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Title: Alzheimer's disease and type 2 diabetes: The association study of polymorphisms in tumor necrosis factor-alpha and apolipoprotein E genes

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Abstract

Type 2 diabetes (T2D) and Alzheimer's disease (AD) are two progressive disorders with high prevalence worldwide. Polymorphisms in tumor necrosis factor-alpha (TNF-α) and apolipoprotein E (ApoE) genes might be associated with both T2D and AD, representing possible genetic markers for the development of the AD in subjects with T2D. The aim was to determine ApoE and G-308A TNF-α gene polymorphisms in unrelated Croatian Caucasians: 207 patients with sporadic AD, 196 T2D patients and 456 healthy controls. Patients with AD had higher frequency of ApoE4 allele compared to T2D patients and controls. The significant association, observed between ApoE2 allele and T2D, disappeared after the data were adjusted for age and sex. The genotype or allele frequencies of G-308A TNF-α gene polymorphism were similar among the patients with AD, T2D and healthy controls. In conclusion, these results do not support the hypothesis that the A allele of G-308A TNF-α gene polymorphism is associated either with AD or T2D. Our data confirm the association between the ApoE4 allele and AD, and point out the E2 allele of ApoE gene as the possible risk factor for T2D.

Key words: Alzheimer's disease; apolipoprotein E; diabetes type 2; gene; polymorphism; tumor necrosis factor- alpha

Introduction

Type 2 or non-insulin-dependent diabetes (T2D) and Alzheimer's disease (AD) are two progressive, disability-inducing disorders with high prevalence across the world. Although these diseases have different aetiology and pathophysiology, such as characteristic degeneration of pancreatic beta cells and/or insulin resistance in T2D (ADA 2011) and progressive atrophy of neocortical/limbic brain areas in AD (Perl 2010), the high incidence of AD was observed in T2D patients (Irie et al. 2008) and inversely T2D in patients with AD (Janson et al. 2004).

The common underlying biochemical and genetic dysfunctions in these disorders are unclear (Zhao and Townsend 2009). The findings of two main neuropathological hallmarks of AD, beta-amyloid (Aβ) plaques and neurofibrillary tangles (Perl 2010) in pancreas of T2D patients (Miklossy et al. 2010), and involvement of ApoE protein in both disorders, suggest ~~s~~ common underlying mechanisms. The glycoprotein apolipoprotein E (ApoE), with its three isoforms E2, E3 and E4 (Achaya et al. 2002), plays an important role in the lipid metabolism, and transport of cholesterol and phospholipids (Schipper 2011). The role of ApoE4 isoform in the formation of Aβ plaques and its importance as a risk factor for development of AD, have been often discussed (Messier 2003; Kim et al. 2009; Schipper 2011). Since insulin resistance is known to be associated with metabolic dyslipidemia (Sijbrands et al. 1994), ApoE isoforms with their functions in lipid metabolism could also play an important role in pathogenesis of T2D (Anthopoulos et al. 2010).

Other plausible common pathogenic component in both AD and T2D is inflammation (Bierhaus and Nawroth 2009). It has been shown that hyperinsulinemia, induced by peripheral insulin administration, elevated cerebrospinal fluid (CSF) levels of tumor necrosis factor-alpha (TNF-α), IL-1α, IL-1β and IL-6, and stimulated amyloidogenesis in healthy volunteers

(Fishel et al. 2005). TNF-α is a pro-inflammatory cytokine that regulates inflammatory and immune responses. It has an important role in the pathophysiology of several disorders, such as coronary heart disease (Vendrell et al. 2003), obesity (Dandona et al. 1998) and AD (Di Bona et al. 2009). The transcription and secretion of TNF-α are regulated by the promoter G-308A polymorphism of the TNF-α gene, with –308A allele related to high TNF–α values (Wilson et al. 1997).

Since there is a lack of data on the association between ApoE and TNF-α genetic variants and their correlation with T2D and AD, the aim of the study was to determine the genotype and allele frequencies of G-308A TNF-α promoter polymorphism and ApoE gene polymorphism in AD and T2D patients and healthy control subjects. The hypothesis was that patients with T2D and AD have higher frequency of the TNF-α rare -308A allele and the ApoE4 allele than healthy controls.

