ORIGINAL PAPER

Variation in *RTN3* and *PPIL2* Genes Does not Influence Platelet Membrane *β*-Secretase Activity or Susceptibility to Alzheimer's Disease in the Northern Irish Population

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Abstract *β*-site amyloid precursor protein cleaving enzyme (BACE1) is the rate-limiting enzyme for production of beta-amyloid peptides (A*β*), which are proposed to drive the pathological changes found in Alzheimer's disease (AD). Reticulon 3 (RTN3) is a negative modulator of BACE1 (*β*-secretase) proteolytic activity, while peptidylprolyl isomerase (cyclophilin)-like 2 (PPIL2) positively regulates BACE1 expression. The present study investigated whether there was any association between genetic variation in *RTN3* and *PPIL2,* and either risk for AD, or levels of platelet *β*-secretase activity, in a large Northern Irish case-control sample. Four hundred and sixty-nine patients with a diagnosis of probable AD (NINCDS-ADRDA criteria) and 347 control individuals (MMSE > 28/30) were genotyped. SNPs in both genes were selected by downloading genotype data from the International HapMap Project (Phase II) and tags selected using multimarker approach in Haploview, where $r^2 > 0.8$ and LOD > 3.0. Non-synonymous SNPs of interest were also included. Genotyping was performed by Sequenom iPLEX and TaqMan technologies. Alleles, genotypes and multi-marker haplotypes were tested for association with AD, and platelet *β*-secretase activities were measured for a subset of individuals ($n = 231$). Eight SNPs in *RTN3* and 7 in *PPIL2* were genotyped. We found no significant associations between allele, genotype or haplotype frequencies and risk of AD. Further, there was no effect of genotype on platelet membrane *β*-secretase activity. We conclude that common or potentially functional genetic variation in these BACE1 interacting proteins does not affect platelet membrane *β*-secretase activity or contribute to risk of AD in this population.

Keywords Alzheimer's disease · *β*-secretase · Genetic polymorphism · Haplotype · Peptidylprolyl isomerase (cyclophilin)-like 2 · Peptidyl-prolyl cis-trans isomerase-like 2 · Reticulon 3

Introduction

Several lines of evidence (reviewed by Hardy 2006) support the amyloid cascade hypothesis (Hardy and Allsop 1991), which proposes that A*β* peptides play a major role in driving the pathogenesis of Alzheimer's disease (AD). A*β* is generated by the sequential cleavage of amyloid precursor protein (APP) by *β*- and *γ*-secretases (Haass 2004). Two '*β*-secretase' proteases have been identified: *β*-site APP cleaving enzymes 1 and 2 (BACE1/memapsin 2/EC 3.4.23.46 and BACE2/memapsin 1/EC 3.4.23.45) (reviewed by Vassar 2004). Elevations in *β*-secretase activity have been found in brains of individuals with AD (Fukumoto et al. 2002; Holsinger et al. 2002; Li et al. 2004; Stockley et al. 2006; Zhao et al. 2007), in cerebrospinal fluid (Holsinger et al. 2004), and in platelets (Johnston et al. 2008). Although inhibition of *β*-secretase activity is a major therapeutic target in AD (Citron 2004), little is currently known about endogenous regulation of *β*-secretase activity. BACE1 has been reported to interact with a number of other proteins and the present study investigated the genetics of two molecules reported to influence BACE1 and *β*-secretase activity, RTN3 and PPIL2.

