

E-LETTER

CYP19 haplotypes increase risk for Alzheimer's disease

R Huang, S E Poduslo

J Med Genet 2006;000:1-7. doi: 10.1136/jmg.2005.039461

Cytochrome P450 aromatase, an enzyme that catalyses the conversion of androgens to oestrogen, is expressed at high levels in the gonads and in the brain. Aromatase activity is increased in the nucleus basalis of Meynert during aging and in Alzheimer's disease (AD), making the gene (*CYP19*), at 15q21.1, a potential candidate risk factor. We examined 18 single nucleotide polymorphisms spanning the 5'-untranslated region and the entire coding region of *CYP19* in 227 patients with AD and 131 control spouses. We found that the gene region could be divided into two haplotype blocks; a haplotype in block 1 and a haplotype in block 2 increased the risk of developing the disease by twofold in *APOE 4* carriers. The implication of two haplotypes conferring increased risk for AD warrants further investigation of the regulation of aromatase activity in brain.

Alzheimer's disease (AD) is a complex neurodegenerative disorder characterised by severe cognitive impairment. *APOE 4* is an established risk factor for late onset AD. Other genetic variants (www.alzgene.org) have been implicated; however, successful replication has been difficult owing to the potential multiple genetic interactions that may exist for this disease. Recently a particular haplotype in the gene for cytochrome P450 aromatase has been suggested to increase risk for the disease by 60%.¹

Cytochrome P450 aromatase is an enzyme that catalyses the conversion of androgens, such as testosterone, to oestrogens, which act as sex steroid hormones but also function during growth and differentiation. There are high levels of expression in both the gonads and the brain. The gene (*CYP19a1*) is localised on chromosome 15q21.2 and spans 123 kb. There is a large 5' flanking regulatory region of 93 kb with at least nine tissue specific promoters. Each tissue expresses a unique first exon or 5' untranslated region (UTR), which splices into a common splice acceptor site (AG/GACT) in exon II. The coding region and the translated protein product are the same in all tissues.² While there is a brain specific promoter (I.f), other promoters may also regulate expression in the brain in a region specific manner.³

We investigated 18 SNPs covering the major portion of the gene. Two haplotype blocks were defined, based on our genotype data; one block resided solely in the 5'-UTR while the second block covered a small section of the 5'-UTR and the coding region. We found that two haplotypes, one in each block, increased the risk of developing the disease by twofold in *APOE 4* carriers. Interestingly, haplotype 1 in block 1 was protective in *APOE 4* carriers.

METHODS

Subjects

There were 227 patients with AD (primarily white; 157 women and 70 men; mean (SD) age of onset, 71.1 (8.0) years, range, 50-92 years; 181 patients with age of onset >64 years) and 131 control spouse subjects (white; 77

women and 54 men; average age at enrolment, 72.0 (8.1) years, range 50-88 years). The clinical diagnosis of probable AD was made according to NINCDS-ADRDA criteria,⁴ after a review of the medical records to verify a documented progressive decline in cognition and appropriate blood work to rule out other medical conditions, including thyroid and vitamin B12 deficiencies. In addition, we included a computed tomography and/or magnetic resonance imaging scan of the brain, which showed cortical atrophy but no evidence of strokes or tumours. The patients were of European descent. As a group, spouses had a similar age, ethnic background, and environment, which controlled for unmeasured risk factors in addition to age and race. All participants or those who were the authorised representatives for the patients gave consent for the study, in accordance with institutional review board guidelines.

