# MOLECULAR GENETIC ANALYSIS OF TAU RELATED NEURODEGENERATION

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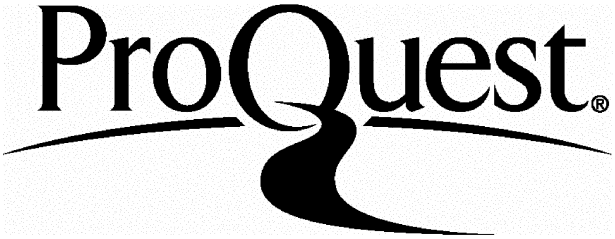

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### Chapters 4 and 5 Allelic association studies and sequence analysis of tau

Pathological analysis of PSP and FTD cases, referred to in this thesis, was performed by Dr. Sue Daniel, Dr. Tamas Revesz, Prof. Peter Lantos and Nadeem Khan, at the UK PDS Brain Research Centre, Department of Neuropathology, Institute of Neurology and Department of Neuropathology, Institute of Psychiatry. Patients were referred by the PDS Brain Research Centre, the Dementia Research Group, Institute of Neurology and neurologists at the National Hospital and throughout the UK. DNA extraction from FTD and PSP brain was performed personally, DNA extraction from PD and control brain was performed by Dr. Oliver Bandmann, Institute of Neurology. The majority of DNA extraction from blood was performed personally, the remainder by the Neurogenetics Lab, Institute of Neurology. In the haplotype/sequence analysis of Pick's disease 11 cases were analysed personally, these were combined with data from a further 33 cases worldwide to generate a larger series. The protein blot analysis of PSP cases was performed by Dr. Graham Gibb and Dr.Tamas Revesz.

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## PUBLICATIONS RESULTING FROM THIS WORK

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Janssen, PL Mcgeer, MN Rossor, AJ Lees , PL Lantos, M Hutton Analysis of tau haplotypes in Pick's disease provisionally accepted Neurology

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**ABBREVIATIONS**

| | |
|---|---|
| 4R:3R ratio | Ratio of four repeat to three repeat tau (RNA or protein) |
| ApoE | Apolipoprotein E |
| α-syn | Alpha synuclein |
| AD | Alzheimer's disease |
| APP | Amyloid precursor protein |
| BAC | Bacterial artificial chromosome |
| Bp | Base pair |
| CBD | Corticobasal degeneration |
| CJD | Creutzfeld-Jakob disease |
| DDPAC | Dementia disinhibition parkinsonism amyotrophy complex |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| FMSTD | Familial multiple system tauopathy with presenile dementia |
| FTD | Frontotemporal dementia |
| FTDP-17 | FTD with parkinsonism linked to chromosome 17 |
| FTD-NLS | FTD without distinctive cellular inclusions |
| FTD-Ub | FTD with ubiquitin positive, tau negative inclusions |
| GRPE | Guam retinal pigment epitheliopathy. |
| HbS | Sickle variant haemoglobin |

| | |
|---|---|
| HDDD | Hereditary dysphasic disinhibition dementia family |
| HFTD | Hereditary frontotemporal dementia |
| Kbp | Kilo base pairs |
| LRT | Likelihood ratio test |
| Mbp | Mega base pairs |
| MPTP | 1-methyl-4-phenyl-1,2,5,6- tetrahydropyridine |
| MSA | Multiple system atrophy |
| NFT | Neurofibrillary tangle |
| NINDS | National Institute for Neurological Diseases and Stroke |
| PCR | Polymerase chain reaction |
| PD | Parkinson's disease |
| PDC | Parkinsonism dementia complex |
| PEP | Post-encephalitic parkinsonism |
| PiD | Pick's disease |
| PPND | Pallido-pontine-nigral degeneration |
| PSG | Progressive subcortical gliosis |
| PSP | Progressive supranuclear palsy |
| PTP | Post traumatic parkinsonism |
| RPFTD | Rapidly progressive fronto-temporal dementia |
| RNA | Ribonucleic acid |
| RT-PCR | Reverse transcriptase PCR |

| | |
|---|---|
| SCA | Spinocerebellar ataxia |
| SNP | Single nucleotide polymorphism |
| SPpc | Substantia nigra pars compacta |
| TD | Touchdown |
| Tm | Melting temperature |
| UPSIT | University of Pennsylvania smell identification test |
| US | United States |

In this thesis to distinguish between genes and their protein products, genes are named using *italics*.

Standard single letter and three letter codes are used for nucleotides and amino acids in this thesis.

