ATP7B ALLELE FREQUENCIES DISTINCT SUBTYPES IN ALZHEIMER'S DISEASE PATIENTS

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INTRODUCTION

Meta-analyses show that serum copper non-bound-to-ceruloplasmin (Non-Cp-Cu) is higher in patients with Alzheimer's disease (AD). If Non-Cp-Cu pool becomes expanded this copper becomes toxic. This is the case of Wilson's disease, the paradigmatic disease of Non-Cp-Cu toxicosis or accumulation, when it does greatly. Moreover, a number of studies demonstrated that the size of Non-Cp-Cu pool correlates negatively with measures of cognition, cerebrospinal fluid AD markers and positively with the rate of cognition loss over time, and with the risk of conversion of patients from mild cognitive impaired status, the precursor state to AD, to full AD. *ATP7B* gene variants associate with AD, modulating the size of Non-Cp-Cu pool. However, a dedicated genetic study comparing AD patients after the stratification for a copper biomarker to demonstrate the existence of a copper subtype of AD has not yet been carried out. An independent patient sample of 287 AD patients was assessed for Non-Cp-Cu serum concentrations, **rs1801243, rs1061472, rs732774** *ATP7B* genetic variants and APOE4 genotype. Patients were stratified in two groups based on a Non-Cp-Cu cut off (1.9 µM).

Table1: Characteristics of the AD groups stratified for Non-Cp-Cu

	AD patients, High Non-Cp-Cu	AD patients, Normal Non-Cp-Cu	Significance of the comparison between groups (p)	whole AD group
Number of subjects	176 (61.3)	111 (38.7)		287
Sex [n of F] (F%)	118 (67)	76 (68.5)	p^1=0.80	194 (67.6)
Age [years] Mean (SD)	80.7 (6.9)	81.5 (6.8)	p=0.34	81.0 (6.8)
APOE4 allele frequency [%]	24.7	31.5	p^1=0.092	27.4
Carriers of at least one APOE4 allele [n] (%)	74 (42)	58 (52)		132 (46)
MMSE [score] Mean (SD)	11.9 (7.5)	11.5 (6.7)	p=0.59	11.7 (7.2)
Education [years] Mean (SD)	6.2 (3.6)	6.2 (3.4)	p=0.97	6.2 (3.5)
Non-Cp-Cu [µmol/L] Mean (SD)	2.5 (0.5)	1.6 (0.3)	**p<0.0001**	2.1 (0.6)

p indicates t-test significance, and p^1 indicates χ^2 test non-parametric test significance.

RESULTS

Main demographic and clinical characteristics of the patients participating to this study are reported in **Table 1**. One-hundred-76 AD with high Non-Cp-Cu and 111 AD patients with normal Non-Cp-Cu AD were recruited.

The two AD subgroups did not differ for age, sex, MMSE score, *APOE4* frequency allele, while they differed for Non-Cp-Cu concentrations in serum **(Table 1)**, allele, genotype and haplotype frequencies of rs1061472 A>G and rs732774 C>T after multiple testing corrections **(Table 2)**. AD patients with a GG genotype had a 1.76-fold higher risk of having a Non-Cp-Cu higher than 1.9 µmol/L (p = 0.029), and those with a TT genotype for rs732774 C>T of 1.8-fold (p = 0.018;**Table 2**). rs1801243 A>C (Exon 2), rs1061472 A>G (Exon 10), and rs732774 C>T (Exon 12) were in Linkage Disequilibrium, with a high association among them **(Figure 1)**, confirming previous reports and data reported for HapMap populations. After 100,000 permutations for multiple testing corrections, the haplotype containing the alleles AC resulted more frequent in AD patients with normal Non-Cp-Cu [43% vs. 33%; Pm = 0.03], while the haplotype containing the risk alleles GT was more frequent in the higher Non-Cp-Cu AD (66% vs. 55%; Pm= 0.01; **Table 3**). A second step of the study investigated whether the ATP7B variants under study could modulate the size of the Non-Cp-Cu pool. We classified all the AD under study in carriers or non-carriers of at least one allele of each ATP7B risk allele. The groups were compared for Non-Cp-Cu by ANOVA separately. While rs1801243 A>C (exon 2) had no effect on the size of the Non-Cp-Cu pool, carriers of at least one G in rs1061472 A>G (exon 10) and of one T in rs732774 C>T (exon 12) had increased values of Non-Cp-Cu **(Table 4).**