SNP selection

The HapMap project had genotyped 41 single nucleotide polymorphisms (SNPs) on *CYP19a1* (as of September 2004; www.hapmap.org) which spanned the complete 5'-UTR through exon 9, but not exon 10 through the 3'-UTR (fig 1). Two haplotype blocks and nine tagSNPs were identified, using a haplotype based method.⁵ Block 1 extended from I.4 to 5' of I.2 and had three tagSNPs (fig1). Block 2 extended from 5' of I.6 through exon 9 and had six tagSNPs. Thus, block 1 covered most of the 5'-UTR while block 2 covered the 3' part of the 5'-UTR and the coding region. We genotyped 18 SNPs spanning the gene, with a focus on the block 2 coding region. Block 1 (table 1) consisted of the 3 tagSNPs (SNP 2.2, rs7181886; SNP 3.8, rs6493494; and SNP 4.1, rs2008691) and one additional SNP used in the previous study (SNP 4, rs1008805).¹ Block 2 had the six tagSNPs (SNP 4.8, rs1062033; SNP 5, rs767199; SNP 6, rs727479; SNP 6.5, rs700518; SNP 6.8, rs9944225; SNP 7.5, rs2899472), plus the three SNPs genotyped in the previous study (SNP 7, rs1065778; SNP 8, rs1143704; SNP 9, rs10046).¹ In addition, there were seven SNPs in the exon area posted on the NCBI website (www.ncbi.nlm.nih.gov; as of September 2004); four were non-synonymous and three were synonymous. We genotyped the four non-synonymous SNPs (SNP 5.8, rs2236722; SNP 7.1, rs1803154; SNP 8.3, rs700519; SNP 8.8, rs2304462) and two synonymous SNPs (SNP 6.5, rs700518 and SNP 8.8, rs2304461). The one synonymous SNP that we did not genotype overlapped with the two non-synonymous SNPs (rs2304462 and rs700519). We designed 17 pairs of primers for the 18 SNPs, with two of the SNPs (rs2304462 and rs700519) sharing one pair of primers because of the short distance between them. The primer sequences are listed in table 1.

Abbreviations: AD, Alzheimer's disease; EM, expectation maximisation; LD, linkage disequilibrium; SBE, single base extension; SNP, single nucleotide polymorphism; UTR, untranslated region

Genotyping

APOE was genotyped as described previously.⁶ SNPs were genotyped using fluorescent detected single base extension.⁷ The 17 pairs of primers were divided into three groups, based on the efficiency of multiplex PCR. The first group had five pairs of primers, while the second and third groups each had six pairs of primers. Multiplex PCR was performed for each group of primers to obtain products of 100–500 bp (60 ng of genomic DNA, 0.6–4.8 pmol of each primer in 10 µl reaction volume containing 1 µl 10× reaction buffer, 3.3 nmol dNTP, and 0.7 U *Taq* polymerase (Qiagen)). The PCR program was 94°C for 5 minutes, then 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 45 seconds, and a final extension of 72°C for 10 minutes. The PCR products from the second and third multiplex reactions were pooled. Shrimp alkaline phosphatase/*ExoI* enzymatic treatment followed, with incubation at 37°C for 75 minutes with 1 U of *ExoI* (New England Biolabs) and 2.5 U of shrimp alkaline phosphatase (Amersham Pharmacia Biotech). Single base extension (SBE) reaction was then performed (SNaPshot Multiplex kit; Applied Biosystems) for the first group and the pooled second and third groups separately. The SBE primers were ~20 nucleotides in length. Primers that ended one nucleotide 5'- of the SNPs were selected; ATCG tails of different sizes were added to the 5'- end of the primers to make their length vary by 7–8 nucleotides from each other (table 1). The 5 µl SBE reaction consisted of 2.5 µl of reaction mix, 1.5 µl of the treated PCR product from the previous step and 0.2 µmol/l of each SBE primer. The reaction program was 38 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C

for 30 seconds. Shrimp alkaline phosphatase (0.5 U) treatment was carried out at 37°C for 75 minutes, followed by inactivation at 75°C for 15 minutes. The SBE products from the first group of SNPs were separated on one gel, and those for the pooled second and third groups were separated on another gel. For the separation, 1 µl aliquots of the samples were denatured at 95°C for 5 minutes in 1.5 µl of loading buffer containing the Liz 120 size standard (Applied Biosystems) and separated on 5% Long RangerJ 6 mol/l urea polyacrylamide gels, using the ABI Prism 377 sequencer system (Applied Biosystems). Each sample was run at least twice to minimise genotyping error, and each batch of reactions had both positive and negative controls. There were no missing data. Samples were analysed using GeneScan (version 3.7) and Genotyper (version 3.7) software (Applied Biosystems).