**ABSTRACT**

Tau protein is deposited as neurofibrillary tangles in Alzheimer's disease and in a range of other neurodegenerative diseases, designated tauopathies, including progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick's disease (PiD), some forms of frontotemporal dementia (FTD) and the parkinsonism dementia complex of Guam (PDC). The role of the *tau* gene in these conditions is analysed, using molecular genetic techniques. In a series of British patients PSP is associated with the *tau* A0 allele and the A0 allele occurs on a segment of genetic variability designated the H1 haplotype, spanning 100 kb of DNA. PSP is not associated with genetic markers flanking *tau* and is not associated with variability in ApoE or α-synuclein. Clinically diagnosed FTD, and pathologically diagnosed PiD and Parkinson's disease are not associated with *tau.* Neither ApoE nor *tau* has any effect on the age at onset of PSP. Cases of pathologically diagnosed PSP with atypical clinical presentations and atypical tau protein deposition patterns have a lower frequency of the *tau* PSP susceptibility haplotype.

*Tau* was sequenced in 22 families with FTD of whom 11 had mutations in *tau:* exon 10 +14, exon 10 +16 and the P301S mutation. Pathologically the FTD cases were split into FTD with tau inclusions, FTD with ubiquitin inclusions and FTD lacking distinctive histopathology. The presence of *tau* mutations correlated with the presence of tau pathology. In general, pathologically defined PiD cases did not have *tau* mutations, however two individuals with the G398R mutation were identified. Both of these cases had atypical immunohistochemical characteristics. In general, PSP cases did not have *tau* mutations although one young onset individual clinically diagnosed to have PSP was identified to have a *tau* exon 10 +16 mutation.

The clinical and pathological features of amyotrophic lateral sclerosis (ALS) and PDC on Guam were analysed. PDC shares clinical similarities with PSP and other tauopathies. PDC and ALS usually appear as separate clinical and pathological entities. A genome wide association study of PDC was carried out

which provided evidence for the association of PDC with chromosome 14 and chromosome 20. These regions will need to be investigated in a second data set and evaluated in a family based study before their significance can be determined.

# CHAPTER 1 Neurodegeneration

## 1.1 Summary

Neurodegeneration is likely to be an increasingly important public health concern. Although environmental hypotheses have been successful in explaining diseases such as lathyrism and kuru they have not provided a major insight into neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Evidence suggests that AD and PD are separate conditions which are not inevitable accompaniments of normal ageing. The neurochemical analysis of neurodegenerative disease has led to partially successful treatments for PD, and more recently for AD. The combination of molecular pathological and genetic approaches has been particularly successful in explaining the aetiology of these conditions. An evolutionary interpretation of ageing relates to the trade off between cellular maintenance and its metabolic demands. This provides a background to the molecular genetic analysis of tau in neurodegeneration and a possible explanation for the role of age in neurodegenerative disease.

## 1.2 Neurodegeneration in human disease

The extension of human lifespan brought about by improvements in nutrition, childbirth and control of infectious diseases in the developed world has changed the profile of diseases regarded as major health concerns. The focus of health care planning has shifted to diseases which cause death and morbidity in later life, particularly cancer, atherosclerosis and the late onset neurodegenerative diseases. These diseases are increasingly prevalent in an ageing population. The United States (US) now spends a third of a trillion dollars on health care for those aged over 65 and the number of elderly people is expected to double by the year 2050.[1] While improvements have been made in treatments for cardiovascular diseases and cancers, neurodegenerative diseases remain largely untreatable and place an increasing demand on supportive care. The commonest

dementia, Alzheimer's disease (AD) now affects around four million people in the US,[2] and the annual US expenditure on AD is estimated to be 100 billion dollars.[3] The future personal and financial burden of these diseases, without new disease modifying treatments, will be immense.

Neurodegeneration is a disease state resulting from chronic progressive neuronal dysfunction and loss. Ageing can be defined by the time dependent accumulation of biological alterations, such as loss of skin elasticity and muscle bulk, together with the increased likelihood of organism death.[4] It remains unclear whether neurodegeneration reflects one aspect of the normal ageing process or a distinct disease entity. In other words, do all old people undergo the neuronal loss of neurodegeneration to some extent, or does it only affect a subgroup of elderly individuals? The risk of neurodegeneration is certainly age dependent, AD prevalence doubling every 5 years after the age of 65.[2] Although age is a risk factor for late onset neurodegenerative diseases it does not necessarily follow that they are part of the ageing process, or that other age related phenomena necessarily relate to neurodegeneration. This is an important issue since if AD is a part of typical ageing, then general research into ageing phenomena and longevity would be expected to provide an explanation for the aetiology of diseases such as AD. Furthermore, the anticipated continued improvements in longevity would inevitably lead to a large number of elderly individuals suffering from neurodegeneration.