Methods

Study population

This study included 859 unrelated Croatian Caucasian subjects: 207 patients (mean age ± SD, 80.3 ± 7.1 years; 132 female) with AD hospitalized at University Psychiatric Hospital, Vrapce, 196 patients (64.8 ± 9.3 years; 56 female) with T2D treated in Vuk Vrhovac University Clinic in Zagreb, the WHO collaborating center for diabetes in Croatia (Alberti et al. 1998) and 456 healthy older volunteers (77.4 ± 5.4 years; 186 female) participants in the longitudinal epidemiological study at Institute for Medical Research and Occupational Health in Zagreb. The probable AD was diagnosed according to the criteria of the National Institute of Neurologic and Communicative Disorders and Stroke and the Alzheimer‘s Disease and

Related Disorders Association (NINDS-ADRDA) (McKhann et al. 1984) and Diagnostic and Statistical Manual of Mental Disorders Fourth Edition (DSM-IV, APA 1994). Patients with vascular dementia were excluded. Patients with diabetes were carefully assessed and diagnosed as type 2 according to the recommendations of the World Health Organization (WHO) and Standards of Medical Care in Diabetes from the American Diabetes Association (ADA 2011). The cognitive status was evaluated by Mini Mental State Examination (MMSE) (Folstein et al. 1975), translated and validated for Croatian population (www.parinc.com). MMSE score in controls and patients with T2D was in normal range (26-30), while the range in patients with AD was 0-20.

All participants underwent a clinical interview for evaluation of current and past medical status. Exclusion criteria for all participants were diagnoses of severe somatic diseases and major psychiatric disorders. Patients with AD were treated with acetylcholinesterase inhibitors. Oral hypoglycaemic drugs and insulin were used in 54 and 141 T2D patients, respectively.

The study was approved by the local Ethics Committees and was in agreement with the ethical standards laid down in the 1964 Declaration of Helsinki. Informed consent was obtained from participants or in the case of AD from their guardians.

Blood collection and DNA extraction

Venous blood samples (4 ml) were drawn from all subjects into plastic syringe with 1 ml of acid citrate dextrose anticoagulant. Genomic DNA was extracted from whole blood samples using salting out procedure (Miller et al. 1988). Genotyping of TNF-α (rs1800896) and ApoE (rs7412 and rs429358) was performed on ABI Prism 7000 Sequencing Detection System

(ABI) using a Taqman SNP genotyping assays in conditions recommended by Applied Biosystems (Foster City, CA, USA).

Statistical analysis

The genotype and allele distributions and deviations from Hardy - Weinberg equilibrium were determined by a χ^2-test. The univariate and multiple (adjusted for age and sex) logistic regression were implemented for testing the association of AD or T2D with of TNF-α and ApoE polymorphisms. Differences in age and glucose levels between groups were assessed by Kruskal-Wallis one way analysis of variance on ranks followed by Mann-Whitney test for between groups comparison. The statistical packages used were GraphPad Prism and Sigma Stat 3.5 (Jandell Scientific Corp. San Raphael, California, USA). The level of significance was set to $\alpha = 0.05$, with 2-tailed *p* values.

Results

As expected, glucose levels differed significantly (H=101.01; df=2; p <0.001, Kruskal-Wallis ANOVA) among groups, with the highest values in T2D patients (8.7 ± 3.1 mmol/l; mean ± SD) compared to AD patients (6.4 ± 8.0 mmol/l) and healthy controls (6.4 ± 2.4 mmol/l). Significant (H=286.97; df = 2; $p < 0.001$) age difference was observed among groups. Patients with AD were significantly (p<0.05) older than controls and patients with T2D, while controls were older than T2D patients. No significant deviation from the Hardy-Weinberg equilibrium was found in G-308A TNF-α genotypes for patients with AD (χ^2 = 0.45; df = 1; p= 0.505),

patients with T2D (χ^2 = 0.90; *df* = 1; *p* = 0.343) and healthy control subjects (χ^2 = 0.857; *df* = 1; *p* = 0.354). The distribution of the ApoE polymorphism genotypes (rs7412 and rs429358) did not deviate from the Hardy-Weinberg equilibrium in AD patients (χ^2 = 4.52; *df* = 1; *p* = 0.211), T2D patients (χ^2 = 3.27; *df* = 1; *p* = 0.353) and healthy subjects (χ^2 = 4.60; *df* = 1; *p* = 0.204).

