Arq Neuropsiquiatr 2005;63(1):1-6

IS MTHFR POLYMORPHISM A RISK FACTOR FOR ALZHEIMER'S DISEASE LIKE APOE?

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ABSTRACT - *Background*: The role of methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms as risk factors for the occurence of Alzheimer's disease (AD) is still controversial: *Objective*: To verify the association between MTHFR and apolipoprotein E (APOE) polymorphisms and Alzheimer's disease. *Method:* This work was conducted as a case-control study. Cases included thirty patients with probable AD. Controls were constituted by 29 individuals without dementia according to neuropsychological tests paired to age, sex, race and educational level. DNA was isolated from peripheral leukocytes of anticoagulated venous blood. Genotyping of APOE and MTHFR were performed by DNA amplification and digestion. The frequences of APOE and MTHFR genotypes were submitted by chi-square test corrected by Fisher test; the APOE genotypes, to chi-square linear tendency test and the frequences of MTHFR mutant and AD, by stratificated anlysis adjust by Mantel-Haenszel method. *Results:* There was significant difference about APOE4 and APOE2 in the groups. (*p=0.002*) The odds ratio increased exponentially with the increased number of E4 allele (χ^2 linear tendency test). No significant difference was detected on MTHFR genotypes in both case and control groups. *Conclusion:* The APOE4 is a risk factor and demonstrated a dose-depenent effect while APOE2 allele conferred a protection to AD. The MTHFR mutation had no correlation with AD.

KEY WORDS: Alzheimer's disease, apolipoprotein E, methylenetetrahydrofolate reductase, PCR-RFLP, risk factors.

Polimorfismo da MTHFR é um fator de risco para demência de Alzheimer como APOE?

RESUMO - *Introdução*: O papel do polimorfismo do gene da metilenotetrahidrofolato redutase (MTHFR) como um fator de risco para demência de Alzheimer (DA) é controverso ainda. *Objetivo*: Verificar a associação entre os polimorfismos da MTHFR e apolipoproteína E (APOE) e DA. *Método:* O trabalho foi conduzido como um estudo caso-controle. Trinta pacientes com DA provável foram incluídos no grupo caso. Vinte e nove indivíduos sem demência comprovadas por testes neuropsicológicos, emparelhados pela idade, sexo, cor e nível educacional constituíram o grupo controle. DNA foi isolado de leucócitos periféricos extraídos de sangue venoso anticoagulado. Genótipos de APOE e MTHFR foram realizados por amplificação de DNA e digestão. As freqüências dos genótipos da APOE e MTHFR foram submetidas ao teste do chi-quadrado corrigido pelo teste de Fisher; os genótipos da APOE, ao teste do chi-quadrado com tendência linear e as freqüências da MTHFR mutante e DA à análise estratificada corrigida pelo método de Mantel-Haenszel. *Resultados:* Houve diferença significativa entre APOE4 e APOE2 nos grupos (*p=0,002*). O *odds ratio* aumentou exponencialmente com o aumento do número de alelo E4 (teste χ^2 com tendência linear). Nenhuma diferença significativa foi detectada nos genótipos da MTHFR em ambos grupos caso e controle. *Conclusão:* O alelo APOE4 é um fator de risco e demonstrou efeito dose-dependente enquanto o alelo E2 conferiu proteção para DA. A mutação da MTHFR não teve correlação dom DA.

PALAVRAS-CHAVE: doença de Alzheimer, apolipoproteína E, metilenotetrahidrofolato redutase, fatores de risco.

The number of elder people has raised with the increase of life expectancy and as consequence, the prevalence of age-related disease. Alzheimer´s disease (AD) is the leading cause of dementia in the elderly. It is a multifactorial pathology resulting of the interaction of both genetics and environmental factors. AD is an illness resulting from selective damage of specific neuronal circuits in the neocortex, hippocampus, and basal forebrain cholinergic system. Affected regions show senile plaques, comprised of neurites displayed around extracellular deposits of β-amyloid peptides; and many neurons develop neurofibrillary tangles, which reflect the local accumulation of abnormal intracytoplas-

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Received 23 April 2004, received in final form 22 July 2004. Accepted 21 September 2004.