Normal ageing may involve the deposition of proteins which are also deposited in neurodegenerative disease. With respect to AD in particular, immunohistochemical techniques suggest that normal ageing may involve the formation of neocortical senile plaques, and neurofibrillary tangles (NFTs), particularly in the hippocampus, which occur in a more severe form and widespread distribution in clinically diagnosed AD.[5, 6] This supports the idea that AD is a part of normal ageing. However, other evidence suggests that AD is distinct from normal ageing: i.) longitudinal studies of the elderly show that a substantial number of the very elderly remain healthy, experiencing little or no decline in cognitive function,[7] ii.) individuals from prospective longitudinal studies with a high density of diffuse neocortical senile plaques, involving the

deposition of Aβ amyloid, have ante-mortem evidence of early cognitive impairment, suggesting that "normal" neocortical senile plaque formation is relatively uncommon,[8] iii.) detailed neuropathological studies suggest that both early PD and AD are associated with substantial neuronal loss in specific areas which are relatively unaffected in normal ageing, the lateral ventral tier of the substantia nigra pars compacta,[9] and layer II of the entorhinal cortex.[10] There is an apparent inconsistency between the statements that some Aβ amyloid may be deposited in normal ageing and that individuals with low level Aβ amyloid deposition have early cognitive impairment. This may reflect differences between longitudinal prospective studies on the one hand, and brain bank series reliant on retrospective clinical data on the other. Although some proteins such as Aβ amyloid and tau may be deposited as part of normal ageing, particularly in the hippocampus, there seem to be quantitative and qualitative differences between neurodegeneration and normal ageing. This distinction is reinforced by the identification of families with young onset, genetically mediated forms of AD and PD and by differential susceptibility to genetic risk factors in sporadic AD. These pathological differences and the occurrence of autosomal dominant forms suggests that it is reasonable to search for specific aetiological agents.

## 1.3 Early pathological descriptions of neurodegeneration

The original descriptions of neurodegenerative disease were based on clinical and pathological description of individual cases. In 1907 Alzheimer published a case report of a woman affected with pre-senile dementia, and using the novel technique of silver staining he described the classical hallmark lesions of NFTs and senile plaques.[11] He hypothesized that the dementia was due to the neuropathological lesions seen and Kraepelin subsequently named this condition Alzheimer's disease (AD).[12] Later pathological studies mapped the topography of affected and unaffected brain areas, and provided some pointers towards clinico-pathological correlation.[12] Eighty-three years later genetic studies of familial AD revealed that the amyloid component of the senile plaque itself has a central role in the disease pathogenesis.[13]

Similarly, the earliest studies of PD were able to identify the characteristic eosinophillic intraneuronal inclusions (the Lewy body) and the pathologists Trétiakoff and Hassler emphasised the substantia nigra cell loss.[14, 15] Eighty-five years after the description of the Lewy body, one of its components, α-synuclein was shown to have a causative role in some families with PD.[16, 17] The interval between the classic pathological descriptions of the hallmark features of both AD and PD, and genetic indication of the importance of their protein composition, has included investigations of environmental factors in neurodegeneration and an increased understanding of their neurochemistry.

## 1.4 Environmental approaches to neurodegeneration

### Kuru and Lathyrism

In the 1960s and 1970s environmental factors were shown to be relevant in two neurodegenerative diseases, kuru and lathyrism. Kuru is a spongiform encephalopathy characterized by abnormal protein deposition, which was investigated by Gadjusek and colleagues in Papua New Guinea.[18, 19] The high prevalence of this disease on Papua New Guinea was unexplained until it was connected with ritual cannibalism. Primate transmission studies showed that the brain tissue contained an infectious agent, which was later shown to be an abnormal prion protein.[18, 19] This work established that a neurodegenerative disease could be due to an transmissible agent, and that affected brain was a reservoir of infection. Subsequently Gajdusek and co-workers attempted to demonstrate that other neurodegenerative diseases could also be due to infectious agents but they were unsuccessful in demonstrating tissue infectivity from patients with AD, PD and the parkinsonism dementia complex of Guam (PDC).[20]

Another neurodegenerative disease which has been convincingly explained by an environmental factor is lathyrism. Lathyrism is a form of motor neuron disease which occurs in West Africa and India, following consumption of the chickling pea (*Lathyrus sativus*). In 1986 Spencer and co-workers demonstrated, with animal toxicity studies, that this was likely to be due to an excitatory amino acid neurotoxin (beta-N-oxalylamino-L-alanine, BOAA).[21] Although lathyrism and

kuru are rare conditions, until the description of genetic mutations responsible for specific neurodegenerative diseases, these environmental exposures to an infectious agent and an excitatory neurotoxin were the only certain aetiological factors described in neurodegeneration. Against this background, environmental hypotheses for the aetiology of PD and AD gained credibility.