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Reticulons are a family of endoplasmic reticulum (ER)-associated proteins which interact directly with BACE1 (He et al. 2004). Four mammalian genes (RTN1-4) exist in various spliced isoforms containing a unique N-terminal domain and a conserved C-terminal reticulon homology domain containing 2 putative transmembrane regions (Yan et al. 2006). RTN3 is highly expressed in neurons (Moreira et al. 1999) and has been shown to modulate secretory pathway protein trafficking between the Golgi and ER (Wakana et al. 2005). RTN3 has been shown to oligomerise and accumulate in a subpopulation of dystrophic neurites in AD postmortem brain (Hu et al. 2007). He et al. (2004) identified interactions between RTN3 and BACE1 by co-immunoprecipitation in human brain, and showed that the two proteins co-localised to similar sub-cellular compartments in neurons. Over-expression of RTN3 reduced β-secretase activity and Aβ production (He et al. 2004; Murayama et al. 2006), whilst Aβ production increased when expression of RTN3 was decreased by RNA interference (He et al. 2004). This interaction occurs between C-terminal regions of both proteins (He et al. 2006). RTN3 is therefore proposed to negatively regulate β-secretase activity, by interacting with BACE1 and preventing access to APP.

Peptidylprolyl isomerase (cyclophilin)-like 2 (PPIL2, EC 5.2.1.8) is a member of the cyclophilin family of peptidylprolyl isomerases and is also known as peptidyl-prolyl cis-trans isomerase-like 2 or cyclophilin 60. It is localized in the cell nucleus where it regulates gene expression and interacts with the proteinase inhibitor eglin c (Wang et al. 1996). Reduction of PPIL2 by RNA interference in a cell-based APP processing assay resulted in reduced levels of BACE1 and Aβ, whilst over-expression of PPIL2 caused an increase in BACE1 mRNA levels (Espeseth et al. 2006). PPIL2 is therefore proposed to positively regulate BACE1 expression and β-secretase activity.

RTN3 (78.4 kb) is localized on chromosome 11q13 and contains 7 exons coding for a 236 amino acid protein (Moreira et al. 1999). PPIL2 (31.9 kb) maps to chromosome 22q11.21 and codes for a 520 amino acid protein. As far as we are aware, there are no previous association studies between RTN3 or PPIL2 and AD. Recent studies by our group showed that platelet membrane β-secretase activity is increased in AD (Johnston et al. 2008) and in mild cognitive impairment (Liu et al. 2007) but found no association between BACE1 polymorphisms and platelet membrane β-secretase activity or overall susceptibility to AD (Todd et al. 2008). The present study aimed to determine whether common genetic variation in RTN3 or PPIL2 affects platelet membrane β-secretase activity or susceptibility to AD in the Northern Irish population.

Methods

Study Subjects

All subjects were Caucasian with grandparents born in Northern Ireland. Informed written consent for the study was obtained from patients or their main carer and ethical approval was obtained from the Research Ethics Committee, Queen's University Belfast. Patients were recruited from out-patient memory clinics at Belfast City Hospital, Mater Infirmorum and Holywell Hospital. A total of 469 patients with a diagnosis of probable AD according to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (McKhann et al. 1984) were included in the study. The control group consisted of 347 age-matched individuals with MMSE scores exceeding 28/30 (Folstein et al. 1975). Control subjects with a family history of dementia were excluded from the study.

SNP Selection

Putatively functional SNPs (pfSNPs) in RTN3 and PPIL2 were identified using Ensembl (Hubbard et al. 2007). In particular, non-synonymous SNPs (nsSNPs) were considered as these affect the amino acid sequence of the protein. Tag SNPs were identified using an aggressive multi-marker tagging function available with Haploview (version 3.2) (Barrett et al. 2005). For each gene, sample-based genotypes from the CEU population (Utah residents with ancestry from northern and western Europe) were downloaded from the International HapMap Project (for RTN3, chr11:63200498-63288916; for PPIL2, chr22:20345276-20385711; data release #21, the International HapMap Consortium, 2007) into Haploview. These genomic regions included 5 kb up and downstream of the genes. Within Haploview, downloaded SNPs were screened for a minor allele frequency (MAF) > 0.05, Hardy–Weinberg equilibrium (HWE) P value > 0.001, and genotype success rate > 95%. The criteria for tag SNP selection were $r^2 > 0.8$ and LOD threshold 3.0.

