learning and memory (Tyler et al., 2002). The BDNF gene encodes a precursor peptide (pro-BDNF) which is secreted and cleaved by extracellular protease to form the mature BDNF protein (Seidah et al. 1996). An SNP at nucleotide 196 (G/A) producing a non-conservative amino acid substitution at codon 66 (Val/Met) has been identified (Ventriglia et al., 2002). Although this SNP is located in the 5' pro-BDNF sequence and does not affect the function of the mature BDNF, it has a major impact on the intracellular trafficking and regulated secretion of pro-BDNF (Egan et al., 2003).

Aims

- The aim of our investigation was to provide data on APOE polymorphism in the Hungarian population to further confirm the role of ε4 allele in AD.

- We tested the hypothesis whether the BACE1 C786G and PLAU Pro141Leu polymorphisms are associated with AD, either alone or in genetic interaction.

- With reference to the Dreses-Werringloer paper (2008) we tried to support their findings, that CALHM1 Leu86 allele can increase the risk for developing AD.

- The aim of our study was to test the hypothesis that the rs638944 and rs600491 polymorphisms of the DHCR24 gene encoding Seladin1 influence the susceptibility to AD.

- We investigated the possible role of IFNG T874A, PLA2G4A Ban*I* and PTGS2 G-765C polymorphisms in AD. Our study was undertaken to confirm the hypothesis that the above-mentioned variants of these genes, either alone or in epistasis, may represent a risk factor for AD.

- The aim of our study was to test the hypothesis that the CHRFAM7A -2bp deletion polymorphism confers predisposition to AD.

- We investigated the possible role of HTR2A T102C and 5HTTLPR polymorphisms in AD either alone or in genetic interaction.

- We tested the hypothesis that the BDNF Val66Met polymorphism influences the risk for developing AD.

Subjects and Methods

Patients and controls

A total of 495 Hungarian Caucasian subjects were enrolled in this study. The study included 250 patients with late onset AD recruited from the Memory Clinic of the Department of Psychiatry, University of Szeged. The diagnosis of probable AD fulfilled the criteria for DMS-IV and NINCDS/ADRDA (McKhann et al., 1984). All AD cases were defined as sporadic since in their family history there was no first or second degree relative with dementia. The clinical diagnosis of probable AD was supported by psychiatric and neurological examinations, basic clinical tests such as Mini-Mental State Exam (MMSE), Addenbrooke's Cognitive Examination including Clock Drawing and Verbal Fluency tests.

The AD patients all had experienced a progressive loss of cognitive functions (in more than two cognitive domains indicative of cortical dysfunction) for at least 1 year with memory loss as the most significant symptom. Brain CT or MRI images were also evaluated.

As a healthy control (HC) group we studied 245 elderly, cognitively intact, healthy individuals that were selected from visitors of patients at the Memory Clinic, Department of Psychiatry, University of Szeged. All of them were medication free and lack of any significant illnesses and any signs of dementia. Informed consent was obtained from the subjects participated in this study and all protocols were approved by the local ethics committee. MMSE was used as a measure of global cognitive performance. MMSE scores in the HC group were higher than 28 points (mean ± SD: 29.1 ± 0.9) and none of the probands had any verified symptoms of dementia. The mean MMSE score in the AD group was 18.5 ± 5.9 (mean ± SD).

Genetic analyses

Blood samples were taken by venous puncture. Genomic DNA was extracted from peripheral blood leukocytes according to a standard procedure using the Roche kit. The genetic analyses were performed by polymerase chain reaction (PCR) based methods or direct sequencing. PCR products and digested fragments were separated by agarose or polyacrilamide gel electrophoresis with ethidium bromide staining. Bands on gels were detected and documented by Quantity One 1-D Analysis software of BioRad GelDoc System.

APOE genotypes were determined by a previously described PCR-restriction fragment length polymorphism (RFLP) method with the restriction enzyme *Cfo*I (Kálmán et al., 1997). Genotyping of the BACE1 polymorphism was done by PCR amplification and enzymatic digestion with restriction enzyme *Bcl*I (Cai et al., 2005). Genotyping of the PLAU polymorphism was assessed by restriction enzyme *Alu*I with the same method described by Pesaresi et al (Pesaresi et al., 2006).