Figure. 1

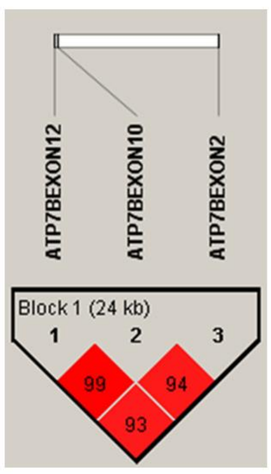

Figure 1: rs1801243 A>C (Exon 2), rs1061472 A>G (Exon 10), and rs732774 C>T (Exon 12) were in linkage disequilibrium (LD), with a high association among them.

Table 3: Frequency and Associations between Haplotypes of ATP7B and copper dyshomeostasis risk in AD

	rs1801243 (A>C)	rs1061472 (A>G)	rs732774 (C>T)	AD patients High Non-Cp-Cu	AD patients Normal Non-Cp-Cu	p value	OR (95% CI)	Pm value
ATP7B Haplotypes	C	G	T	0.53	0.45	Reference	Reference	0.07
	A	A	C	0.33	0.43	0.026	1.55 (1.06 - 2.27)	**0.03**
	A	G	T	0.12	0.09	0.71	0.90 (0.50 - 1.61)	0.61
	C	A	C	0.010	0.011	0.93	1.09 (0.18 - 6.62)	1
		G	T	0.66	0.55	Reference	Reference	**0.01**
		A	C	0.34	0.43	0.015	1.57 (1.09 - 2.24)	**0.024**
		A	T	0.006	0.018	0.13	3.75 (0.67 - 21.15)	0.26

After 100,000 permutations for multiple test corrections, four haplotypes with a frequency higher than 1% resulted from the ATP7B rs1801243 (A>C), rs1061472 (A>G) and rs732774 (C>T) in Linkage Disequilibrium considering a three SNP haplotype block analysis. When considering a two SNP haplotype block [from rs1061472 (A>G) to rs732774 (C>T)], three haplotypes resulted (gray).Pm values are obtained by 100,000 Permutation multiple (Pm) for multiple tests correction.
A p value < 0.03 is considered significant according to multiple test correction, according to Single Nucleotide Polymorphism Spectral Decomposition (SNPSpD)

CONCLUSION

High Non-Cp-Cu AD patients had increased frequencies of certain ATP7B genetic variants than Normal Non-Cp-Cu AD. This comparison for demographic, clinical, biological or genetic variables after the stratification for a specific copper biomarker identifies a subtype of disease, as recently discussed, providing an independent replication of the results of a previous study. These results sustain the existence of genotype/phenotype correlation for a copper metabolic endophenotype in AD patients. Even though copper dysfunction cannot be assumed as the determinant of the disease, its causative, rather than associated, role in AD pathology can be claimed, in terms of a risk factor of the disease, which can be counteracted by means of specific strategies.

METHODS

Subjects

287AD patients (NINCDS–ADRDA criteria) with an MMSE score ≤ 25

The study was approved by the local IRB, and all participants or legal guardians signed an informed consent.

Biochemical and molecular investigations

Non-Cp-Cu was measured with a new CE certified test [C4D test, 2012 CE certified test code n. 1211662]. The upper reference limit (95%) for the healthy population has been set to 1.90 µmol/L [its 90% confidence interval is equal to 1.78-2.06] and so this value has been taken as the cut-off for AD stratification.

SNPs genotyping

Genomic DNA was extracted from whole blood using a method based on an organic deproteinization reagent. The ATP7B and APOE SNPs were genotyped with one SNaPshot assay, according with the manufacturer's instruction with specific primers and evaluated on an ABI3100xl Genetic Analyzer.