Genotyping

Genomic DNA was extracted from peripheral blood leucocytes by the salting-out method (Miller et al. 1988), quantified using nanodrop spectrophotometry and diluted with sterile water to 5 ng/µl. Genotyping was performed by a combination of techniques: TaqMan assay (Applied Biosystems, Foster City, CA, USA) and Sequenom iPLEX (Sequenom, Hamburg, Germany). All plates included no template controls (NTCs) and duplicate samples. Genotyping by TaqMan was

performed on 10 ng genomic DNA using an ABI 7900 sequence detection system and genotypes were called using SDS v2.2 software. MassARRAY Typer v3.4 was used to generate genotypes from the iPLEX data. Full details of primers and reaction conditions are available from the authors.

Platelet Membrane β-Secretase Activity

Platelet membrane β-secretase activity was assayed as previously described (Johnston et al. 2008). In brief, platelets recovered from blood samples by centrifugation were homogenised, and platelet membranes prepared by ultracentrifugation. Five microgram of membrane fraction protein samples were assayed in triplicate for β-secretase activity using a fluorogenic peptide substrate (10 μM)

based on the APP wild-type sequence (Calbiochem β-secretase Substrate I) in 50 mM sodium acetate, pH 4.5 at 37°C. Initial rates were calculated over the 8–25 min linear phase, and results were expressed as pmol MCA/min/μg protein.

Data Analysis

SNP genotype frequencies were tested for deviations from HWE using a χ^2 goodness-of-fit test. Allele and genotype frequencies were compared between cases and controls using χ^2 analysis (significance level $P < 0.05$), followed by permutation adjustment ($n = 10,000$) for positive results, to correct for multiple testing bias. For a sample of this size (1,632 alleles), there is >80% power to detect a doubling of
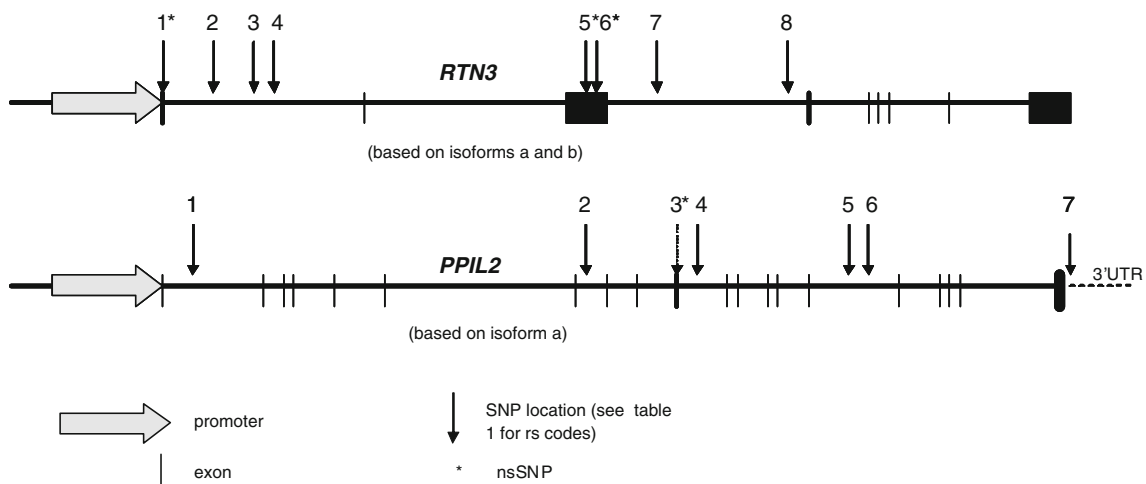

Fig. 1 Gene structures of *RTN3* and *PPIL2* and location of selected SNPs

Table 1 The exact chromosome locations, minor allele frequencies (MAF), genotyping success rates and HWE results for all SNPs genotyped