Significant differences in the frequencies of ApoE genotypes, alleles and E2 or E4 carriers were observed among controls and AD or T2D patients (Table 1). Between groups comparison showed significant differences in genotype (χ^2 =18.16, *df* = 5, *p* = 0.003) or allele (χ^2 = 8.82, *df* = 2, *p* = 0.012) frequencies of the ApoE polymorphism (Table 1) between AD patients and controls. AD patients also differed from T2D patients in ApoE genotype (χ^2 =14.35, *df* = 5, *p* = 0.014) and allele (χ^2 = 6.66, *df* = 2, *p* = 0.036) frequencies. There was significant (χ^2 = 6.22, *df* = 2, *p* = 0.047) difference in the ApoE allele, but not in the genotype (χ^2 =10.04, *df* = 5, *p* = 0.074) frequencies between T2D patients and controls. The comparison of ApoE2 carriers and non-carriers between groups revealed significantly (χ^2 = 6.31, *df* = 1, *p* = 0.012) more ApoE2 carriers among T2D patients than in controls and comparable distribution of ApoE2 carriers in AD and T2D patients (χ^2 = 3.61, *df* = 1, *p* = 0.057), or in AD patients and controls (χ^2 = 0.000, *df* = 1, *p* = 0.982). The assessment of ApoE4 carriers and non-carriers between groups revealed more ApoE4 carriers among AD patients when compared to controls (χ^2 = 6.28, *df* = 1, *p* = 0.012) or to T2D patients (χ^2 = 4.01, *df* = 1, *p* = 0.045), but similar frequency of ApoE4 carriers in controls and T2D patients (χ^2 = 0.01, *df* = 1, *p* = 0.915).

The G-308A TNF-α polymorphism genotype, or allele frequencies did not differ significantly between or within the studied groups (Table 2). No significant differences were detected in the genotype frequencies between AD (χ^2 = 2.188, *df* = 2, *p* = 0.335) or T2D (χ^2 =

1.975, $df = 2$, $p = 0.373$) patients when compared to healthy controls or AD and T2D patients ($\chi^2 = 2.188$, $df = 2$, $p = 0.335$). Allele frequencies between controls and AD ($\chi^2 = 1.186$, $df = 1$, $p = 0.276$) or T2D subjects ($\chi^2 = 0.178$, $df = 1$, $p = 0.673$), or between AD and T2D patients ($\chi^2 = 1.775$, $df = 1$, $p = 0.183$) showed no significance. The frequency of A allele carriers (Table 2) was similar between healthy subjects and AD patients ($\chi^2 = 0.580$, $df = 1$, $p = 0.446$) or T2D patients ($\chi^2 = 0.585$, $df = 1$, $p = 0.444$), or between AD and T2D patients ($\chi^2 = 1.812$, $df = 1$, $p = 0.178$).

Potential risk factors for AD and T2D were tested separately using univariate logistic regression model. It revealed a significant ($p < 0.0001$) effect of sex and age in T2D and AD. The multivariate logistic regression model was used to predict association of -308A allele of TNF-α gene with T2D, with data adjusted for well known risk factors to exclude their confounding effect (Table 3). While univariate model showed a significant ($p = 0.009$) association of ApoE2 allele with T2D, statistical significance was lost in the multivariate logistic regression model when adjusted for age, sex and TNF-α. Both univariate ($p = 0.010$) and multivariate ($p = 0.001$) analyses (adjusted for age, sex and TNF- α) have shown that ApoE4 significantly predicted a risk for AD, but not for T2D. In both models allele -308A TNF-α showed no association with T2D or AD when data were adjusted for age, sex and presence of ApoE2 or ApoE4 allele (Table 3).

Discussion

Our results confirmed the previously described association of E4 allele and AD, (Farrer et al. 1997) supporting the ApoE gene variant as a risk factor for the development of AD (Leduc et al. 2010). ApoE4 allele might play an important role in the pathophysiology of AD (Leduc et al. 2010; Perl 2010). The E4 carriers exhibit reduced clearance with

consequently higher deposition of toxic Aβ, enhanced formation of neurofibrillary tangles, and insufficient neuronal damage repair, compared to E2 and E3 carriers (Perl 2010).

The relationship between ApoE gene variants and T2D is unclear. The ethnic differences in the frequency of ApoE alleles have been described, such as the higher frequency of E4 allele in Caucasians than Chinese (Burman et al. 2009). However, our data showed no difference in ApoE allele frequencies in Croatian compared to Chinese T2D patients (Ma et al. 2008). In addition, our hypothesis that T2D patients, like AD patients, have higher frequency of ApoE4 allele compared to healthy controls, has not been confirmed. The reason could be a difference in patients' age. In the association meta-analysis between ApoE alleles and age in healthy population (Farrer et al. 1997), the frequencies of E2 and E3 alleles increased, while the frequency of E4 allele decreased with aging. Nevertheless, we cannot exclude the possibility that younger E4 carriers among T2D patients might develop AD later in life. Since T2D ApoE4 allele carriers are at higher risk for the earlier occurrence of cognitive decline and dementia compared to E2 or E3 allele carriers (Irie et al. 2008), a longitudinal follow-up study would be necessary to evaluate the association between E4 allele and incidence of AD in our population of T2D patients.