**Aluminium and AD**

The aluminium hypothesis for the aetiology of AD originated from a number of separate lines of evidence which received considerable publicity and culminated in a "health scare".[22] The initial finding was that aluminium injections into the brains of rabbits led to a form of neurofibrillary degeneration, and this was linked with dialysis encephalopathy, a condition in which patients receiving haemodialysis for renal failure developed cognitive impairment related to unfiltered aluminium in tap water.[22] It was then suggested that environmental aluminium exposure could be a specific cause of AD.[23] This was supported by reports of higher aluminium levels in the brains of patients with AD, the finding of aluminium in NFTs, and epidemiological evidence relating the incidence of Alzheimer's disease to the amount of aluminium in local drinking water.[24, 25] This theory was also used to explain the parkinsonism dementia complex of Guam (PDC), in which it was thought that low levels of calcium in the drinking water induced secondary hyperparathyroidism and led to neural aluminium deposition.[25] However, each part of the evidence linking AD with aluminium has come under critical review: i.) unlike AD, aluminium related rabbit neurofibrillary degeneration relates to neurofilament protein deposition primarily in motor cortex, ii.) the dialysis encephalopathy syndrome differs clinically and pathologically from AD, iii.) the original observation of increased aluminium in AD brain used a comparison with a younger control group, and although subsequent studies have demonstrated an increase in neural aluminium content with age, they have not demonstrated an association with AD, iv.) molecular analytical studies demonstrating aluminium within NFTs have not been replicated using newer techniques, v.) there is no evidence for secondary hyperparathyroidism in Guamanian individuals with PDC.[22, 26, 27] Furthermore, the original epidemiological study linking water aluminium content to the

prevalence of AD has not been confirmed and the original authors of this study have failed to replicate their findings in a more recent case control study.[28]

**MPTP and Parkinson's disease**

Another line of evidence supporting the role of environmental factors in neurodegeneration, is the MPTP theory of Parkinson's disease. This developed from the description of an outbreak of Parkinsonism in a group of Californian intravenous drug users who had used a synthetic meperidine analogue designed to replicate the effects of fentanyl which was contaminated with small quantities of 1-methyl-4-phenyl-1,2,5,6- tetra-hydro-pyridine (MPTP).[29] These patients developed rigidity, tremor and bradykinesia which was responsive to L-DOPA.[29, 30] The link between MPTP and PD was strengthened by positron emission tomography (PET) which provided evidence of a dopaminergic deficit in patients exposed to MPTP, and nigral cell loss with specific deficits in tyrosine hydroxylase and aromatic acid decarboxylase in animals exposed to this neurotoxin.[31, 32] MPTP is metabolised to $MPP^+$ by the enzyme mono-amine oxidase type B (MAO-B) and $MPP^+$ can be shown in vitro to be a mitochondrial complex I toxin. Langston and others suggested that patients with PD may have been exposed to low levels of an environmental neurotoxin with similarities to MPTP, and that PD was likely to be due to environmental toxin related mitochondrial dysfunction.[30, 33] The MPTP/PD theory has been related to other lines of research suggesting that there was a specific mitochondrial defect in PD, and that free radicals generated by mitochondrial dysfunction might mediate neuronal damage in PD.[34, 35] The MPTP/PD theory gained some clinical credibility with the DATATOP study and the suggestion that selegeline, a MAO-B inhibitor might delay disease progression in PD, and that this therapeutic effect related to the inhibition of an environmental MPTP analogue.[36, 37] Subsequent re-analysis of the DATATOP data suggested that the study may have neglected the small treatment effect provided by selegeline and the disease modifying effect of selegeline in PD has not been confirmed.[38] While MPTP has been useful in developing pharmacological models of PD treatment, substantive data supporting a widespread environmental MPTP analogue in the aetiology of PD has been difficult to establish. However, recent work has shown that the