	SNP ID	Chromosome position	SNP type	Genotyping method	MAF	%Genotype success	HWE P value
RTN3							
1	rs11551941	63205698	nsSNP	Sequenom	0	99.1	1
2	rs520908	63209752	intronic	Sequenom	0.032	98.3	0.879
3	rs7943876	63211715	intronic	Sequenom	0.128	99.1	0.606
4	rs7928900	63212052	intronic	TaqMan	0.176	99.3	0.912
5	rs542998	63243962	nsSNP	Sequenom	0.208	99.5	0.769
6	rs7936660	63244051	nsSNP	Sequenom	0.006	99.9	1
7	rs10897445	63253388	intronic	TaqMan	0.307	99.4	1
8	rs17657473	63273401	intronic	Sequenom	0.084	99.8	1
PPIL2							
1	rs1103229	20346559	intronic	Sequenom	0.153	91.9	0.384
2	rs1669114	20360505	intronic	Sequenom	0.229	99.5	0.107
3	rs5749645	20363748	nsSNP	Sequenom	0.002	100	0.004
4	rs396913	20364417	intronic	TaqMan	0.289	99.1	0
5	rs5754659	20370665	intronic	TaqMan	0.084	99	0.013
6	rs12170039	20371341	intronic	Sequenom	0.394	99.4	1
7	rs1045681	20377368	3′UTR	Sequenom	0.414	99.5	1

the risk allele frequency, from 5% in controls to 10% in cases. Multi-marker haplotypes as indicated in the Haploview tag SNP selection process were tested for association with AD. In addition, haplotypes were also defined according to linkage disequilibrium (LD) between paired SNPs using Gabriel's method (Gabriel et al. 2002), and association tests on haplotype frequencies carried out. One-way analysis of variance (ANOVA) was used to determine any effect of *RTN3* or *PPIL2* genotypes on platelet membrane β-secretase activity.

Results

A total of 469 AD patients (64.9% female, mean age 77.9 years (SD 7.25)) and 347 control subjects (67.2% female, mean age 74.7 years (SD 8.99)) were included in the study.

According to dbSNP build 129, 472 SNPs have been identified within *RTN3* (accessed 29/04/09). Of 80 SNPs downloaded from HapMap into Haploview, 25 met the criteria for MAF, HWE and genotype success rate. Eight

Table 2 Genotype and allele frequencies for individual *RTN3* and *PPIL2* SNPs in the control and AD groups

SNP	Genotype	AD n (%)	Controls n (%)	P value	Allele	AD n (%)	Controls n (%)	P value
RTN3								
rs520908	CC	423 (92)	327 (96)	0.14	C	882 (96)	669 (98)	0.05
	CT	36 (8)	15 (4)		T	36 (4)	15 (2)	
	TT	0 (0)	0 (0)					
rs7943876	AA	354 (76)	258 (75)	0.89	A	813 (87)	596 (87)	0.95
	AG	105 (23)	80 (23)		G	119 (13)	88 (13)	
	GG	7 (1)	4 (1)					
rs7928900	CC	16 (3)	8 (2)	0.64	C	168 (18)	117 (17)	0.54
	CG	136 (29)	101 (29)		G	760 (82)	573 (83)	
	GG	312 (67)	236 (68)					
rs542998	CC	284 (61)	223 (65)	0.45	C	732 (78)	553 (83)	0.23
	CT	164 (35)	107 (31)		T	204 (22)	133 (13)	
	TT	20 (4)	13 (4)					
rs7936660	CC	0 (0)	0 (0)	0.86	C	6 (1)	3 (0.4)	0.57
	CG	6 (1)	3 (1)		G	930 (99)	689 (99.6)	
	GG	462 (99)	343 (99)					
rs10897445	CC	47 (10)	29 (8)	0.32	C	283 (30)	215 (31)	0.7
	CT	189 (41)	157 (46)		T	649 (70)	473 (69)	
	TT	230 (49)	158 (46)					
rs17657473	CC	6 (1)	0 (0)	0.05	C	77 (8)	59 (9)	0.83
	CT	65 (14)	59 (17)		T	857 (92)	633 (91)	
	TT	396 (85)	287 (83)					
PPIL2								
rs1103229	CC	306 (75)	235 (69)	0.21	C	707 (86)	562 (83)	0.07
	CT	95 (23)	92 (27)		T	113 (14)	116 (17)	
	TT	9 (2)	12 (4)					
rs1669114	AA	27 (6)	24 (7)	0.7	A	206 (22)	165 (24)	0.39
	AG	152 (33)	117 (34)		G	726 (78)	525 (76)	
	GG	287 (62)	204 (59)					
rs5754659	AA	5 (1)	7 (2)	0.53	A	75 (8)	60 (9)	0.7
	AG	65 (14)	46 (13)		G	847 (92)	632 (91)	
	GG	391 (85)	293 (85)					
rs12170039	AA	74 (16)	52 (15)	0.8	A	373 (40)	266 (39)	0.53
	AG	225 (48)	162 (47)		G	557 (60)	424 (61)	
	GG	166 (36)	131 (38)					
rs1045681	CC	82 (18)	57 (17)	0.88	C	391 (42)	281 (41)	0.62
	CT	227 (49)	167 (48)		T	541 (58)	409 (59)	
	TT	157 (34)	121 (35)					