The DNA samples were genotyped for CALHM1 polymorphism through direct sequencing of a 291 bp long PCR product. The PCR products were purified by ethanol precipitation and bidirectionally sequenced. Direct sequencing of the PCR products was performed using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) on the ABI 3100 Genetic Analyzer (Applied Biosystems). Obtained sequences were analyzed using Applied Biosystem Sequencing Analysis v.3.7 software.

Genotyping of the DHCR24 rs600491 polymorphism were assessed by PCR amplification, and the PCR products were digested with *Aci*I restriction enzyme. The

DHCR24 rs638944 was determined by allele specific primers in two parallel PCR reactions. Genotyping of the IFNG polymorphism was done by PCR amplification with allele specific primers as described by Raitala and co-workers (Raitala et al., 2005). Genotyping at PLA2G4A loci was assessed by restriction enzyme Ban*I* with the same method described by Chowdari et al (Chowdari et al., 2004). The two alleles of the PLA2G4A Ban*I* polymorphism are designated as A1 (with nucleotide A) and A2 (with nucleotide G, the Ban*I*-cut allele). Genotyping of the PTGS2 polymorphism was done by PCR amplification and enzymatic digestion with restriction enzyme Aci*I*, (Papafili et al., 2002).

Genotyping of the CHRFAM7A -2bp deletion polymorphism was done by PCR amplifications, and the two bp difference between the wild and the -2bp deletion alleles was revealed by 10% polyacrilamide gel electrophoresis (Lai et al., 2001). The genotype lacking the -2bp deletion was designated genotype 1, genotype having one copy of the -2bp allele was designated genotype 2 and genotype with two copies of the -2bp allele was designated genotype 3.

Genotyping of the 5HTTLPR polymorphism was carried out as previously described (Sundaramurthy et al., 2000). The 44 bp difference between the 5HTTLPR L and the S alleles was revealed by 2% agarose gel electrophoresis with ethidium bromide staining. The HTR2A primers used in the amplification were described by Virgos et al (2001). After amplification, the PCR products were digested with *Msp*I restriction enzyme at 37 ℃ for 12 hours. Genotyping of the BDNF Val66Met polymorphism was done by PCR amplification and enzymatic digestion with restriction enzyme *Pma*CI (Chen-Jee Houg et al., 2003).

Statistical analyses

The program SPSS 15.0 was used for all statistical analyses, and the significance level was set at $p<0.05$. Fisher's exact and Pearson's χ2 tests were used to compare gender, Hardy-Weinberg equilibrium (HWE), allele and genotype frequencies between the AD and HC groups. The mean age of the AD and HC groups was compared by using the t-test for independent samples. Analysis of variance was carried out to determine possible effect of the different genotypes on age at onset of AD. A logistic regression model was applied to test for interactions between the investigated polymorphisms and to estimate crude and adjusted odds ratios (ORs) with 95% confidence intervals (95% CI) in testing for possible associations between genotypes or alleles and the risk for AD.

Results

As expected from many previous studies the genotypes with the APOE ε4 allele were significantly over-represented in the AD group as compared to the controls ($p<0.0001$). The ratio of the ε3/ε4 carriers was significantly higher in AD than in HC group (AD: 34.4%, HC: 12.2%). The occurrence of the ε4 allele were significantly over-represented in AD as compared to HC (AD: 27.8%, HC: 7.1%, $p<0.0001$). The OR for AD was reduced in ε4 negative carriers of ε2 allele, and was higher in the ε4 positive individuals as compared to those with ε3/ε3 genotype. The ε4 homozygotes have significantly increased risk for AD (OR=4.29), than ε4 heterozygotes (OR=16.97), which further supports the previous results of the allele dose dependent risk of APOE ε4 allele for AD.