Statistical analysis

Hardy-Weinberg equilibrium tests were performed separately for patient and control groups for each SNP with HelixTree (version 4.1.0); no significant deviations were observed. Single locus analyses were performed with SAS 9.1. Age, sex, and *APOE* adjusted P values for the single locus analysis were obtained using logistic regression. Pairwise linkage disequilibrium (LD) (D') for the SNPs was evaluated with HelixTree, using all of the subjects. Haplotype blocks were then defined, based on the pairwise D' values. Haplotypes were inferred for each individual for each block by use of the expectation maximisation (EM) algorithm, implemented in HelixTree. The haplotype data for the individuals with EM probabilities

Table 2 Allele distribution in all the subjects and in *APOE 4* carriers and non-carriers

Allele	All subjects				<i>APOE 4</i> carriers			<i>APOE 4</i> non-carriers		
	Controls*	AD patients*	p		Controls*	AD patients*	p	Controls*	AD patients*	p
2.2	C	13 (4.96)	25 (5.51)	0.6088	5 (7.58)	14 (4.43)	0.2930	8 (4.08)	11 (7.97)	0.1429
	G	249 (95.04)	429 (94.49)		61 (92.42)	302 (95.57)		188 (95.92)	127 (92.03)	
3.8	C	142 (54.20)	270 (59.47)	0.0698	31 (46.97)	186 (58.86)	0.0807	111 (56.63)	84 (60.87)	0.3432
	T	120 (45.80)	184 (40.53)		35 (53.03)	130 (41.14)		85 (43.37)	54 (39.13)	
4	C	99 (37.79)	197 (43.39)	0.2600	20 (30.30)	146 (46.20)	0.0201	79 (40.31)	51 (36.96)	0.6871
	T	163 (62.21)	257 (56.61)		46 (69.70)	170 (53.80)		117 (59.69)	87 (63.04)	
4.1	C	41 (15.65)	75 (16.52)	0.1543	9 (13.64)	41 (12.97)	0.9045	32 (16.33)	34 (24.64)	0.0800
	T	221 (84.35)	379 (83.48)		57 (86.36)	275 (87.03)		164 (83.67)	104 (75.36)	
4.8	C	125 (47.71)	199 (43.83)	0.2200	35 (53.03)	139 (43.99)	0.1856	90 (45.92)	60 (43.48)	0.6013
	G	137 (52.29)	255 (56.17)		31 (46.97)	177 (56.01)		106 (54.08)	78 (56.52)	
5	C	127 (48.47)	240 (52.86)	0.2728	30 (45.45)	168 (53.16)	0.2587	97 (49.49)	72 (52.17)	0.6653
	T	135 (51.53)	214 (47.14)		36 (54.55)	148 (46.84)		99 (50.51)	66 (47.83)	
5.8	T	262 (100)	454 (100)	N/A		N/A			N/A	
	C	0	0							
6	G	87 (33.21)	168 (37.0)	0.1581	15 (22.73)	116 (36.71)	0.0286	72 (36.73)	52 (37.68)	0.9700
	T	175 (66.79)	286 (63.0)		51 (77.27)	200 (63.29)		124 (63.27)	86 (62.32)	
6.5	A	124 (47.33)	239 (52.64)	0.0942	26 (39.39)	166 (52.53)	0.0551	98 (50.00)	73 (52.90)	0.5488
	G	138 (52.67)	215 (47.36)		40 (60.61)	150 (47.47)		98 (50.00)	65 (47.10)	
6.8	G	240 (91.60)	412 (90.75)	0.7958	59 (89.39)	282 (89.24)	0.9708	181 (92.35)	130 (94.20)	0.7607
	T	22 (8.40)	42 (9.25)		7 (10.61)	34 (10.76)		15 (7.65)	8 (5.80)	
7	A	123 (46.95)	240 (52.86)	0.0677	26 (39.39)	166 (52.53)	0.0546	97 (49.49)	74 (53.62)	0.4413
	G	139 (53.05)	214 (47.14)		40 (60.61)	150 (47.47)		99 (50.51)	64 (46.38)	
7.1	A	262 (100)	454 (100)	N/A		N/A			N/A	
	T	0	0							
7.5	G	189 (72.14)	361 (79.52)	0.0115	43 (65.15)	251 (79.43)	0.0126	146 (74.49)	110 (79.71)	0.1806
	T	73 (27.86)	93 (20.48)		23 (34.85)	65 (20.57)		50 (25.51)	28 (20.29)	
8	A	118 (45.04)	235 (51.76)	0.0549	25 (37.88)	164 (51.90)	0.0400	93 (47.45)	71 (51.45)	0.4157
	T	144 (54.96)	219 (49.24)		41 (62.12)	152 (48.10)		103 (52.55)	67 (48.55)	
8.3	C	252 (96.18)	431 (94.93)	0.2043	63 (95.45)	304 (96.20)	0.7921	189 (96.43)	127 (92.03)	0.0759
	T	10 (3.82)	23 (5.07)		3 (4.55)	12 (3.80)		7 (3.57)	11 (7.97)	
8.4	G	262 (100)	454 (100)	N/A		N/A			N/A	
	A	0	0							
8.8	C	262 (100)	454 (100)	N/A		N/A			N/A	
	T	0	0							
9	C	119 (45.42)	231 (50.88)	0.0890	25 (37.88)	161 (50.95)	0.0553	94 (47.96)	70 (50.72)	0.5197
	T	143 (54.58)	223 (49.12)		41 (62.12)	155 (49.05)		102 (52.04)	68 (49.28)	
e4-	e4-	228 (87.02)	257 (56.61)	<0.0001		N/A			N/A	
	e4+	34 (12.98)	197 (43.39)							