tag SNPs (mean r^2 of 1.0) in 8 tests were selected to capture 100% of alleles with $r^2 > 0.8$. Three of these were nsSNPs and the remaining 5 were intronic, as shown in Fig. 1.

Three hundred and fifty-seven SNPs have been identified within *PPIL2*. Genotype data from 104 SNPs in the HapMap CEU population were downloaded into Haploview. Of these, 30 met the criteria for tag SNP selection. Six tag SNPs (mean r^2 of 0.976) in 9 tests were selected to capture 100% of alleles with $r^2 > 0.8$. As shown in Fig. 1, five of the tag SNPs were intronic and one was located in the 3′ untranslated region (UTR) of *PPIL2*. These tag SNPs and the nsSNP rs5749645 were genotyped in this study.

Table 1 gives exact chromosome locations, minor allele frequencies (MAF), % genotyping success rates and Hardy–Weinberg equilibrium results (cut-off $P > 0.001$) for all SNPs genotyped in the study. The mean concordance rate for the SNPs genotyped by Sequenom iPLEX was 99%, based on repeat analysis of 350 samples. One *PPIL2* SNP (rs396913), genotyped using Taqman, only generated two genotype clusters. Sequencing of a sub-set of the products revealed that this was due to an inability to differentiate between the heterozygote cluster and one of the homozygote clusters. SNaPshot primers were designed for this variant, but again the heterozygotes were difficult to distinguish and were not called. *RTN3* SNP rs11551941 was monomorphic in our population. One intronic SNP was observed with MAF of 3.2% (originally reported in CEU population at 6.7 and 5.2%). Table 2 shows the results of single marker association tests. The *RTN3* SNPs, rs520908 and rs17657473, were on the threshold of significance for allelic and genotypic association respectively ($P = 0.05$). However, these associations were not consistent between comparisons of genotype and allele frequencies, and did not withstand correction for multiple testing bias. There were no significant associations between any of the *PPIL2* polymorphisms and AD.