Previous studies of AD and T2D were focused primarily on the frequency of E4 allele, providing insufficient data about the other ApoE variants. In agreement with the literature (Farrer et al. 1997), we found that E3 allele was the most common ApoE allele in healthy subjects, as well as in AD and T2D patients. In addition, a higher frequency of the rare E2 allele carriers in T2D patients compared to controls and AD patients, was also found. However, logistic regression analysis suggested that the observed association between the E2 variant and T2D was related to sex and age differences. To our knowledge, there is only one study (Errera et al. 2006) that reported a higher frequency of E2 allele in the small number of male and female diabetic patients (type 1 and type 2) of African-Brazilian and European-

Brazilian origins (Errera et al. 2006). Unfortunately, this study (Errera et al. 2006) did not evaluate the effects of sex and age in order to confirm the association between E2 allele and diabetes. Although E2 might be protective against the development of AD (Farrer et al. 1997), it could be detrimental for T2D. The role of ApoE gene variants, especially E2, in T2D is unknown. E2 might be related to altered lipid metabolism in T2D (Dallongeville et al. 1991). Since E2 variant has a low affinity for the low density lipoprotein cholesterol (LDL-C) receptors (Brown and Goldstein 1983) and induces their up-regulation in liver (Brown and Goldstein 1983), it could be associated with altered plasma cholesterol levels (Schiele et al. 2000), high triglyceride levels (Dallongeville et al. 1991), high ApoE levels (Schiele et al. 2000), insulin resistance, hyperlipidemia (Dallongeville et al. 1991) and obesity (Dandona et al. 1998). It has also been shown that ApoE2 allele carriers have different composition of LDL-C levels, including very dense intermediate-density proteins, compared to the other genotypes (Murdoch et al. 2007).

Our results show that allele frequencies of the G-308A TNF-α polymorphism in Croatian control subjects are in line with the frequencies observed in other healthy populations (Vendrell et al. 2003). The distribution of genotypes and alleles of the G-308A TNF-α polymorphism observed in our AD patients is in accordance with the recent meta-analysis (Di Bona et al. 2009) showing no association of TNF-α gene variants with the occurrence of AD.

The studies about the association between G-308A TNF-α gene polymorphism and T2D presented inconsistent results (Hamann et al. 1995; Koch et al. 2000; Kubaszek et al. 2003). We found no association between TNF-α gene variants and T2D, which is in agreement with the previous data showing a lack of increased risk for A allele carriers to develop T2D compared to G allele carriers (Hamann et al. 1995), and a lack of association between TNF-α gene variants and insulin sensitivity or secretion in the first-degree relatives

of T2D patients (Koch et al. 2000). In contrast, other studies (Kubaszek et al. 2003) reported a correlation between A allele and development of T2D, probably due to the higher transcriptional activity and increased TNF-α production in A allele carriers.

Several limitations of our study should be mentioned. Taking into account aging as a risk factor for AD, the possibility that our T2D patients might develop AD in older age could not be excluded, since they were younger than AD patients and healthy controls. Although only 9% of T2D patients were E4 carriers, a longitudinal follow-up study would be necessary to confirm E4 allele as a risk factor for the occurrence of AD in T2D patients. Moreover, we did not determine plasma lipid levels and prevalence of coronary heart disease in control subjects, AD and T2D patients due to the technical reasons. The advantages of the present study were well-defined groups of unrelated Caucasians exposed to similar environmental and lifestyle factors.

Conclusions

The E2 allele of the ApoE gene might be the risk factor for T2D. An association between the E4 allele and AD, but not T2D was found. The genotype or allele frequencies of G-308A TNF-α gene polymorphism were similar between patients with AD, T2D and healthy controls. The hypothesis that AD and T2D patients have similar distribution of the TNF-α gene and the ApoE gene variants was not confirmed.

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Conflict of Interest statement. All authors declare no conflicts of interest.

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Table 1. Genotype, allele and E4 carrier count (N) and frequencies (%) of ApoE gene polymorphism (rs7412 and rs429358) in healthy controls, patients with type 2 diabetes (T2D) and patients with Alzheimer's disease (AD).

