Vol.2, No.3, 117-121 (2013)
http://dx.doi.org/10.4236/aad.2013.23015

BLMH and *APOE* genes in Alzheimer Disease: A possible relation

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Received 12 June 2013; revised 22 July 2013; accepted 31 July 2013

ABSTRACT

Alzheimer disease (AD) is a progressive and irreversible neurodegenerative disorder that is characterized by cognitive decline, memory loss and confusion. The E4 allele of the apolipoprotein E gene (*APOE*) is associated with AD and it is the main genetic risk factor for disease. Although the exact physiological function is unknown, bleomycin hydrolase (*BLMH*) may also be associated with AD development, although previous immunohistochemical findings have been inconsistent. Therefore, the purpose of this study was to evaluate the genotypic and allele frequencies of the *APOE* gene and *BLMH* 1450 G > A polymorphism and assess *BLMH* expression using PCR-RFLP and RT-qPCR analyses of blood samples from patients with Alzheimer disease (AD), healthy elderly adults (EC) and healthy young subjects (YC). *BLMH* expression was significantly different among groups ($p = 0.015$) and there was substantial reduction with age and with AD. The *APOE* and *BLMH* genotype frequency did not diverge from the Hardy-Weinberg equilibrium. There was a higher frequency of genotype 3/3 in all subjects (61.1%) and the AD group demonstrated a higher frequency of allele 4; however, differences in genotype and allele distributions were statistically different among groups.

Keywords: Alzheimer Disease; Bleomycin Hydrolase; Apolipoprotein E; Expression Gene; Polymorphism

1. INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia and both environmental and genetic factorsare contributing to risk, which increases with age [1]. Prevalence of Alzheimer's disease is 0.8% in individuals aged 65 - 69 years and upwards of 28.5% in persons aged 90 years and older [2]. Brain anatomy of patients with AD has two hallmark neuropathological characteristics, neuritic plaques and the neurofibrillary tangles. Amyloid-β (Aβ) peptide is the major plaque component, while hyperphosphorylated tau protein is the major tangle component [3-5].

The apolipoprotein E (*APOE*) gene is the most important genetic risk factor for sporadic AD and it is located on chromosome 19q13.2 and consists of 4 exons. The 3 *APOE* (ε2, ε3, and ε4) are defined by 2 SNP, which encode 3 protein isoforms (E2, E3, and E4). Only about 20% - 25% of the general population carries one or more ε4 alleles, whereas 50% - 65% of people with AD are ε4 carriers [6,7].

Bleomycin hydrolase (BLMH) enzyme is a cysteine protease of the papain superfamily encoded by the *BLMH* gene. This enzyme is expressed in most human tissues, including the hippocampus and amygdala. Although the exact physiological function is unknown, *BLMH* is associated with AD and the expression of this enzyme is substantially lower in AD brains [10,11]. *BLMH* gene is located on chromosome 17q BLMH 11.2 and its enzyme exposes 455 amino acids [8]. *BLMH* 1450 G > A is its main polymorphism, in which there is the substitution of nucleotide Adenine by Guanine at position 1450. This subsequently leads to either isoleucine or valine as the amino acid in position 443 (Ile443Val) [9]. In patients

with the polymorphism, the biotransformation is inefficient and resulting in an accumulation and toxic action of the bleomycin hydrolase [9,19].

Although bleomycin hydrolase is widely distributed in most human tissues, previous studies have demonstrateedthat the *BLMH* gene is associated with AD. It is hypothesized that the *BLMH* gene alters the processing of amyloid precursor protein (APP) and significantly increases the release of its proteolytic fragment, Amyloid-β (Aβ) [10,11]. Despite this potential relationship, the specific role of this altered gene expression has not been fully elucidated and the relationship between *BLMH* and the development of AD remains a source of controversy [10-12].

Given the potential relationship between *APOE*, *BLMH* and the development of AD, the purpose of this paper was to determine whether differences exist in the *APOE* allele frequency, *BLMH* genotype distribution, and *BLMH* gene expression in healthy and pathological subject groups. PCR-RFLP and qRT-PCR techniques were to compare allele frequencies, genotypes distribution and gene expression in the peripheral blood of healthy young (YC), healthy elderly (EC) and AD subject groups. We hypothesized that the significant differences would exist between the AD and control groups and, in particular, subjects in the AD group would demonstrate less *BLMH* expression.

2. MATERIALS AND METHODS

2.1. Samples

Ninety-one subjects with AD (31 ♂ and 60 ♀, mean age of 74.54 ± 7.58 years), 93 EC subjects (38 ♂ and 55 ♀, mean age of 71.52 ± 8.02 years) and 86 YC subjects (31 ♂ and 55 ♀, mean age of 24.60 ± 1.92 years) participated in this study. *BLMH* expression was analyzed in 164 subjects: 56 subjects with AD (13 ♂ and 43 ♀, mean age of 74.54 ± 7.66 years), 59 EC (22 ♂ and 35 ♀, mean age of 71.52 ± 8.22 years) and 52 YC (23 ♂ and 28 ♀, mean age of 21.18 ± 1.83 years). The three subject groups had similar ethnic origins, 95% of the total sample were of European origin, 2.5%, were of Japanese origin and 2.5% were multi-ethnic. The ethnic origins were determined by self-report and by the geographical origin of family.