Table 3 Haplotype frequencies for *RTN3* and *PPIL2* in the AD and control groups

	Freq.	Case, control ratios	χ^2	P value
RTN3 haplotype				
Block 1				
CT	0.486	448.5:487.5, 342.5:349.5	0.392	0.5314
CC	0.306	283.5:652.5, 215.0:477.0	0.117	0.7322
TT	0.207	203.3:732.7, 133.0:559.0	1.519	0.2177
PPIL2 haplotype				
Block 1				
CG	0.769	724.4: 209.6, 525.6:166.4	0.57	0.4503
TA	0.154	132.1:801.9, 118.2:573.8	2.64	0.1042
CA	0.074	74.1:859.9, 47.0:645.0	0.758	0.3839

Haploview was used to determine pairwise linkage disequilibrium (D′ > 0.8) between SNPs and haplotype blocks defined. As shown in Fig. 2, blocks were generated in both *RTN3* and *PPIL2*, but there were no significant differences in haplotype frequencies between cases and controls (Table 3). Multi-marker analyses were also performed in accordance with the selection of tagSNPs; there were no significant differences observed (rs12170039 and rs1669114, results not shown).

Platelet membrane β-secretase activity was measured for a subset of 231 subjects (118 cases and 113 controls). For each SNP, a one-way ANOVA was used to compare mean β-secretase activities between genotype groups. The analysis was performed on the whole subset and for cases and controls separately. As shown in Table 4, there were no significant effects of genotype on β-secretase activity in any group.

Discussion

We have carried out a robust genetic study to assess the contribution of common genetic variants and pfSNPs in

Fig. 2 Haplotype blocks in **a** *RTN3* and **b** *PPIL2*. Haplotype blocks were defined by Gabriel's method where dark grey shading indicates strong evidence of LD, light grey shading indicates uninformative and white indicates strong evidence of recombination
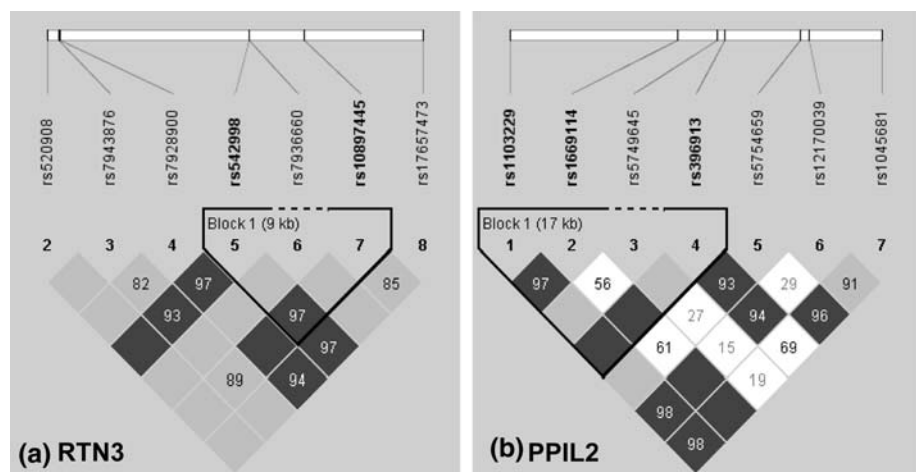
Table 4 Mean platelet membrane β-secretase activity expressed as pmol MCA/min/μg protein (±SD) for each genotype analysed in a subset of control and AD individuals