*n (%). N/A, not available.

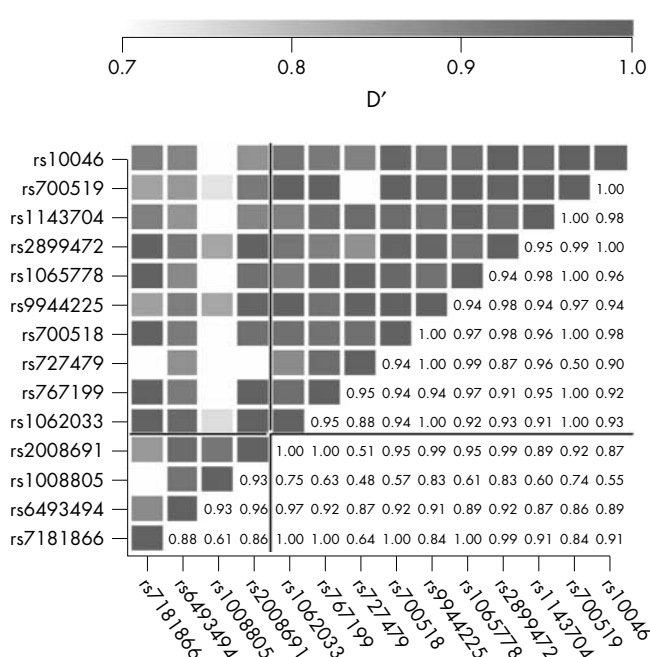


Figure 2 Linkage disequilibrium. Numbers in the figure are D' . The strength of the linkage disequilibrium is shown by the increasing darkness of shading for higher D' values.

greater than 90% were exported to SAS for logistic regression analysis to determine the risk associated with particular haplotypes. Each haplotype was compared with the combination of the other haplotypes.

RESULTS

Allelic frequencies

APOE 4 was significantly increased in Alzheimer's patients, as previously reported ($p < 0.0001$, table 2). Among the 18 SNPs that were genotyped for *CYP19a1*, four (SNP 5.8, rs2236722; SNP 7.1, rs1803154; SNP 8.4, rs2304462; SNP 8.8, rs2304461) exhibited no heterozygosity and were excluded from further analysis. The SNP 7.5 (rs2899472) had the most significant allelic frequency associated with the disease (T allele, $p = 0.0115$) (table 2), after adjustment for age, sex, and *APOE* status. The two flanking SNPs, SNP 7 (rs1065778) (G allele, $p = 0.0677$; odds ratio (OR) 1.67; 95% confidence interval (CI) 1.12 to 2.49) and SNP 8 (rs1143704) (T allele, $p = 0.0549$) approached significance in frequency associated with the disease, suggesting a high risk haplotype with the disease.

We then divided the sample into two groups, based on the *APOE 4* genotype. The odds ratio of the SNP 7.5 T allele was increased from 1.67 to 2.08 ($p = 0.0126$; 95% CI 1.17 to 3.70) in *APOE 4* carriers. SNPs 7 and 8 also exhibited greater

significance in *APOE 4* carriers ($p = 0.0546$ and $p = 0.04$, respectively). Two additional SNPs (SNP 4, rs1008805 and SNP 6, rs727479) were also significant ($p = 0.0201$ and $p = 0.0286$, respectively) in *APOE 4* carriers. None of the SNPs were significant in *APOE 4* non-carriers.