Statistical Analysis

Student's t-test and the chi-square ($\chi 2$) test were used to compare the characteristics of High Non-Cp-Cu AD and normal Non-Cp-Cu AD groups using the package SPSS 21.0. To account for multiple testing, we used the Single Nucleotide Polymorphism Spectral Decomposition (SNPSpD) program to correct the significance threshold taking into account linkage disequilibrium (LD) between SNPs. Distribution of haplotypes was compared in normal and high Non-Cp-Cu AD groups with $\chi 2$ test in HaploView and Plink. Permutation tests were used to correct multiple testing errors with 100,000 simulations. ORs and 95% CIs were computed for each haplotype and compared to the most common haplotype with Plink. Haplotypes with frequencies greater than 1% were considered.

Table2: Allele and genotype distribution of ATP7B variants AD groups stratified for Non-Cp-Cu.

ATP7B genetic variant	AD patients, High Non-Cp-Cu (n=176)	AD patients, Normal Non-Cp-Cu (n=111)	p value ($\chi 2$)	OR (95%CI) p value
rs1801243 A>C				
Allele A n (%)	161 (45.7)	117 (52.7)	p= 0.10	
Allele C n (%)	191 (54.3)	105 (47.3)		
AA n (%)	36 (20)	27 (24)	p= 0.15	1.25 (0.71 – 2.20); p= 0.44[1]
AC n (%)	89 (51)	63 (57)		1.75 (0.98 – 3.11); p= 0.057[2]
CC n (%)	51 (29)	21 (19)		
rs1061472 A>G				
Allele A n (%)	121 (34.4)	100 (45)	**p= 0.01**	
Allele G n (%)	231 (65.6)	122 (55)		
AA n (%)	20 (11)	22 (20)	**p= 0.037**	1.93 (0.99 – 3.73); p= 0.051[1]
AG n (%)	81 (46)	56 (50)		**1.76 (1.06-2.91); p= 0.029[2]**
GG n (%)	75 (43)	33 (30)		
rs732774 C>T				
Allele C n (%)	119 (33.8)	97 (43.7)	**p= 0.017**	
Allele T n (%)	233 (66.2)	125 (56.3)		
CC n (%)	20 (11)	19 (17)	p=0.047	1.61 (0.82 – 3.18); p=0.17[1]
CT n (%)	79 (45)	59 (53)		**1.84 (1.11 - 3.44); p= 0.018[2]**
TT n (%)	77 (44)	33 (30)		

1 Dominant Model (wt/mut + mut/mut vs wt/wt). 2 Recessive model (mut/mut vs wt/mut + wt/wt).
A p value < 0.03 is considered significant according to multiple test correction, according to Single Nucleotide Polymorphism Spectral Decomposition (SNPSpD)

Table 4: ATP7B modulation of the size of the Non-Cp-Cu pool in AD patients. Carriers of the risk allele/genotype at each SNP were compared for Non-Cp-Cu levels

ATP7B genetic variant	n. of AD carriers (%)	Non-Cp-Cu (µmol/L) mean (SD)	p value (χ^2)
rs1061472 A>G			
Non-carriers of G allele	42 (14.6)	1.94 (0.46)	**F(1,285) = 5.854; p= 0.016**
At least one G allele	245 (85.4)	2.17 (0.61)	
AA genotype	42 (14.6)	1.94 (0.46)	F(2,284) = 3.022; p= 0.05
AG genotype	137 (47.7)	2.16 (0.61)	
GG genotype	108 (37.7)	2.19 (0.61)	
rs732774 C>T			
Non-carriers of T allele	39 (13.6)	1.94 (0.48)	**F(1,285) = 5.080; p= 0.025**
At least one T allele	248 (86.4)	2.17 (0.61)	
CC genotype	39 (13.6)	1.94 (0.48)	F(2,284) = 2.814; p= 0.062
CT genotype	138 (48.1)	2.15 (0.61)	
TT genotype	110 (38.3)	2.20 (0.61)	

A p value < 0.03 is considered significant according to multiple test correction, according to SNPSpD