SNP ID		AD and control combined			AD			Control		
RTN3										
rs520908	Genotype (*n*)	CC (212)	CT (16)	TT (0)	CC (106)	CT (11)	TT (0)	CC (106)	CT (5)	TT (0)
	Mean (SD)	0.17 (0.05)	0.18 (0.05)		0.18 (0.06)	0.19 (0.05)		0.15 (0.05)	0.17 (0.03)	
	P value	0.37			0.85			0.5		
rs7943876	Genotype (*n*)	AA (180)	AG (46)	GG (0)	AA (95)	AG (21)	GG (0)	AA (85)	AG (25)	GG (0)
	Mean (SD)	0.17 (0.05)	0.17 (0.05)		0.19 (0.06)	0.17 (0.05)		0.15 (0.05)	0.16 (0.04)	
	P value	0.49			0.15			0.22		
rs7928900	Genotype (*n*)	CC (4)	CG (76)	GG (149)	CC (3)	CG (38)	GG (76)	CC (1)	CG (38)	GG (73)
	Mean (SD)	0.19 (0.02)	0.17 (0.05)	0.17 (0.05)	0.18 (0.03)	0.18 (0.06)	0.18 (0.06)	0.2	0.15 (0.04)	0.15 (0.05)
	P value	0.83			0.99			0.59		
rs542998	Genotype (*n*)	CC (136)	CT (90)	TT (5)	CC (66)	CT (49)	TT (3)	CC (70)	CT (41)	TT (2)
	Mean (SD)	0.17 (0.06)	0.17 (0.05)	0.2 (0.06)	0.18 (0.06)	0.18 (0.05)	0.22 (0.06)	0.15 (0.05)	0.16 (0.04)	0.17 (0.05)
	P value	0.52			0.58			0.87		
rs7936660	Genotype (*n*)	CC (0)	CG (3)	GG (227)	CC (0)	CG (1)	GG (116)	CC (0)	CG (2)	GG (111)
	Mean (SD)		0.18 (0.06)	0.17 (0.05)		0.25	0.18 (0.06)		0.14 (0.01)	0.15 (0.05)
	P value	0.82			0.24			0.66		
rs10897445	Genotype (*n*)	CC (20)	CT (106)	TT (103)	CC (10)	CT (51)	TT (56)	CC (10)	CT (55)	TT (47)
	Mean (SD)	0.16 (0.06)	0.17 (0.05)	0.17 (0.05)	0.19 (0.05)	0.18 (0.05)	0.18 (0.06)	0.12 (0.04)	0.16 (0.05)	0.16 (0.04)
	P value	0.49			0.88			0.06		
rs17657473	Genotype (*n*)	CC (2)	CT (37)	TT (192)	CC (2)	CT (14)	TT (102)	CC (0)	CT (23)	TT (90)
	Mean (SD)	0.19 (0.04)	0.16 (0.05)	0.17 (0.05)	0.19 (0.04)	0.18 (0.07)	0.18 (0.06)		0.15 (0.04)	0.15 (0.05)
	P value	0.72			0.96			0.94		
PPIL2										
rs1103229	Genotype (*n*)	CC (163)	CT (58)	TT (6)	CC (83)	CT (29)	TT (4)	CC (80)	CT (29)	TT (2)
rs1669114	Mean (SD)	0.17 (0.05)	0.16 (0.05)	0.16 (0.06)	0.19 (0.06)	0.17 (0.04)	0.16 (0.06)	0.15 (0.04)	0.15 (0.06)	0.16 (0.07)
	P value	0.46			0.29			0.94		
	Genotype (*n*)	AA (12)	AG (87)	GG (131)	AA (7)	AG (47)	GG (64)	AA (5)	AG (40)	GG (67)
	Mean (SD)	0.18 (0.05)	0.17 (0.05)	0.17 (0.05)	0.17 (0.06)	0.18 (0.05)	0.19 (0.06)	0.19 (0.05)	0.15 (0.06)	0.15 (0.04)
	P value	0.6			0.53			0.22		
rs5754659	Genotype (*n*)	AA (4)	AG (42)	GG (184)	AA (1)	AG (23)	GG (93)	AA (3)	AG (19)	GG (91)
	Mean (SD)	0.2 (0.04)	0.17 (0.05)	0.17 (0.05)	0.15 (0.05)	0.19 (0.06)	0.18 (0.06)	0.22 (0.04)	0.16 (0.04)	0.15 (0.05)
	P value	0.43			0.78			0.06		
rs12170039	Genotype (*n*)	AA (32)	AG (124)	GG (75)	AA (16)	AG (62)	GG (39)	AA (16)	AG (62)	GG (35)
	Mean (SD)	0.17 (0.06)	0.17 (0.05)	0.16 (0.05)	0.18 (0.06)	0.19 (0.05)	0.17 (0.06)	0.15 (0.04)	0.16 (0.05)	0.16 (0.05)
	P value	0.42			0.19			0.89		
rs1045681	Genotype (*n*)	CC (36)	CT (121)	TT (74)	CC (17)	CT (60)	TT (41)	CC (19)	CT (61)	TT (33)
	Mean (SD)	0.17 (0.05)	0.17 (0.05)	0.17 (0.05)	0.18 (0.06)	0.19 (0.05)	0.17 (0.06)	0.15 (0.04)	0.15 (0.05)	0.16 (0.05)
	P value	0.66			0.28			0.79		