Stratification according to gender revealed an increased risk for AD in women carrying the ε4/ε4 genotype (OR=22.29; $p=0.003$) as compared to men with the ε4/ε4 genotype (OR=11.61; $p=0.024$). However, the same effect size was calculated for women (OR=4.05; $p<0.001$) and men (OR=4.81; $p<0.001$) carrying the ε3/ε4 genotype. In case of ε4 negative carriers of ε2 allele the OR for AD was reduced - but not significantly - both in women and in men as compared to the ε3/ε3 carriers.

I. Amyloid-β metabolism-related polymorphisms

Comparison of BACE1 genotype frequencies between AD and HC groups did not reach statistically significant difference ($p=0.647$). The percentage of the different PLAU genotypes was similar in AD as compared to HC, and showed no statistically significant difference ($p=0.964$). Logistic regression analysis revealed no effect of interaction between PLAU and BACE1 genotypes ($p=0.716$), and there was also no interaction with APOE ε4 allele on AD risk (BACE1*APOE: $p=0.648$; PLAU*APOE: $p=0.579$).

The CALHM1 Pro/Leu and Leu/Leu genotypes occurred with higher frequency in AD as compared to HC group, although the difference did not show statistical significance ($p=0.153$). Given the relatively low occurrence of the Leu/Leu genotype both in AD cases and in controls, the analysis was also conducted by presence or absence of the Leu allele in the genotypes. The frequency of the Leu+ genotypes (Leu/Leu and Pro/Leu genotypes together) was found to be marginally significantly higher in the AD than in the HC group (AD: 53.8%, HC: 44.6%; $p=0.056$). The Leu+ genotypes had a marginally significantly increased risk for AD (OR=1.45; $p=0.053$) considering Pro/Pro genotype as reference category (OR=1).

The interaction between the CALHM1 Leu and APOE ε4 alleles did not contribute significantly to the logistic regression model (p=0.913). Given the co-localization of PLAU and CALHM1 genes in the 10q21-24 AD linkage region, a possible interaction between PLAU and CALHM1 on prediction of AD was assessed, but no interaction was found (p=0.891).

II. Cholesterol metabolism-related polymorphisms

The DHCR24 rs600491 genotype and allele distributions did not differ significantly between the AD and HC groups (p=0.881 for genotypes; p=0.845 for alleles). Stratification according to gender however, revealed a statistically significant association between T/T genotype and AD risk in men (AD: 32.4%, HC: 16.9%; p=0.028), in contrast with the results in women (AD: 29.6%, HC: 33.8%; p=0.472). Men with the T/T genotype had a significantly increased risk for AD (OR=4.37; p=0.009) considering C/C genotype as reference category (OR=1). Logistic regression analysis revealed no interaction between the DHCR24 rs600491 and APOE polymorphisms (p=0.856).

DHCR24 rs638944 genotype frequencies were similar in the AD as compared to the HC group, and showed no statistically significant difference (p=0.687). Logistic regression analysis revealed no interaction between the DHCR24 rs638944 and APOE polymorphisms (p=0.795).

III. Neuroinflammation-related polymorphisms

The frequencies of the IFNG A/A genotype and the A allele were higher in AD as compared to HC group, although the differences did not reach statistical significance (p=0.174 for genotypes, p=0.139 for alleles). Comparison of PLA2G4A genotype and allele frequencies between AD and HC showed no statistically significant difference, although genotype A1/A1 occurred more frequently in AD than in HC (p=0.143 for genotypes, p=0.269 for alleles).

The PTGS2 G/G genotype was significantly over-represented in AD as compared to HC group (AD: 74.7%; HC: 59.6%), while the G/C and C/C genotypes were significantly more frequent in HC than in AD group (G/C: AD: 23.6%, HC: 33.1%; C/C: AD: 1.7%, HC: 7.3%; p<0.001). The PTGS2 allele distribution also showed statistically significant difference between cases and controls with higher G allele frequency in the AD group (AD: 86.5%, HC: 76.1%; p<0.001). The effect of the G/G genotype on AD risk was significantly increased (OR=5.46; p=0.003) as compared to C/C genotype as reference category (OR=1). The G/G genotype also had a significantly increased risk (OR=2.00; p<0.001) when it was compared to C+ genotypes as reference category (OR=1).