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mic filaments, composed of hyperphosphorylated isoforms of the tau protein[1]. Over one hundred rare, highly penetrant mutations have been described in three genes (APP, PSEN1, PSEN2) for early-onset familial AD[2-4]. In the more common late-onset form, a polymorphism in the apolipoprotein E (APOE) gene has been associated with increased susceptibility[5].

APOE is a plasma protein involved in cholesterol transport. It is produced and secreted in the central nervous system (CNS) by astrocytes. APOE synthesis is increased following injury and is implicated in the growth and repair of nervous system during development or after injury. APOE is bound to extracellular senile plaques, to intracellular neurofibrillary tangles, and at sites of cerebral vessel congophilic angiopathy[6]. The APOE gene has three alleles: E2, E3 and E4. The variant APOE4 is a major risk factor for the development of AD[7-13] However, APOE4 is neither necessary nor sufficient to cause AD. So it is clear that other yet unknown genes, and environmental factors must be involved in its etiology[14].

Low blood levels of folate and vitamin B12, and elevated homocysteine levels were associated with poor cognitive performace in elderly people[15-19]. Possible biochemical interpretation of the putative effects of this low vitamin status and elevated homocysteine levels on cognitive decline can be made on basis of the pathway of one-carbon metabolism[15]. In this pathway, the methylenetetrahydrofolate reductase (MTHFR) is a central enzyme forming the substrate needed for the transferring reaction. Dysfunctional one-carbon metabolism gives rise to various pathogenetic mechanisms: insufficient DNA synthesis, transmethylation insufficiency and toxicity of homocysteine and related compounds[9]. Furthermore, insufficient one-carbon metabolism has been suggested to have a contributory role in the development of dementia and has found to be significant in AD patient[9]. It has been recently reported a C677T mutation on the MTHFR gene which produces a thermolabile variant. In its homozygous form, this variant possesses a reduced overall enzyme activity to less than 30% of normal, resulting on increased serum homocysteine levels[9,20].

We examined both the APOE polymorphism and MTHFR mutation in a group of AD individuals and in controls, to assess whether these genetic factors increase the risk for this illness.

METHOD

Patients – We investigated 29 Caucasian patients (24 women and 5 men) and 1 Afro-Brazilian patient (1 man) all over 55 years old (mean 73.5 ± 18.5) with the diagnosis of Probable AD according to NINCDS-ADRDA criteria and an Inventory of Diagnostic Clinic Features of Disease of Alzheimer Type[21,22]. All cases were sporadic, unrelated and were recruited at an outpatient clinic (Clínica médica Salvador Dali and Serviço de Geriatria Hospital São Lucas da PUCRS) in Porto Alegre city.

Table 1. Sample outline studied.

	Case n=30	Controls n=29	p
Sex, No (%) F	24 (80)	24 (82.7)	
Race, No (%) white	29 (96.9)	29 (100)	
Age, years	73,5±18.5	76±17	
Educational level, <8 years	70%	72.4%	
Discrete periventricular white matter lucencies in neuroimaging (CT or MRI) with encephalic atrophy	70%		
Smoking use, yes	15 (50)	10 (34,5)	0.346
Antinflamatory drugs, yes	17 (56.6)	15 (51.7)	0.905
Familial history, yes	8 (26.7)	5 (17.2)	0.576
Hormonal replacement, yes	9 (12.5)	6 (4.2)	0.304*
Diabetes mellitus, yes	8 (26.7)	2 (6.9)	0.044*
Hypercholesterolemy, yes	18 (60)	14 (48.3)	0.520
Atherosclerosis, yes	12 (40)	15 (55.2)	0.364
Atrial fibrilation, yes	2 (6.7)	6 (20.7)	0.116*
Arterial hypertention, yes	15 (50)	13 (44.8)	0.891

The data were presented in numbers (percentage) and p calculated by χ^2 corrected by Yates test; (*) corrected by Fisher test.

We evaluated 29 Caucasian control individuals (24 women and 5 men), without dementia according to neurophychological tests matched for age, sex and educational level with case group.

The sample outline was demonstrated on Table 1.

