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Association of an extended haplotype in the *tau* gene with progressive supranuclear palsy

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We describe two extended haplotypes that cover the human *tau* gene. In a total of ~200 unrelated caucasian individuals there is complete disequilibrium between polymorphisms which span the gene (which covers ~100 kb of DNA). This suggests that the establishment of the two haplotypes was an ancient event and either that recombination is suppressed in this region, or that recombinant genes are selected against. Furthermore, we show that the more common haplotype (H1) is significantly over-represented in patients with progressive supranuclear palsy (PSP), extending earlier reports of an association between an intronic dinucleotide polymorphism and PSP.

INTRODUCTION

Progressive supranuclear palsy (PSP) is the second most frequent cause of degenerative parkinsonism after Parkinson's disease (PD) (1). In addition to parkinsonism, the clinical symptoms include early postural instability and supranuclear gaze palsy (2). Neuropathologically, PSP is characterized by abundant neurofibrillary tangles (NFTs) and neuropil threads consisting of hyperphosphorylated Tau protein. The tangles observed in PSP differ in both distribution and composition from those associated with Alzheimer's disease (AD). In PSP, the tangles are primarily localized to subcortical regions and are found in both neurons and glia, whereas in AD they are more widespread, largely cortical and are limited to neurons. At the ultrastructural level the filaments that make up the tangles in PSP are straight, in contrast to the paired helical filaments (PHFs) that are the most abundant species associated with the tangles in AD (3,4).

There are six major protein isoforms of Tau in the adult human brain (Fig. 1). These are generated by alternative splicing of exons 2, 3 and 10 (5). Exons 9–12 encode four microtubule binding domains that are imperfect repeats of 31 or 32 residues (6). Alternative splicing of exon 10 generates isoforms with either four (exon 10+; '4-repeat Tau') or three (exon 10–; '3-repeat Tau') microtubule binding domains (7). The NFTs consisting of straight filaments that are observed in PSP contain only 4-repeat Tau isoforms, whereas the NFTs consisting of PHFs found in AD contain all six major Tau isoforms (4-repeat and 3-repeat) (4).

Mutations in the *tau* gene have recently been found to be associated with frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (8–10). In this context, we have recently shown that mutations in the 5′ splice site of *tau* exon 10 increase the incorporation of this exon into *tau* mRNA (9) and thus increase the proportion of 4-repeat Tau isoforms (10). In affected families this increase is associated with the formation of 4-repeat Tau NFTs and leads to frontotemporal dementia. This observation shows that the control of this alternative splice event is critical and that disregulation can result in tangle formation and neurodegeneration (9–11).

The genetics of PSP had not been studied in great detail until recently, as the disease was usually considered to be sporadic in nature. However, Conrad *et al*. (12) demonstrated an association between a polymorphic dinucleotide marker, found between exon 9 and exon 10 of the *tau* gene, and PSP. This initial result has subsequently been confirmed by at least four other studies (13–16). In each case, an over-representation of the most common allele (a0) and genotype (a0a0) in the PSP group was reported. However, due to the nature of the dinucleotide polymorphism it was considered unlikely that this variation was biologically significant in the disease process, but was in fact in disequilibrium with other polymorphisms (12). The identification of mutations in the *tau* gene that influence alternative splicing of exon 10 and lead to the development of FTDP-17 (9,10) has led to speculation that the location of the dinucleotide polymorphism (between exons 9 and 10) might be significant and an indication that if the polymorphism itself was not functionally relevant, the critical variation would be nearby.

During the sequencing of the *tau* gene in FTDP-17 cases, we and others identified a series of polymorphisms scattered through the gene (8,9). Here we investigate the extent of disequilibrium between these polymorphisms and their association with PSP as a step towards defining the precise mechanism of genetic susceptibility for PSP at the *tau* locus.

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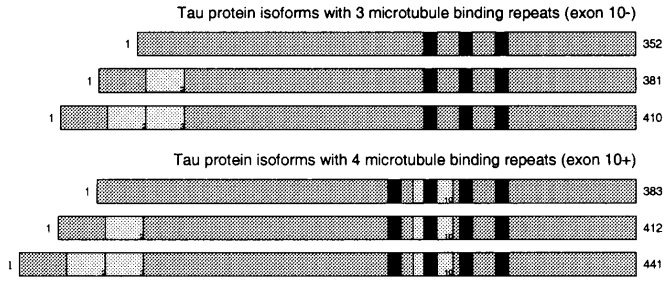

Figure 1. The six major isoforms of Tau. Regions encoded by alternatively spliced exons 2, 3 and 10 are indicated by light shaded boxes. Microtubule binding repeats encoded by exons 9–12 are indicated by vertical black bars.