In accordance with the genotype frequencies, IFNG A/A, PLA2G4A A1/A1, PTGS2 G/G genotypes were considered as possible risk factors for AD. The interaction between the possible risk factors were investigated in pairs, and none of them contributed significantly to the logistic regression model (IFNG*PLA2G4A: p=0.877; IFNG*PTGS2: p=0.245; PLA2G4A*PTGS2: p=0.872). Logistic regression analysis also revealed no effect of interaction with APOE ε4 allele on AD risk (IFNG*APOE: p=0.132; PLA2G4A*APOE: p=0.733; PTGS2*APOE: p=0.482).

IV. Neuronal dysfunction

IV.1. Cholinergic system-related polymorphism

Genotype 1 was significantly over-represented in AD as compared to HC (AD: 36.0%, HC: 24.0%), while the frequency of genotype 3 was significantly lower in AD, than in HC (AD: 12.6%, HC: 22.3%). The differences were statistically significant (p=0.011). The CHRFAM7A genotype 1 had a significantly increased risk on AD (OR=2.66; p=0.012) as compared to genotype 3 as reference category (OR=1).

According to the genotype frequencies CHRFAM7A genotype 1 and APOE ε4 allele were considered as possible risk factors for AD. Simultaneous presence of APOE ε4 allele and CHRFAM7A genotype 1 occurred more frequently in AD as compared to HC (AD: 12.6%, HC: 4.0%). The ORs for the presence of ε4 allele with the CHRFAM7A genotype 1 (OR=6.03, p<0.001) or without the CHRFAM7A genotype 1 (OR=6.11, p<0.001) were same values, therefore it is unlikely that the combination of these genetic variants would be involved in AD.

IV.2. Serotonergic system-related polymorphisms

Compared with the controls, there was a higher frequency of HTR2A C/C genotype and lower frequency of T/T and T/C genotypes in the AD group without statistically significant difference (p=0.108). Individuals with at least one T allele were grouped together. The analysis was also performed by the presence or absence of the T allele in the genotypes, thereby T- (C/C) and T+ (C/T and T/T) genotype categories were investigated, and a statistically significant difference was found between cases and controls (p=0.037). The effects of C/C and C/T genotypes on AD risk were not significant considering T/T genotype as reference category (OR=1). However, as compared to the T+ genotypes as reference category (OR=1) the HTR2A C/C genotype had a significantly increased risk for AD (OR=1.53; p=0.035).

The frequency of the different 5HTTLPR genotypes was similar in AD as compared to HC, and there was no statistically significant difference (p=0.883). The 5HTTLPR allele distribution also did not show statistically significant difference between the two investigated groups (p=0.768).

The presence of the HTR2A C/C and the 5HTTLPR L/L genotypes were considered as possible risk factors for AD. Both AD and HC groups were divided into four subgroups according to the presence or absence of the HTR2A C/C and 5HTTLPR L/L genotypes. Logistic regression analysis revealed an interaction between the 5HTTLPR and the HTR2A T102C polymorphisms (p=0.032). The L/L and C/C genotype carriers had a significantly increased risk for AD (OR=2.50; p=0.016).

IV.3. Brain-derived neurotrophic factor polymorphism

The frequency of the BDNF Val/Val homozygous genotype was significantly higher in AD than in the HC group (AD: 58.8%, HC: 31.7%; p<0.0001). There were also robust differences in the distribution of the Val/Met and Met/Met genotypes in AD versus HC (Val/Met: AD: 35.0%, HC: 48.2%; Met/Met: AD: 6.2%, HC: 20.1%; p<0.0001). The effect of the Val/Val genotype on AD risk was significantly increased (OR=5.97; p<0.001) as compared to Met/Met genotype as reference (OR=1). The Val/Val genotype also had a significantly increased risk (OR=3.07; p<0.001) when it was compared to Met+ genotypes as reference (OR=1). The Val allele was significantly over-represented in AD as compared to HC (AD: 76.2%, HC: 55.8%; p<0.0001).