Linkage disequilibrium and haplotype blocks

LD across the gene was examined for the 14 markers and all of the subjects. The markers were all in LD with most of the other SNPs with a $D' > 0.85$; the exception was rs1008805, which was only in LD with the two adjacent markers (fig 2). "Strong" LD was defined as having a pairwise $D' \geq 0.85$.⁵ The haplotype block was defined as having strong LD pairs ≥ 0.9 . The boundary of the two blocks was between SNP 4.1 (rs2008691) and 4.8 (rs1062033). The SNPs in block 1 were in LD as were the SNPs in block 2. Thus, the linkage disequilibrium extended over 37.4 kb, which covered the entire coding region in block 2. The linkage disequilibrium further supports the high risk haplotype.

Haplotypes

Block 1 had three major haplotypes with frequencies $> 5\%$, whereas block 2 had four major haplotypes (tables 3 and 4). Logistic regression, accounting for age, sex, and *APOE* status, was performed, using subjects with EM probability of haplotype estimation $> 90\%$. The H1 haplotype (GTTT) in block 1 ($p = 0.0567$) and the H2 haplotype in block 2 (CTTGGTTCT) ($p = 0.0856$) approached significance, after adjustment for age, sex, and *APOE* status. However, when the samples were stratified according to *APOE 4* status, the H2 haplotype in block 1 in *APOE 4* carriers had an OR of 1.927 ($p = 0.0262$; 95% CI 1.081 to 3.436). Moreover, the H1 haplotype in block 2 (GCGAGAGACC) in *APOE 4* carriers had an OR of 1.949 ($p = 0.0473$; 95% CI 1.008 to 3.768). Interestingly, the H1 haplotype in block 1 (GTTT) in *APOE 4* carriers seemed to be protective with an odds ratio of 0.579 ($p = 0.0452$; 95% CI = 0.34 to 0.988). No haplotypes in either block were significant for *APOE 4* noncarriers. There was no detectable interaction with age or sex between the haplotypes. Thus, risk associated with these haplotypes is independent of age and sex, but is dependent upon *APOE 4* status.

DISCUSSION

Cytochrome P450 aromatase activity has been localised to neurons and glia in the basal forebrain and hypothalamic nuclei.³ While aromatase activity was increased in the nucleus basalis of Meynert during ageing and during the AD process, it was decreased in the hypothalamic nuclei in AD.³ Interestingly, there was no sex difference in immunostaining. In our studies, we found no sex effect on the risk for the disease associated with the aromatase haplotypes.

Table 3 Haplotype association: block 1: 2.2, 3.8, 4, 4.1

	All subjects				<i>APOE 4</i> carriers				<i>APOE 4</i> non-carriers			
	H1	H2	H3	Others	H1	H2	H3	Others	H1	H2	H3	Others
Controls	116 (44.97)	95 (36.82)	28 (10.85)	19 (7.36)	35 (53.02)	19 (28.79)	5 (7.58)	7 (10.61)	81 (42.19)	76 (39.58)	23 (11.98)	12 (6.25)
Patients	176 (39.11)	187 (41.56)	50 (11.11)	37 (8.22)	124 (39.49)	138 (43.95)	27 (8.60)	25 (7.96)	52 (38.24)	49 (36.03)	23 (16.91)	12 (8.82)
	H1 v all the others OR=0.71 95% CI 0.50 to 1.01 $p=0.0567$				H1 v all the others OR=0.58 95% CI 0.34 to 0.99 $p=0.0452$				H1 v all the others OR=0.82 95% CI 0.52 to 1.30 $p=0.3866$			
	H2 v all the others OR=1.21 95% CI 0.85 to 1.71 $p=0.2950$				H2 v all the others OR=1.93 95% CI 1.09 to 3.44 $p=0.0262$				H2 v all the others OR=0.90 95% CI 0.56 to 1.43 $p=0.6462$			
	H3 v all the others				H3 v all the others				H3 v all the others			

H1, GTTT; H2, GCCT; H3, GCTC.