RESULTS

Identification of extended haplotypes in the tau gene

Sequence analysis of the coding region and flanking intronic sequences in the *tau* gene, primarily in FTDP-17 families (8,9), identified a series of single nucleotide polymorphisms (SNPs) in exons 1, 2, 3, 9 (three polymorphisms), 11 and 13 (Table 1) which were present in both controls and patients. Analysis of the occurrence of these polymorphisms (Table 2) revealed that they are in complete disequilibrium with each other ($P < 1 \times 10^{-100}$). Thus, there are two extended haplotypes (designated H1 and H2) that cover the entire *tau* gene (~100kb; Fig. 2). We did not identify a single recombinant event within these haplotypes in any of the unrelated patient and control individuals tested (>200 total). The absence of recombinant events within the *tau* gene in this large group of unrelated individuals implies strongly that these haplotypes were established early in the history of the Caucasian population tested. One rare SNP (SNP 9iii in Tables 1 and 2) in exon 9 is present only on the more common H1 haplotype, suggesting that it arose by an independent mutagenic event after the establishment of this haplotype. In addition, the dinucleotide polymorphism (a0), which had been shown previously to be associated with PSP, is also inherited with the two extended haplotypes: dinucleotide polymorphism alleles a0 (11 repeats), a1 (12 repeats) and a2 (13 repeats) are inherited with the H1 haplotype, whereas the a3 (14 repeats) and a4 (15 repeats) alleles are inherited with the H2 haplotype. Given that a0 and a3 are the most common alleles (70.5 and 23%, respectively, in our control individuals; Table 3), it seems highly likely that a0 and a3 were present when the extended haplotypes were established, and that a1, a2 and a4 arose from subsequent slippage events (Fig. 2). This observation provides further testament to the conservation and antiquity of the two haplotypes.

Association of the extended *tau* haplotypes with PSP

We tested each of the polymorphisms (SNP and dinucleotide) and, thus, the extended haplotypes for association with PSP. A total of 65 cases of PSP were employed in this analysis (mean age 65.3 years); 18 were autopsy-confirmed cases while the remainder conformed to National Institute of Neurological Disorders and Stroke (NINDS) criteria for probable PSP. Aged caucasian controls (n = 135, mean age 63 years) were used for comparison.

Table 1. Details of SNPs

SNP	Primer sequence and name		Product (bp)	Digestion enzyme	Digestion products	
					Allele A (bp)	Allele B (bp)
1	CAACACTCCTCAGAACTTATC	1F	229	*Alu*I	229	183 and 46
	CAGTGATCTGGGCCTGCTGTG	1R				
2	CAGCTCCACAGGACACTGCTC	2F	298	*Bsa*HI	201 and 97	298
	GGAGTGAGCACATCTCTCAG	2R				
3	GGGCTGCTTTCTGGCATATG	3F	297	*Ban*II	165 and 132	165, 68 and 64
	CCTCACTTCTGTCACAGGTC	3R				
9i	CCACCCGGGAGCCCAAGAAGGTGCC	9iF[a]	152	*Msp*I	147 and 5	128, 19 and 5
	CTGGTGCTTCAGGTTCTCAGTG	9iR				
9ii	CGAGTCCTGGCTTCACTCC	9F	370	*Mae*II	370	127 and 243
	CTTCCAGGCACAGCCATACC	9R				
9iii	CGAGTCCTGGCTTCACTCC	9F	370	*Bst*NI	257, 54, 26,	201, 56, 54, 26,
	CTTCCAGGCACAGCCATACC	9R			20 and 6	20 and 6
11	GCTCATTCTCTCTCCTCCTC	11F	173	*Alu*I	173	24 and 149
	CCAGGACTCCTCCACCCCATGCAGC	11R[a]				
13	ACTTCATCTCACCCTCCCTC	13F	597	*Tsp*509I	276, 94, 78, 72,	370, 78, 72,
	CCTCTCCTTCTCCCTCTTCTAC	13R			62 and 15	62 and 15

[a]Mismatch primer sequence, designed to create an artificial restriction site. SNP numbering denotes the exon in which each is observed. SNPs were originally identified through sequence analysis of the *tau* gene primarily in FTDP-17 families (8,9).