RTN3 and *PPIL2* to the risk of AD. *RTN3* and *PPIL2* are strong biological candidate genes for AD because they code for proteins which have been reported to modulate β-secretase activity, the rate-limiting step for production of Aβ peptides from APP. The RTN3–BACE1 interaction has been shown to decrease β-secretase activity and Aβ production (He et al. 2004), whilst PPIL2 can positively regulate BACE1 expression and β-secretase activity/Aβ production (Espeseth et al. 2006).

Tag SNPs were selected in both genes using data from the International HapMap Project by an aggressive multi-marker tagging method. Strict criteria for MAF, HWE and genotyping success rate were applied to this selection process. This generated a list of SNPs and multi-marker

haplotypes which captured all variants of interest and greatly increased the efficiency of the study. In addition, 3 nsSNPs in *RTN3* and one in *PPIL2* were included. Recruited individuals were restricted to those whose grandparents had been born in Northern Ireland to minimise population heterogeneity. Cases and controls were reliably phenotyped by a clinician using NINCDS-ADRDA criteria and MMSE scores. Significance values were corrected for multiple testing bias using the permutation procedure in Haploview.

Following correction for multiple testing, there were no significant associations between single SNPs or haplotypes in *RTN3* or *PPIL2* and risk for AD, or platelet β-secretase activity, in this population. This is the first genetic study of *RTN3* or *PPIL2* in AD. The SNPs genotyped in this case-control collection have effectively examined sequence variation across these two genes. As tag SNPs were selected from the International HapMap Project, it is possible that additional common variants not genotyped in the CEU population may be involved in genetic predisposition to AD. The selection of all the SNPs included was dependent on data submitted to public repositories and resequencing of *RTN3* and *PPIL2* would be required to exhaustively assess all variants in these genes.

We also investigated whether common genetic variation in *RTN3* or *PPIL2* influenced platelet β-secretase activity. Our data did not demonstrate a significant genetic contribution of *RTN3* or *PPIL2* to platelet β-secretase activity, whether considered across all samples or specifically within the AD or control groups. RTN3 is present in platelets, as it has been detected in studies of the platelet proteome (PlateletWeb Knowledgebase; http://plateletweb.bioapps.bio zentrum.uni-wuerzburg.de). The rationale for studying PPIL2 in this context is less strong, as there is no proteomic evidence to date that PPIL2 is present in platelets. In addition, it is predominantly located in the cell nucleus (which platelets lack) and the proposed interactions between BACE1 and PPIL2 at gene transcription level (Espeseth et al. 2006) may therefore be absent in platelets. However, it should be noted that platelet proteome studies have not identified BACE1 either (PlateletWeb), even though several independent groups have detected both BACE1 protein and activity in platelets directly (Colciaghi et al. 2004; Johnston et al. 2008; Tang et al. 2006). In addition, a range of other proteins associated with nuclear functions (for example, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, NFκB1; and oestrogen receptor 2, ER2) have been identified in the platelet proteome (PlateletWeb). We have therefore included the data analysis for PPIL2 SNPs and platelet membrane β-secretase activity, with the caveat that it is currently unknown whether the BACE1-PPIL2 interaction is physiologically relevant in this cell type.

In summary, our results suggest that common genetic variation in the BACE1-interacting proteins, RTN3 and PPIL2, does not influence platelet β-secretase activity or susceptibility to AD in this population.

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