Consent for participation in the study was provided by the subjects themselves or their legal guardians.

Ethics – The research protocol was approved by Scientific and Ethics Committes of the University.

Laboratory methods – DNA from each patient was isolated from peripheral leukocytes of 1 mL venous blood, anticoagulated with ethylenediaminetetraacetic acid (EDTA) by kit GFX Genomic Blood DNA Purification (Amersham Biosciences, USA). Blood samples were stored at 20°C for analysis.

APOE genotyping was performed by amplification of the third exon of the APOE gene by polymerase chain reaction (PCR) with primers described by Wenham et al.[23], followed by an enzymatic clivage with the restriction enzyme *Hha*I (RFLP)[20]. Fragments of 72bp and 48bp are produced in APOE4, fragments of 91bp and 83bp are produced in APOE2 an 91bp and 48bp are generated in APOE3. (Fig 1).

To detect the mutant allele of MTHFR, the same leukocytes DNA samples were examined. The PCR was performed with the primers described by Nishiyama et al. using *Hinf*I restriction enzyme to identify the mutation[20]. The mutant allele generated two fragments: 175bp and 23bp, while the wild-type is not clived and is identified by a 198bp fragment (Fig. 2).

Data analysis – Allele frequences for patients with AD and control subjects were estimated by counting alleles and calculating sample proportions.

Frequences of APOE and MTHFR genotypes in cases and controls were compared using the chi-square test corrected by Fisher test (Stat calc/Epi Info 6).

The APOE genotypes were submitted to chi-square linear tendency test.

The frequences of MTHFR mutant and AD were evaluated after stratificated analysis adjust by Mantel-Haenszel method.

RESULTS

The APOE alleles frequencies in our control sample were 83% E3, 10% E4 and 7% E2 and in case sample, 63% E3, 35% E4 and 2% E2. This diference

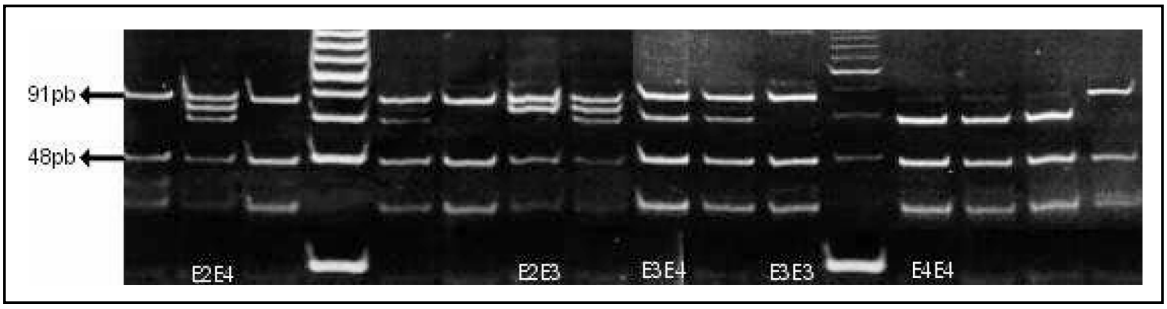

Fig 1. APOE genotypes: polyacrilamide (15%) gel electrophoresis stained with ethidium bromide. Some representative genotypes and relevant molecular sizes are indicated.