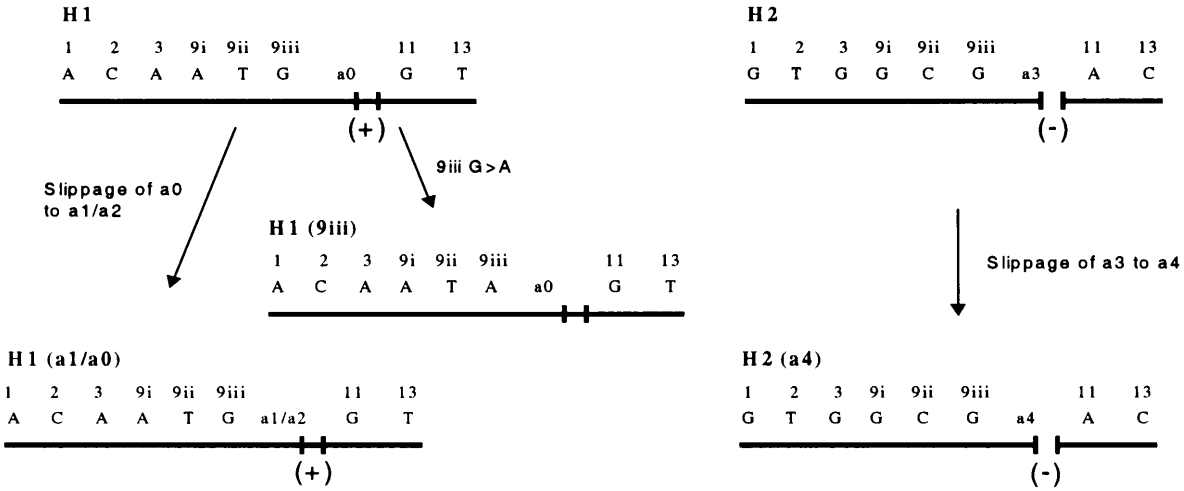

Figure 2. Human *tau* haplotypes. Schematic representation of the development of various human *tau* gene haplotypes. Ancestral haplotypes H1 and H2 are modified by subsequent mutational events (slippage of the dinucleotide polymorphism and the appearance of the exon 9iii polymorphism) but are not altered by recombination. SNPs that were typed in this study are shown at points along the *tau* gene with the nucleotide present in each haplotype indicated. The 238 bp deletion between exons 9 and 10 is shown by a break in the gene in the H2 haplotype. The presence or absence of this region is denoted by (+) or (–), respectively.

Table 2. Genotype frequencies of the common *tau* SNPs in PSP and control series

SNP	Genotype frequency % (number of individuals with each genotype)						χ^2	*P*-value
	H1 H1		H1 H2		H2 H2			
	PSP	Control	PSP	Control	PSP	Control		
exon 1	88.9 (56)	62.8 (91)	11.1 (7)	30.3 (44)	0	6.9 (10)	15.214	0.0005
exon 2	87.3 (55)	62.8 (91)	12.7 (8)	31.0 (45)	0	6.2 (10)	13.944	0.0009
exon 3	88.0 (44)	62.2 (89)	12.0 (6)	30.8 (44)	0	7.0 (10)	12.102	0.0024
exon 9i	88.5 (54)	63.8 (74)	11.5 (7)	30.2 (35)	0	6.0 (7)	12.952	0.0015
exon 9ii	88.3 (53)	63.0 (92)	11.7 (7)	30.8 (45)	0	6.2 (9)	13.753	0.0010
exon 9iii[a]	95.0 (57)	91.0 (131)	5.0 (3)	9.0 (13)	0	0	0.951	0.6217
exon 11	88.4 (38)	64.4 (87)	11.6 (5)	28.9 (39)	0	6.7 (9)	9.456	0.0088
exon 13	88.7 (47)	63.3 (83)	11.3 (5)	29.8 (39)	0	6.9 (9)	12.317	0.0021

[a]9iii is a rare polymorphism that occurs only on the H1 haplotype. The number of individuals typed with each genotype for individual polymorphisms is given in parentheses. Chi-squared analysis testing association between individual *tau* polymorphism genotypes and PSP is presented. H1 allele frequencies (not shown) also displayed a significant association with PSP.