Table 4 Haplotype association: block 2: 4.8, 5, 6, 6.5, 6.8, 7, 7.5, 8, 8.3, 9

	All subjects					APOE 4 carriers					APOE 4 non-carriers				
	H1	H2	H3	H4	Others	H1	H2	H3	H4	Others	H1	H2	H3	H4	Others
Controls	78 (30.71)	64 (25.20)	53 (20.87)	20 (7.87)	39 (15.35)	13 (21.67)	17 (28.33)	14 (23.33)	6 (10.00)	10 (16.67)	65 (33.50)	47 (24.23)	39 (20.10)	14 (7.22)	29 (14.95)
Patients	154 (35.16)	85 (19.41)	95 (21.69)	39 (8.90)	65 (14.84)	106 (34.87)	58 (19.05)	66 (21.71)	32 (10.53)	42 (13.84)	48 (35.83)	27 (20.15)	29 (21.64)	7 (5.22)	23 (17.16)
	H1 v all the others OR=1.33 0.92 to 1.93					H1 v all the others OR=1.94 1.01 to 3.77					H1 v all the others OR=1.08 0.67 to 1.73				
	H2 v all the others OR=0.69 0.46 to 1.05					H2 v all the others OR=0.59 0.31 to 1.11					H2 v all the others OR=0.75 0.43 to 1.30				
	H3 v all the others OR=1.03 0.67 to 1.58					H3 v all the others OR=0.92 0.48 to 1.79					H3 v all the others OR=1.15 0.65 to 2.02				
	H4 v all the others OR=0.92 0.49 to 1.75					H4 v all the others OR=0.92 0.48 to 1.79					H4 v all the others OR=0.85 0.32 to 2.23				
	p=0.1284					p=0.0856					p=0.0473				
	p=0.0856					p=0.0856					p=0.0994				
	p=0.9066					p=0.9066					p=0.8113				
	p=0.8052					p=0.8052					p=0.8113				

Only the subjects with EM probability of haplotype estimation higher than 90% were used for haplotype association analysis. *95% CI. H1, GCGAGAGAC; H2, CTGGGGTCT; H3, CTGGGGTCT; H4, GCTATAGACC.

A genome wide linkage disequilibrium study of late onset AD found that a microsatellite marker (D15S659) located at 15q21.1 was associated with the disease in patients from Finland.⁸ As *CYP19a1* is approximately 2 cM from the marker, this group analysed nine SNPs spanning the gene and found that the haplotype A1 (CACTTTGTT) increased the risk for the disease.¹

Given that the reported genetic association with the disease was significant and with the relevant role of aromatase in brain, we investigated SNPs spanning the gene, using the tagSNPs identified through the HapMap project, as well as those SNPs in the report on the patients from Finland.¹ As non-synonymous SNPs in coding sequences are more likely to affect protein function, we also included the four non-synonymous SNPs and two of the three synonymous SNPs identified at the NCBI website.⁹ Our studies indicated an increased risk associated with SNP 7.5 (rs2899472) in the total number of AD patients, which was amplified in *APOE 4* carriers. There was also a significant association with SNPs 4, 6, and 8 (rs1008805, rs727479, rs1143704) in *APOE 4* carriers. SNPs 5–7 were significantly associated with the disease in the patients from Finland, but not in *APOE 4* carriers. None of the SNPs in our study was significant for *APOE 4* non-carriers. Among the six coding SNPs, only two exhibited sufficient heterozygosity for further analysis. The synonymous SNP 6.5 had a trend toward significance in *APOE 4* carriers.

The HapMap project defined two haplotype blocks by genotyping 41 SNPs; similar results were subsequently found by the group from Finland,¹ and confirmed by our genotyped data of 14 SNPs. The boundary between the two blocks is between SNP 4.1 (rs2008691) and SNP 4.8 (rs1062033). Haplotype H2 (GCCT) of block 1 and haplotype H1 (GCGAGAGACC) of block 2 were found to be associated with the disease in *APOE 4* carriers in our study. The group from Finland found that haplotype A1 (CACTTTGTT) increased the risk for the disease. They did not find any interaction with *APOE* in their sample. The A1 haplotype is part of our H2 and H3 haplotypes in block 2. Our haplotype H2 approached significance, with $p = 0.0856$. However, it was the H1 haplotype in block 2 that was significant in *APOE 4* carriers in our study. We also found significant linkage disequilibrium in each block, as described previously.¹ Neither study found evidence of interaction between *CYP19a1* and sex.

Aromatase expression in the brain is controlled by the exon I.f,¹⁰ responsible for transcripts in the amygdala, by the promoter II for the hypothalamus preoptic area, and by promoter I.4 in other areas of the brain. It is thought that regulation of aromatase activity may differ in various brain regions. The transcription factors necessary for expression in the different brain regions have not been well studied. While numerous studies of mRNA expression in various brain regions have been made, the regulatory mechanisms for the expression have not been taken into consideration. The study with the patients from Finland and our study indicate that there may be functional alterations within the coding/intron regions that may also affect expression of the enzyme. With the implication of the two haplotypes conferring increased risk for AD, the regulation of aromatase in brain warrants further investigation.