	Healthy controls	**T2D**	**AD**
	(456)	(196)	(207)
Genotype	N (%)	N (%)	N (%)
E2/2	1 (0.2)	0 (0.0)	2 (1.0)
E2/3	48 (10.5)	35 (17.9)	16 (7.7)
E2/4	2 (0.5)	2 (1.0)	6 (2.9)
E3/3	328 (71.9)	127 (64.8)	135 (65.2)
E3/4	76 (16.7)	30 (15.3)	44 (21.3)
E4/4	1 (0.2)	2 (1.0)	4 (1.9)
		χ^2=29.55, *df*=10, *p*=0.001	
Alele			
E2	52 (5.7)	37 (9.4)	26 (6.3)
E3	780 (85.5)	319 (81.4)	330 (79.7)
E4	80 (8.8)	36 (9.2)	58 (14.0)
		$\chi 2$=15.38, *df*=4, *p*=0.004	
Carriers			
E2 carriers	51 (11.2)	37 (18.8)	24 (11.6)

E2 non carriers	405 (88.8)	159 (81.2)	183 (88.4)
		$\chi 2$=7.65, *df*=2, *p*=0.022	
E4 carriers	79 (17.3)	34 (17.4)	54 (26.1)
E4 non carriers	377 (82.7)	162 (82.6)	153 (73.9)
		$\chi 2$=7.69, *df*=2, *p*=0.021	

Table 2. Genotype, allele and carriers (GG vs. AA+AG) count (N) and frequencies (%) of G-308A TNF-α gene polymorphism in healthy controls, patients with type 2 diabetes (T2D) and patients with Alzheimer's disease (AD).

	Healthy controls (456)	**T2D** (196)	**AD** (207)
Genotype	N (%)	N (%)	N (%)
AA	12 (2.6)	3 (1.5)	2 (1.0)
AG	108 (23.7)	55 (28.1)	46 (22.2)
GG	336 (73.7)	138 (70.4)	159 (76.8)
		$\chi 2$=4.39, *df*=4, *p*=0.356	
Alele			
A	132 (14.5)	61 (15.6)	50 (12.1)
G	780 (85.5)	331 (84.4)	364 (87.9)
		$\chi 2$=2.19, *df*=2, *p*=0.335	
Carriers			
GG	336 (73.7)	138 (70.4)	159 (76.8)
AA + AG	120 (26.3)	58 (29.6)	48 (23.2)
		$\chi 2$=2.13, *df*=2, *p*=0.345	

Table 3. Logistic regression analysis in patients with type 2 diabetes (T2D) and patients with Alzheimer's disease (AD) compared to healthy controls.

	T2D						AD					
	univariate model						**univariate model**					
predictor	N	β	SE β	*p*	OR	CI	N	β	SE β	*p*	OR	CI
age	630	-0.25	0.02	***<0.0001***	0.78	0.745 - 0.810	641	0.08	0.01	***<0.0001***	1.09	1.055 - 1.118
sex	630	1.29	0.18	***<0.0001***	3.63	2.527 - 5.211	663	-1.76	0.24	***<0.0001***	0.17	0.107 - 0.279
-308 TNFα A allele carrier	652	0.16	0.19	0.390	0.85	0.587 - 1.231	663	-0.17	0.20	0.391	0.85	0.576 - 1.241
E2 carrier	652	0.61	0.24	***0.009***	1.85	1.165 - 2.931	663	0.04	0.26	0.877	1.04	0.622 - 1.744
E4 carrier	652	0.00	0.23	0.995	1.00	0.644 - 1.559	663	0.52	0.20	***0.010***	1.68	1.136 - 2.497
	multivariate model						**multivariate model**					
predictor	N	β	SE β	*p*	OR	CI	N	β	SE β	*p*	OR	CI
-308 TNFα A allele carrier *	630	0.18	0.27	0.491	1.20	0.712 - 2.033	641	-0.18	0.21	0.398	0.84	0.551 - 1.267
-308 TNFα A allele carrier #	630	0.16	0.27	0.555	1.17	0.695 - 1.968	641	-0.17	0.21	0.438	0.85	0.556 - 1.289
E2 carrier **	630	0.65	0.34	0.061	1.91	0.972 - 3.751	641	0.08	0.29	0.783	1.08	0.614 - 1.912
E4 carrier **	630	-0.11	0.32	0.729	0.90	0.482 - 1.666	641	0.71	0.22	***0.001***	2.04	1.314 - 3.152

* adjusted for age, sex and ApoE2; # adjusted for age, sex and ApoE4; ** adjusted for age, sex and TNFα A allele