Table 3. Allele and genotype frequencies of the intronic dinucleotide polymorphism in PSP cases and in controls

		PSP (*n* = 64)	Controls (*n* = 139)
Allele frequency %	a0	89.8 (115)	70.5 (196)
(number of chromosomes)	a1	4.7 (6)	5.7 (16)
	a2	0	0.4 (1)
	a3	5.5 (7)	23 (64)
	a4	0	0.4 (1)
		χ^2 = 20.83, *P* = 0.00034 (4 df)	
Genotype frequency %	a0/a0	79.7 (51)	51.1 (71)
(number of individuals)	a0/a1	9.4 (6)	10.1 (14)
	a0/a3	10.9 (7)	28.1 (39)
	a0/a4	0	0.7 (1)
	a1/a3	0	1.4 (2)
	a2/a3	0	0.7 (1)
	a3/a3	0	7.9 (11)
		χ^2 = 18.56, *P* = 0.00497 (6 df)	

Chi-squared analysis testing association between *tau* a0 haplotypes and genotypes and PSP is presented.

Table 4. Extended haplotype and genotype frequencies in PSP and the aged control series

		PSP (n = 64)	Controls (n = 145)
Haplotype	H1	93.7% (120)	78.4% (228)
	H2	6.3% (8)	21.6% (62)
		χ^2 = 14.58, P = 0.00013 (1 df)	
Genotype	H1 H1	87.5% (56)	62.8% (91)
	H1 H2	12.5% (8)	31.0% (45)
	H2 H2	0	6.2% (9)
		χ^2 = 13.85, P = 0.00098 (2 df)	

For analysis of the extended haplotypes, all cases in which two or more of the SNPs had been scored were used, thus for this series more samples (for PSP n = 64) were available. Chi-squared analysis of association between *tau* extended haplotypes and genotypes and PSP is presented.

Initial analysis focused on the dinucleotide TG repeat polymorphism that had previously been shown to be associated with PSP. A significant over-representation of the most common allele (a0) and genotype (a0a0) was observed in the PSP cases compared with controls (Table 3). A proportion of the cases (n = 24) in this study had previously been used to demonstrate the association of this polymorphism with PSP (14); therefore, this observation was anticipated.

In addition, each of the polymorphisms displayed evidence of association with PSP in that the most common allele (H1) and genotype (H1H1) were significantly over-represented in this group compared with controls (Table 2). Since each of these SNPs are in complete disequilibrium with each other in this population, we also analyzed the data for association between each extended haplotype and PSP (Table 4). Again, a significant association with PSP was observed with the most common haplotype [H1, χ^2 = 14.58, P = 0.00013, 1 degree of freedom (df)] and genotype [H1H1, χ^2 = 13.85, P = 0.00098, 2 df). The odds ratio, for developing PSP, with the inheritance of the H1H1 genotype was calculated to be 4.08 [8.79 > CI (95%) > 1.89].

Sequence analysis of *tau* in PSP cases

Exons 9–13 were sequenced in 60 of the PSP cases employed in the association study, 13 of which were autopsy confirmed. These exons were analyzed as all of the mutations identified in *tau* associated with FTDP-17 have been found in this region of the gene. No missense or exon 10 5′ splice site mutations were identified in any of 60 PSP cases sequenced, indicating that typical PSP is not caused by similar mutations to those observed in FTDP-17 families. In 27 of these PSP cases (six confirmed by autopsy), the entire coding region of the *tau* gene was analyzed; again, no mutations were identified.

In addition to sequencing the coding exons, we also examined ~1 kb of intronic sequence 5′ and 3′ of *tau* exon 10. The reason for this analysis is that we consider the region around exon 10 to be the most likely candidate for biologically relevant genetic variation. This is because the tangles in PSP consist of 4-repeat Tau isoforms and when similar inclusions are observed in FTDP-17 families they are associated with missense or splice site mutations in exon 10 (4,9,11,17,18).

Sequence analysis of the introns flanking *tau* exon 10 revealed the presence of a deletion, positioned between –951 and –713 nucleotides upstream of exon 10. This 238 bp deletion is inherited as part of the less common H2 extended haplotype and thus shows a negative association with PSP.

DISCUSSION

We identified a series of eight common SNPs in the *tau* gene and found that these were inherited in complete disequilibrium with each other and with the dinucleotide polymorphism. Together, these polymorphisms define two extended haplotypes that contain at least the entire *tau* gene (exons 1–13; ~100 kb) and have remained uninterrupted by recombination in all the unrelated individuals examined in this study. These data clearly suggest that these two haplotypes were established early in the history of the Caucasian population. Indeed, additional polymorphisms have clearly arisen by independent mutational events rather than recombination since the establishment of the two haplotypes (point mutations and slippage of the dinucleotide polymorphism). The fact that there is little or no recombination over the entire length of the *tau* gene suggests that there must either be suppression of recombination in this chromosomal region, or selection against recombinant alleles.