ACKNOWLEDGEMENTS

We thank the Texas and Georgia families for their active participation in the DNA Bank. DNA was also obtained from the National Cell Repository for Alzheimer's Disease. This research was supported by MCG startup funds and by a cooperative agreement grant U24AG21886 from the National Institute of Aging for the National Cell Repository.

Authors' affiliations

R Huang, S E Poduslo, Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, GA, USA

S E Poduslo, Department of Neurology, VA Medical Center, Augusta, GA, USA

Competing interests: there are no competing interests.

The study was approved by the MCG institutional review board.

Correspondence to: Dr S E Poduslo, IMMAG, Medical College of Georgia, 1120 15th Street, Augusta, GA 30912; spoduslo@mail.mcg.edu

Received 8 November 2005

Revised version received 5 January 2006

Accepted for publication 8 February 2006

REFERENCES

- 1 **Iivonen S**, Corder E, Lehtovirta M, Helisalmi S, Mannermaa A, Vepsäläinen S, Hanninen T, Soininen H, Hiltunen M. Polymorphisms in the *CYP19* gene confer increased risk for Alzheimer disease. *Neurology* 2004;**62**:1170–6.
- 2 **Bulun SE**, Sebastian S, Takayama K, Suzuki T, Sasano H, Shozu M. The human *CYP19* (aromatase P450) gene: Update on physiologic roles and genomic organization of promoters. *J Steroid Biochem and Mol Biol* 2003;**86**:219–24.
- 3 **Ishunina TA**, van Beurden D, van der Meulen, Unmehopa UA, Hol EM, Huitinga I, Swaab DF. Diminished aromatase immunoreactivity in the hypothalamus, but not in the basal forebrain nuclei in Alzheimer's disease. *Neurobiol Aging* 2005;**26**:173–94.
- 4 **McKhann G**, Drachman D, Folstein M, Katzman R, Price D, Stadlan E. Clinical diagnoses of Alzheimer's disease: Report of the NINCDS-ADRDA work group under the auspices of Department of Health and Human Services task force on Alzheimer's disease. *Neurology* 1984;**34**:939–44.
- 5 **Gabriel SB**, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, David Altshuler D. The structure of haplotype blocks in the human genome. *Science* 2002;**296**:2225–9.
- 6 **Poduslo SE**, Neal M, Herring K, Shelly J. The apolipoprotein C1 A allele as a risk factor for Alzheimer's disease. *Neurochem Res* 1998;**23**:361–7.
- 7 **Makridakis NM**, Reichardt JKV. Multiplex automated primer extension analysis: Simultaneous genotyping of several polymorphisms. *BioTechniques* 2001;**31**:1374–80.
- 8 **Hiltunen M**, Mannermaa A, Thompson D, Easton D, Pirskanen M, Helisalmi S, Koivisto AM, Lehtovirta M, Ryyanen M, Soininen H. Genome-wide linkage disequilibrium mapping of late-onset Alzheimer's disease in Finland. *Neurology* 2001;**57**:1663–8.
- 9 **Carlson CS**, Eberle MA, Kruglyak L, Nickerson DA. Mapping complex disease loci in whole-genome association studies. *Nature* 2004;**429**:446–52.
- 10 **Honda S**, Harada N, Takagi N. Novel exon 1 of the aromatase gene specific for aromatase transcripts in human brain. *Biochem Biophys Res Commun* 1994;**198**:1153–60.

Authors QueriesJournal: **Journal of Medical Genetics**Paper: **mg39461**Title: **CYP19 haplotypes increase risk for Alzheimer's disease**

Dear Author

During the preparation of your manuscript for publication, the questions listed below have arisen. Please attend to these matters and return this form with your proof. Many thanks for your assistance

Query Reference	Query	Remarks
1	JMG uses minimal hyphenation and UK English, so some spellings have changed	
2	JMG uses brackets rather than \pm	
3	BMJ uses white in preference to Caucasian unless the population is specifically from the Caucasus	
4	The tables had to be set separately, so are now tables 3 & 4	
5	The 95% CI was removed to the footnote to allow the table to fit on the page	