Subjects with AD were evaluated using Mini-Mental State Examination (MMSE) and Katz index [13,14]. AD patients were diagnosed according to National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria and The Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) for probable AD [15]. All patients were recruited from the Department of Neurology at the Federal University of São Paulo (UNIFESP), São Paulo-SP, Brazil. The Institutional Research Ethics Committee approved this study and all subjects or their legal representatives signed an informed consent according to the Declaration of Helsinki.

2.2. DNA/RNA Extraction and cDNA Synthesis

Genomic DNA was extracted from blood samples using QIAamp DNA Blood Midi Kit (Qiagen, Germany), following the protocol provided by the manufacturer. All samples were stored at −20˚C until analyzed. Total RNA was extracted using the RiboPure™ Blood Kit (Ambion, USA) and RNeasy Lipid Tissue Mini Kit (Qiagen, Germany), respectively, according to the manufacturer's protocol. Total RNA was quantified using Spectrophotometer NanoDrop-2000 (NanoDrop, USA). The concentrations were adjusted and the samples were stored at −80˚C until use. The cDNA synthesis was carried out using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems™, USA), following the protocol provided by the manufacturer. All cDNA was quantified using Spectrophotometer NanoDrop - 2000 (NanoDrop, USA). The concentrations were adjusted and stored at −80˚C.

2.3. Genotyping

Genotypes for the *APOE* gene (rs429358 e rs7412) were determined by polymerase chain reaction (PCR) amplification using the primers 5'-TAA GCT TGG CAC GGC TGT CCA AGG A-3' (sense) and 5'-ACA GAA TTC GCC CCG GCC TGG TAC AC-3' (antisense) according to a previously described protocol (16). The amplified products were digested by RFLP with *Hha*I restriction enzyme (Fermentas, Canada) overnight at 37˚C, producing fragments of 91 bp, 81 bp, 72 bp, 42 bp and 33 bp [16]. Fragments were separated by electrophoresis in 3% agarose gels and visualized by ethidium bromide staining.

To determine the genotypes for the *BLMH* 1450 G > A polymorphism (rs1050565), PCR was carried out using the primers 5'-GTC GTG TTA GAG CAG GAA CCC AAT T-3' (sense) and 5'-CCT GGA TCT GTC CTT TGC AGC TAC G-3' (antisense) according to a previously described protocol [9,17]. The amplified products were digested with MunI restriction enzyme (New England Biolabs, Ipswich, MA) for 3 hours at 37˚C, producing fragments of 130 bp, 106 bp and 24 bp [9,17]. Fragments were separated by electrophoresis in 2.5% agarose gels and visualized by ethidium bromide staining.

2.4. Gene Expression Analysis

All gene expression was measured by RT-qPCR on

the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems™, USA), according to cycling conditions recommended by Applied Biosystems. We used *BLMH* (target; assay id: Hs00166071_m1), *TM*9 (endogenous control; assay id: Hs00207196_m1) and *B2M* (endogenous control; assay id: Hs99999907_m1) inventoried TaqMan® Gene Expression Assays.

The threshold values were uniformly set for all assays. All reactions were performed in duplicate. Replicates with standard deviations (SD) greater than 0.5 for the cycle threshold (CT) value were repeated or excluded from the analysis. The amplification curve of each group was created, and the CT values were obtained for all genes (*BLMH*, *TM*9 and *B2M*). Relative quantification was calculated using the comparative CT method ($2^{-\Delta\Delta CT}$).

2.5. Statistical Analyses

Descriptive analyses were performed using means and standard deviations for continuous variables and proportions (%) for dichotomous variables. A one-way analysis of variance (ANOVA) was used to determine if significant differences existed between in BLMH expression among the three groups. Genotype distribution for each gene was assessed for deviation from the Hardy-Weinberg equilibrium. Differences in genotype and allele frequencies among groups were assessed using χ2-tests. Differences were considered significant with a p value less than 0.05. All statistical analyses were performed with SPSS version 20.0.

3. RESULTS

BLMH expression, as measured using relative quantification values, was significantly different between groups. (**Table 1**) Subjects in the AD group had the lowest mean expression, followed by the EC group. Expression was highest in the YC group (see **Figure 1**).

The distribution of genotypes for the two polymorphisms did not diverge from the Hardy-Weinberg equilibrium (**Table 2**). The frequencies of the *BLMH* genotypes in the all groups were similar to those previously reported in the literature [18]. There were no significant differences in *APOE* genotypes or allele distributions between groups (**Table 2**); however, the genotype 3/3 was most frequent for all subjects (61.1%). Although the distribution was not significantly different, allele 4 was much more common in the AD group (67.4%) compared to the EC (14.0%) and YC (18.6%) groups.