Our association studies have demonstrated that there is a significant over-representation of the more common (H1) haplotype and genotype in PSP cases compared with controls. Thus, the earlier reports (12–16) of an association with the most common allele of the dinucleotide polymorphism (a0), between exon 9 and 10, are a reflection of this association with the broader haplotype. Indeed, using the results from the association study alone, it is not possible to determine any information about the position of the biologically relevant polymorphism that directly influences the risk of developing PSP, since the extended haplotype contains all of the *tau* gene analyzed.

From a biological perspective, there are three possibilities that might explain the association between *tau* and PSP: (i) there may be crucial differences between the two haplotypes in terms of the expression of the Tau protein; (ii) there may be differences in the splicing of the two cognate proteins; or (iii) a pathogenic, but non-coding, mutation has occurred on the H1 background. The fact that the brains of patients with PSP contain NFTs that consist of Tau isoforms with four microtubule binding repeats (4) suggests that there may be disruption of exon 10 alternative splicing in brain regions affected by PSP. Therefore, it would seem that the best candidate for a role in the pathogenesis in PSP is genetic variability that affects the alternative splicing of *tau* exon 10. In order to search for such variability we sequenced 1 kb into each intron flanking exon 10 and located a 238 bp deletion. It is possible that the presence or absence of the deleted region influences the alternative splicing of exon 10 in a neuron-specific manner, thus affecting the risk of developing PSP. Clearly, further studies, such as splicing analysis, will be needed to determine the role of *tau* exon 10 alternative splicing in the development of PSP and, thus, the significance, if any, of this deletion.

The association of polymorphisms in *tau* with PSP demonstrates that Tau dysfunction is probably crucial to the development of this disease. However, it remains to be determined if Tau dysfunction is the primary lesion in the pathogenesis of PSP or if some other initiating event is required first and that variability in the *tau* gene simply influences the sensitivity of specific neurons and glia to this initial insult.

MATERIALS AND METHODS

Patient ascertainment

All patients used in this study were Caucasian. Individuals were diagnosed with probable PSP utilizing guidelines set by the NINDS (2). These criteria have been reported to have an optimal specificity of 100% (2). Neuropathologic findings in 18 cases were also consistent with PSP (NINDS criteria).

PCR and sequence analysis

Tau exons (1–5,7,9–13) were amplified from genomic DNA from individuals with primers designed to flanking intronic sequence (8,9,19). Exons 4A, 6 and 8 are essentially absent in Human *tau* brain mRNA and were therefore not analyzed (19). Twenty-five nanograms of DNA were used in a 50 µl reaction mixture containing 20 pmol of each primer, 0.2 mM dNTPs, 1 U Taq Gold polymerase (Perkin Elmer, Foster City, CA), 1.5 mM $MgCl_2$, 75 mM Tris–HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$ and 0.01% Tween-20. Amplification of exon 9 required the addition of 5% DMSO. Amplifications were performed oil-free in Hybaid Touchdown thermal cyclers (Hybaid, Cambridge, UK). Conditions were 35 cycles of 94°C for 30 s, 60 to 50°C touchdown annealing for 30 s, and 72°C for 45 s with a final extension of 72°C for 10 min. All products were purified using the Qiaquick PCR Purification kit (Qiagen, Chatsworth, CA). For each exon, 100 ng of product was sequenced in both directions using the Big Dye kit (Perkin Elmer) and relevant PCR primers. Sequencing was performed on an ABI377 automated sequencer and processed using Factura and Sequence Navigator software (Perkin Elmer).

Genotyping and polymorphism analysis

Previously reported polymorphisms (8,9) were analyzed by PCR amplification followed by digestion of the product with the diagnostic restriction enzyme (PCR–RFLP). For polymorphisms 9i and 11, where the polymorphic site did not alter an enzyme recognition site, a mismatch primer was designed to create an artificial site that could be used for genotyping (Table 1). In the PSP series, the genotypes in exons 9 and 11 were determined from the sequence analysis. The presence of the intronic 238 bp deletion was determined by visualizing PCR product on an agarose gel. PCR conditions were as previously described, using primer sequences GGAAGACGTTCTCACTGATCTG (sense) and AGGAGTCTGGCTTCAGTCTCTC (antisense). All samples were genotyped for the intronic dinucleotide repeat polymorphism by PCR using a tet-labeled forward primer, followed by analysis on the ABI377 using Genotyper software (Perkin Elmer). Frequency of polymorphisms are shown in Tables 2 and 3. Linkage disequilibrium between loci was determined by the 'Estimated Haplotype Frequencies' program (20).

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