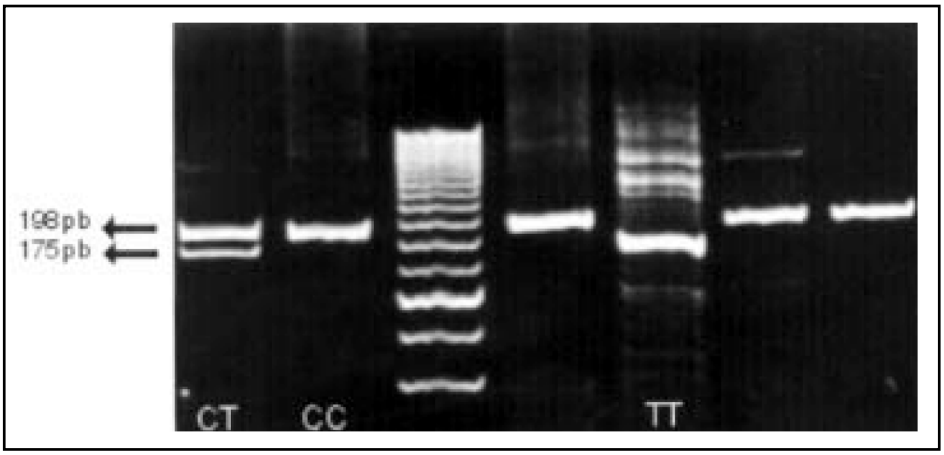

Fig 2. MTHFR genotypes: polyacrilamide (15%) gel eletrophoresis stained with ethidium bromide. Some representative genotypes and relevant molecular sizes are indicated.

was significant (*p=0.002*) calculated by Exact Fisher test.

The genotyping distribution and its impact/significance were analised using the chi-square linear tendency test (Table 2).

The MTHFR alleles frequencies were 65% allele C, 35% allele T in case group while in controls were 71% and 29% respectively. This diference was not significant (*p=0.508* χ^2 test)

The interaction of APOE4 allele and MTHFR T (mutation) was demonstrated in Table 3. The genotypes distribution in cases was 35% CC (wild gene homozigose), 57% CT and 6% TT (mutation gene homozigose) while controls it was 52% CC, 38% CT and 10% TT. This diference was not statistical significant (*p=0.401* Exact Fisher test).

DISCUSSION

The control allele frequencies in our sample were 83% E3, 10% E4 and 7% E2. All controls were Caucasians. In Caucasian populations the E3 allele is the most commonly ocurring: it occurs about 77%. The average frequencies of E4 and E2 are 15% and 8% respectively. The E4 allele frequence varies according to the race. It reaches up to 30-35% in African and Asian populations. The north European population presents larger frequencies (Finland 22.7%, Sweden 20.3%) than southern countries (Italy 9.4%)[24]. Andrade et al. found in a populational study in south Brazilian region 81% E3, 11.5% E4 and 7.5% E2 allele frequencies in caucasians while for Afrobrazilians they found 70% E3, 22.5% E4 and 7.5% E2 allele frequencies[25]. Schwanke et al. found in an elderly population in Veranópolis (south Brazilian region) 84% E3, 11% E4 and 5% E2 allele frequencies[26]. The control allele frequencies in our sample were similar to others Caucasian population described in literature.

The E4 allele frequencies were significantly higher in case group than control.The E4 allele frequence was 10% in control group against 35% in cases and similar to values reported by Andrade *et al* (11.5% in controls and 39% in cases)[25]. Almeida in São Paulo population find 22.1% E4 in cases and 8.9% in controls[27].

Our study confirms the E4 allele presence as an AD risk factor for this Caucasians sample according to literature data[7,8,10,12,13,20,28-30]. Based on these, it is obvious that APOE4 has a massive impact on the development of AD.

The E2 allele presence was significantly different in both groups (*p=0.002* Exact Fisher Test). Only 2% of the demented presented E2 alleles whereas 7% of normal individuals presented it.

It was demonstrated that AD patients with E2E3 genotype compared with E3E3 presented less β-amiloid densities in brain cortex moreover reduced amiloid angiopathy[31]. Review articles have suggested a protective effect of E2 allele in AD patients[10,12]. Nevertheless, Molero in an aging study performed in Maracaibo (Venezuela), Almeida in São Paulo (Brazil) and Andrade in Porto Alegre (Brazil) did not find significant association between E2 allele in cases and controls[25,27,28]. In our sample the E2 allele presence suggested a protective effect for AD.

Our genotipic distribution showed a strong tendency to increase the risk (OR) to develop AD dependent on genotype. The E2E3 genotype presented like the most protective genotype for AD (OR=1) The genotypes that brings near the E4 homozigose determined an exponential increase in odds ratio (E3E3 OR=4^1 E3E4 OR=4^2 ; E4E4 OR=4^3) (Table 2).