4. DISCUSSION

We hypothesized that subjects with AD would demonstrate reduced BLMH expression. This hypothesis was supported by our results and corroborated previous immunohistochemical findings. Immunohistochemical analyses have found a predominantly astrocytic expression of BH with a reduction in signal intensity [10,11]. However,

Table 1. *BLMH* expression in Alzheimer disease (AD), Early Control (EC) and Young Control (YC) groups.

Groups	N°	RQ mean (±SD)	Standard Error	IC 95%
YC*	51	1.211 ± 0.372	0.052	1.107 - 1.316
EC*	57	1.108 ± 0.497	0.066	0.976 - 1.240
AD*	56	0.965 ± 0.432	0.058	0.849 - 1.080
Total	164	1.091 ± 0.448	0.035	1.022 - 1.160

Table 2. Genotype and allele frequencies for *BLMH* and *APOE* in Alzheimer disease (AD), Early Control (EC) and Young Control (YC) groups.

Gene	Genotype/Alleles	AD (n = 91/33.7%)	EC (n = 93/34.4%)	YC (n = 86/31.9%)	Total n = 270
***BLMH* 1450 G > A**	AA	44 (34.6%)	44 (34.6%)	39 (30.7%)	127 (47%)
	AG	41 (34.2%)	37 (30.8%)	42 (35%)	120 (44.4%)
	GG	6 (26.1%)	12 (52.2%)	5 (21.7%)	23 (8.5%)
APOE	2/2	0	0	1 (100%)	1 (0.4%)
	2/3	3 (11.1%)	6 (22.2%)	18 (66.7%)	27 (10%)
	2/4	3 (50.0%)	0	3 (50%)	6 (2.2%)
	3/3	38 (23.0%)	75 (45.5%)	52 (31.5%)	165 (61.1%)
	3/4	39 (62.9%)	12 (19.4%)	11 (17.7%)	62 (23%)
	4/4	8 (88.9%)	0	1 (11.1%)	9 (3.3%)
***APOE* (*alleles*)**	2	6 (17.1%)	6 (17.1%)	23 (65.7%)	(6.5%)
	3	118 (28.2%)	168 (40.1%)	133 (31.7%)	(77.6%)
	4	58 (67.4%)	12 (14.0%)	16 (18.6%)	(15.9%)

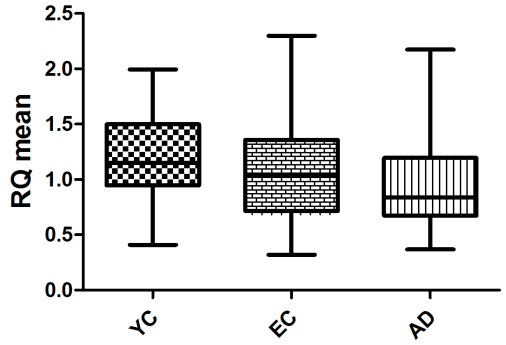

Figure 1. Box-plots representing mean, interquartile, and total range of BLMH expression for the three groups.

this reduction in signal intensity was not statistically significant, owing to the large variability among controls subjects [11]. While immunohistochemical analysis can be used to identify BH availability in specific tissues and determine regional and cellular distribution, our approach analyzed peripheral blood samples to identify systemic differences in *BLMH* expression. Our results support findings from the previous immunohistochemical studies because we also found a decrease in *BLMH* expression.

Reduced *BLMH* expression may affect the activity of the APP pathway in patients with AD. In particular, reduced expression of BLMH may alter the development of amyloid peptides Aβ1-40 and Aβ1-42, which are major components of plaques in AD brain [20,21]. However, this theory requires additional research as APP processing is complex and includes APP posttranslational modifications and the activity of at least three secretases [12,22]. Future work should evaluate the relationship between *BLMH* and *APP* expression in patients with AD to determine if lower *BLMH* expression occurs with a concomitant increase *APP* expression.

The *BLMH* 1450 G > A is localized in the C-terminal domain of the gene, which gives rise to Val443Ill isoforms and covers the active site of the enzyme [9,19]. In our analysis of the frequency of single nucleotide polymorphisms (SNP), allele 4 was most common in the AD groups, while for all subjects collectively, the 3/3 genotype was most frequent. Although different groups had different alleles that were most common, there were no statistically different distribution frequencies among the three groups in this study.

Previous studies investigating *BLMH* 1450 G > A in AD and control subjects have found that subjects with the G/G alleles and without *APOE* allele 4 had four times greater likelihood to have AD [23-25]. However, this association between *BLMH* genotypes and AD was not found by other researchers [18,26-28]. The relationship between *BLMH* expression and Val443Ill isoforms with amount of patients bigger than our study, it still is an alternative for another researchinasmuch as the 1450 G > A covers the BH's active and it can have an influence about the disease.

In conclusion, our study was the first to identify a relationship between *BLMH* expression and Alzheimer's disease using RT-qPCR. *BLMH* expression was significantly lower in subjects with the disease compared to healthy elderly adults and a healthy young sample. These findings corroborate with previous results and may offer a new direction of study to identify the underlying causes of pathological changes in individuals with AD.

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