The examined groups were divided into subgroups according to the presence of the ε4 allele (one or two) and the presence of the Val/Val genotype. Genotypes containing both the ε4 allele and BDNF Val/Val genotype occurred more frequently in AD than in HC (AD: 20.6%, HC: 1.8%). The OR for the presence of both the ε4 allele and BDNF Val/Val genotype in the AD group was 26.05 as compared to patients with neither the ε4 nor the BDNF Val allele. Since in AD, the OR for the occurrence of the ε4 allele and BDNF Val/Val genotype was much higher than the OR for the presence of the ε4 allele without the Val/Val genotype, we propose a synergistic effect of the two SNPs on risk for AD.

Discussion

We found the expected statistically significant APOE ε4 allele elevation in AD as compared to the HC group. These results are in line with former findings for the Hungarian population (Kálmán et al., 1997; Janka et al., 2002; Juhász et al., 2005) and with results on other ethnic groups (Lung et al., 2005; Murrell et al., 2006; Sando et al., 2008). The reported ε4 allele frequencies for patients with AD show a wide range of variance from 19 to 55% even within Europe, however these are the end values and they are generally between 30 and 45%. The Caucasian population is not homogeneous and the Hungarian population is genetically different from other European ethnic groups. According to our results, the ε4 allele frequency in Hungarians is lower as compared to the average ε4 allele frequency of other Caucasian populations. The different association studies including our study are consistent however, since significantly higher ε4 allele frequency was found in AD compared to healthy controls in each population.

Our findings confirm the results of genetic and epidemiological studies reporting that the ε4 allele is the most important known risk factor for late-onset sporadic AD. Our data partly confirmed the effect of gender on AD risk, since we found an increased predisposition to AD in ε4/ε4 homozygote women as compared to ε4/ε4 homozygote men, but the same risk for AD in women carrying the ε3/ε4 genotype as compared to men with the ε3/ε4 genotype. Our results support the dose dependent risk of APOE ε4 allele for AD, in view of the fact that we found significantly increased risk for AD in ε4 homozygotes as compared to ε4 heterozygotes.

I. Amyloid-β metabolism-related polymorphisms

Our findings failed to support the hypothesis that BACE1 C786G and PLAU Pro141Leu polymorphisms confer susceptibility to AD, and we did not detect genetic interaction between the BACE1 and the APOE genotypes or between the PLAU and the APOE genotypes in the development of AD. We also failed to support the hypothesis that CALHM1 rs2986017 polymorphism is associated with late onset AD. Only a very modest, marginally significant effect of the Leu+ genotypes on AD was observed.

Dreses-Werringloer and co-workers (2008) examined this polymorphism in five Caucasian AD and control samples, and found that the Leu allele is associated with the risk for AD (Dreses-Werringloer, et al, 2008). Further studies including ours, however, did not corroborate this observation. Bertram et al (2008) investigated several independent datasets of

Caucasian patients and controls and found no association between rs2986017 polymorphism and AD (Bertram et al, 2008). The CALHM1 genotype distribution reported by Minster and co-workers (2009) looks very similar to that found by Sleegers et al (2009) and Beecham et al (2009) without any statistically significant difference comparing AD and HC groups.

Although, the effect of the CALHM1 Leu allele on AD in itself in our study was found to be small and only marginally significant, we hypothesized that in combination with the APOE ε4 allele it may increase the effect of the ε4 allele occurring on its own. We failed to detect a genetic interaction between the CALHM1 Leu allele and the APOE genotypes in the development of AD. Despite the modest correlation between the presence of the Leu allele and AD, this study does not support the involvement of CALHM1 rs2986017 polymorphism in the aetiology of AD.