Austin Bradford-Hill describes 8 elements that make evident an effect-cause association. Among

Table 2. APOE genotype distribuition.

	n	Cases n=30	Controls n=29	OR*	*p* Fisher
E4E4	4	4 (13.3)	0 (0.0)	63.0	0.029
E4E3	17	12 (40.0)	5 (17.2)	15.9	0.049
E4E2 e E3E3	35	14 (46.6)	21 (72.4)	4.7	0.283
E3E2	3	0 (0.0)	3 (10.3)	1.0	

χ^2 linear tendence test, p< 0.001; * Agresti ajusted.

Table 3. Association between MTHFR allele T and AD after adjust to stratifed analysis to allele E4 effect.

Allele status	Cases	Controls	OR (IC95%)
E4 Allele (+)			
T Allele (MTHFR)	n=17	n=6	
Positive	10 (58.8)	4 (66.7)	0.71 (0.07 a 6.95)
Negative	7 (41.2)	2 (33.3)	p=0.999
E4 Allele (-)			
T Allele (MTHFR)	n=13	n=23	
Positive	9 (69.2)	10 (43.3)	2.92 (0.57 a 15.94)
Negative	4 (30.8)	13 (56.6)	p=0.254
Total			1.80* (0.51 a 6.30) p=0.473

Data are presented as number (percentage). *Odds ratio adjusted by Mantel-Haenszel method.

them, temporality (causes precede the effects), association force (a high related or absolute risk), a dose-effect relation (high doses are related with effect variations) and consistence (other studies, in different times, in different places, with different patients come to the same evidence). The E4 allele presence precedes AD manifestation, the 63 *odds ratio* of E4E4 genotype was remarkable absolute risk, the E4 allele quantity increases exponentially the AD risk and a lot of studies in different parts of the world comes to the same evidence. So our data confirm a predisposition to develop AD in individuals that present E4 allele and make clear several Bradford-Hill elements[32]. Sixty-five percent of AD individuals did not present APOE4 allele in our sample. In this cases other risk factors (genetics or environmental) would be acting in the AD development.

Regland et al. related 40% of heterozigose prevalence in general population (CT), 11% of polymorphism homozigose (TT) and 49% wild gene homozigoses (CC)[9]. Nishiyama et al. describe 39% CC, 45% CT and 15% TT similar to Japanese and Canadian population data[20]. Our results (38%CT, 10%TT and 52%CC) resemble genotypic distribution related by Regland[9].

The MTHFR allele frequence did not show significant difference in control and case groups. Nishiyama et al found an increased proportion of senile demented individuals in the MTHFR mutation group, particularly in men. This mutation was more associated with AD than with vascular dementia[20]. In our work no association with MTHFR mutation and AD was found. It could be probably because of the high polymorphism prevalence in general population associated to a limited group sample (30 cases and 29 controls). It would be need 60 cases and 120 controls to express really diferences.

Regland et al. found an inverse correlation between MTHFR mutation and APOE4, suggesting that MTHFR mutation is 1.8 times more frequent in the absence of APOE4[9]. The association rate of MTHFR T allele and AD cases after adjusted for allele E4 effect was not significant, despite it suggested a small tendency of allele T individuals to develop AD (OR=1.80) (Table 3).

Clark et al. Postiglione et al. Brunelli et al. and Prince et al. did not find a significant relation between MTHFR mutation and AD either[19,33-35]. Seripa et al found no difference in MTHFR polymorphism distribuition between AD cases and elderly controls in both American cohort and Italian cohort[36]. Religa et al. found that plasma total homocysteine is increased in AD patients and depended on the MTHFR T/T genotype (mutation homozigoze) in the presence of low folate levels, however the distribuition of MTHFR C677T polymorphism in the Polish population does not differ in AD and controls[37]. Our negative results, in individuals with Probable AD, confirmed the lack of association between AD and C/T polymorphism in the MTHFR gene.

The main conclusions of this study were: APOE4 allele was a risk factor for AD; APOE2 allele was a protective factor for AD; the presence of allele E4 demonstrated a dose-dependent effect, increasing exponentially compared with genotype E2E3; there was a discreet tendency of MTHFR allele T presence in AD patients adjusted to allele E4 efect, but it was not significant; the MTHFR mutation did not demonstrate significant difference in cases and controls.

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