II. Cholesterol metabolism-related polymorphisms

Our results indicate a gender dependent effect of DHCR24 rs600491 polymorphism on the susceptibility to AD. A statistically significant correlation was found in men between the T/T genotype and the risk for AD, whereas this association was not observed in the whole population or in women. Men with the T/T genotype had a significantly increased risk for AD as compared to those men carrying the C allele. Our findings on DHCR24 rs600491 polymorphism are in line with the study of Lämsä and co-workers (2007) that was also reported and increased risk for AD in men carrying the T/T genotype.

We failed to support the hypothesis that DHCR24 rs638944 polymorphism influences the predisposition to AD, since we did not observe statistically significant differences neither in genotype nor in allele distribution comparing the AD and the HC groups. We also failed to detect genetic interaction between the DHCR24 and the APOE genotypes in the development of AD.

III. Neuroinflammation-related polymorphisms

Our results indicate that PTGS2 G/G genotype can confer susceptibility to AD and the possession of the C allele can have a protective effect. Our findings do not confirm the hypothesis that IFNG and PLA2G4A polymorphisms are associated with late onset AD. We did not detect an epistasis between the investigated polymorphisms in the development of AD. The genotype distribution of PTGS2 in this study is very similar to that was found by Abdullah and co-workers in a Caucasian case-control sample from Florida (Abdullah et al., 2006). We confirmed their findings in a Hungarian sample.

The investigated PTGS2 promoter polymorphism has functional consequence, since the C allele was reported to have a significantly lower promoter activity in human lung fibroblasts (Papafili et al., 2002). In agreement with this observation, higher PTGS2 expression was measured in monocytes from probands with G/G genotypes as compared to probands having G/C or C/C genotypes (Cipollone et al., 2004). Possible explanation for the protective effect of the PTGS2 C allele could be that due to its reduced promoter activity, in the early stages of AD the PTGS2 expression in individuals with the C allele is not as high as in patients with the G/G genotype. The intensity of PTGS2 expression can influence the extent of inflammatory response, which may interact with other pathological processes in AD.

IV. Neuronal dysfunction

IV.1. Cholinergic system-related polymorphism

The CHRFAM7A genotype without the -2bp allele was significantly over-represented in AD as compared to the controls. It was demonstrated that CHRFAM7A are transcribed, but there is no evidence whether this transcript is translated or not (Gault et al, 1998; Riley et al, 2002). It is unlikely that this hybrid gene functions as a nicotinic receptor due to the absence of signal peptide, glycosylation site and part of the ligand-binding site encoded by exons 1-4 (Leonard and Freedman, 2006). It is possible however, that if CHRFAM7A is translated, the gene product is able to interact with α7 polypeptide, since most of the contact regions are encoded in exon 5-10 (Riley et al, 2002).

The mechanism by which the -2bp allele variant of duplicated exon 6 decreases the risk of AD remains to be established. The -2bp deletion results in a stop codon within exon 6, therefore the putative translational product will be truncated. Possible explanation could be that the wild type CHRFAM7A gene product may alter the normal assembly of the α7 nAChR which could be avoided by the truncated gene product (Riley et al, 2002). This hypothesis is supported by the observation that the deletion of the CHRNA7 gene improves cognitive deficits and synaptic pathology in a mouse model of Alzheimer's disease (Dziewczapolski et al., 2009).

IV.2. Serotonergic system-related polymorphisms

Our results indicate that the HTR2A T102C polymorphism may influence the susceptibility to AD, especially in combination with the 5HTTLPR L/L genotype. Although we did not detect statistically significant differences in the HTR2A genotype distribution between AD and HC groups, an association was found between the C/C genotype and the risk for AD when we conducted the C/T and T/T genotypes. Our results confirm the earlier findings of an increased

frequency of the co-presence of the L/L and C/C genotypes in AD as compared to HC group (Micheli et al., 2006). We found that the simultaneous occurrence of the HTTLPR L/L and HTR2A C/C genotypes enhance the risk 2.5-fold for the development of AD.

These findings can be of considerable relevance given the fact that both polymorphisms are functional and both act on the same biological pathway, namely serotonergic transmission. The HTR2A gene activity of the C allele was proved to be significantly decreased as compared to the T allele, therefore in individuals with the C/C genotype less receptor may be available for 5HT than in C/T or T/T carriers. The 5HTTLPR polymorphism determines dose-dependent 5HT reuptake from the synaptic cleft being the S allele less effective than the L allele. In summary, in case of simultaneous presence of the HTR2A C/C and the 5HTTLPR L/L genotypes, the serotonergic transmission may be less effective as compared to the other combinations of HTR2A and 5HTTLPR genotypes.

IV.3. Brain-derived neurotrophic factor polymorphism

Our results revealed that the Val66Met polymorphism of the BDNF gene may be implicated in the susceptibility to AD, since we have found that the Val/Val genotype and the Val allele occurred with significantly higher frequency in AD than in HC. Japanese and Italian studies have also reported this association (Ventriglia et al., 2002; Matsushita et al., 2005), although another Japanese and a Spanish study failed to find the same results (Combarros et al., 2004; Akatsu et al., 2006). BDNF is important for memory-related hippocampal processes. The functional Val66Met polymorphism of BDNF can influence the activity-dependent BDNF secretion, thus it can impact on hippocampal activity.

V. Genetic interactions

Increasing body of evidence supports the complex genetic model of AD, which suggests that polygenic network of susceptibility genes may underlie the disease. Since the predisposing gene variants confer only fractional risk, genetic interactions may have a major role in contributing to neurodegeneration in AD. The interactions were investigated in pairs of polymorphisms, and the pairs were selected on the basis of the same chromosomal localization and/or on the involvement of the same pathogenic mechanism in AD.

The interaction was clearly demonstrable in case of the investigated serotonin-related genes. Logistic regression analysis revealed an interaction between the 5HTTLPR and the HTR2A polymorphisms. The simultaneous presence of the 5HTTLPR L/L and HTR2A C/C genotypes increase the risk for AD 2.5-fold, while the effects of L/L or C/C genotypes on AD risk analyzed separately on the risk for AD were not statistically significant.

According to the logistic regression model there is no interaction between the BDNF and APOE polymorphisms. However, considering the calculated effect sizes, it is presumable that the interaction between the BDNF and APOE polymorphisms would be statistically verifiable by increasing the number of cases. The adjusted ORs for the different BDNF and APOE genotype combinations suggest a synergistic effect between the BDNF and APOE polymorphisms. The OR for the presence of both the APOE ε4 allele and BDNF Val/Val genotype was more than eightfold higher than the ORs for the presence of one of them.

Conclusions

- Our results confirm the previous findings that APOE ε4 allele confers an allele dose dependent risk for AD.

- We failed to detect association between BACE1 C786G, PLAU Pro141Leu and CALHM1 Leu86Pro polymorphisms and the risk for AD.

- Our findings indicate a gender dependent effect on AD risk with increased susceptibility to AD in men carrying the T/T genotype of the DHCR24 rs600491 polymorphism.

- We failed to detect association between IFNG T874A and PLA2G4A Ban*I* polymorphisms and the risk for AD.

- We found that the PTGS2 G/G genotype can confer susceptibility to AD and the possession of the C allele can have a protective effect.

- According to our results the -2bp deletion polymorphism of CHRFAM7A can be implicated in AD.

- Our study demonstrates an interaction between 5HTTLPR L/L and HTR2A C/C genotypes that seems to increase the risk for AD.

- We suggest that the BDNF Val/Val genotype in itself and in combination with the APOE ε4 allele can be risk factor for AD.

- In summary, our results support the involvement of the following genes in AD aetiology: APOE, DHCR24, PTGS2, CHRFAM7A, HTR2A in epistasis with SLC6A4 and BDNF.

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Articles the Thesis is based on

Fehér Á, Juhász A, Rimanóczy Á, Kálmán J, Janka Z. Association between BDNF Val66Met Polymorphism and Alzheimer's Disease, Dementia with Lewy Bodies and Pick's Disease. Azheimer's Disease and Associated Disorders. 2009. 23(3):224-228. **IF$_{2008}$: 3.220**

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Selected abstracts related to the thesis:

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