pesticide rotenone can act as a specific mitochondrial complex I inhibitor and lead to selective nigro-striatal damage in experimental models.[39] There are some very important differences between MPTP induced parkinsonism and PD. MPTP induced parkinsonism develops within days of MPTP exposure, is symmetrical and is associated with the early development of motor complications. Furthermore, recent pathological data have shown that patients with MPTP related parkinsonism do not develop Lewy bodies, do not have abnormal α-synuclein deposition and do not develop the topographic pattern of SNPc cell loss seen in PD.[40] Although these data do not support the role of MPTP-like toxicity in PD, it is possible that these pathological differences relate to the amount of neurotoxin exposure and the age of the affected individual.[40] Additionally, only a proportion of drug abusers exposed to MPTP developed parkinsonism, suggesting that there may be a role for genetic susceptibility to environmental co-factors in the development of this condition. MPTP and other complex I inhibitors undoubtedly have anatomical specificity for the substantia nigra which may still be informative as to the selective sensitivity of this region to damage in PD, perhaps relating to the metabolic activity of the substantia nigra. Some data supports a primary abnormality of complex I in patients with PD.[34]

In summary, although environmental hypotheses have been successful in explaining rare disorders such as kuru and lathyrism and have generated aetiological theories relating to AD and PD they have not so far had a major impact on our understanding of the aetiology of the common neurodegenerative diseases. These comments must be qualified by the difficulty in establishing comparative lifetime environmental exposure risks in patients with neurodegenerative disease and appropriate controls.

## 1.5 Neuro-chemical approaches to neurodegeneration

The determination of dopamine as a neurotransmitter, followed by the finding of dopaminergic depletion in the brains of patients with PD, and then the success of dopaminergic replacement therapy was a triumph in the logical analysis and

treatment of neurodegeneration which was recognised by the award of the year 2000 Nobel prize for Medicine or Physiology to Arvid Carlsson and others. In the last four decades biochemical analysis of neurotransmitter changes has become an increasingly important approach to the investigation of neurodegenerative disease. This has been facilitated by the development of histological techniques, and then immunocytochemistry as a tool to define chemical neuroanatomy. The earliest studies of dopamine deficiency in PD were based on direct quantitation of dopamine and its metabolites in the basal ganglia, following Carlsson's work describing dopamine as a neurotransmitter using histochemical fluorescence techniques.[41] This led to the trials of L-DOPA treatment in PD by Barbeau, Birkmayer, Hornykiewicz and Cotzias.[14, 42] The idea that neurological disease could be due to a neurotransmitter deficiency which could be replaced pharmacologically was an exciting concept in the battle against neurodegenerative diseases. The cholinergic hypothesis of AD was initially based on the quantitation of the enzyme choline acetyl transferase and its depletion in AD.[43, 44] It was reinforced by immunocytochemical studies showing the loss of neurons in subcortical cholinergic nuclei, in particular the nucleus basalis of Meynert.[45] However, the use of pharmacological methods to enhance central cholinergic neurotransmission in AD, has been far less successful than the dopaminergic treatment of PD. This most likely relates to the extent of cell loss in AD, the involvement of several different neurotransmitter systems, in addition to the cholinergic system and possibly also to differences in the physiology of cholinergic and dopaminergic cortical projection systems. Neurotransmitter replacement therapy may be more successful in PD because the SNpc is a tonically active nucleus whose neurotransmitter release can be mimicked by systemic administration of an exogenous drug, flooding the synapse. A deficiency of a temporally patterned neuronal discharge in several different neurotransmitter systems is likely to be much more difficult to replace pharmacologically. The lack of success of neurotransmitter replacement therapy for diseases other than PD, and the problems with the long term use of L-DOPA suggests the need for more fundamental treatments related to the diseases' underlying cause.

## 1.6 Pathological and genetic approaches to neurodegeneration

### Alzheimer's disease

The detailed study of the distinctive neuropathological lesions of PD and AD appeared to be a less promising approach to understanding neurodegeneration. A principle argument against the detailed study of lesions such as senile plaques and NFTs in AD was that they were either the final stages of the neurodegenerative process ("tombstone lesions") or that they were the response of surviving neurons to the underlying insult. In both cases neuropathological studies of these lesions would not be expected to explain the underlying cause of the disease. However, in AD characterisation of the constitution of the senile plaques and NFTs was a major advance in understanding the disease aetiology, and this preceded and guided the genetic breakthroughs. In the 1960s and 1970s new techniques were used to re-examine the properties of the classical pathological lesions of AD. Electron microscopy showed that NFTs consisted of filaments of regularly varying diameter described as paired helical filaments.[46] The constitution of these filaments was then examined using immunohistochemistry. Initially recognition of the NFTs by anti-neurofilament antibodies suggested that the main constituent of the NFT was neurofilament protein.[47] Subsequently they were shown to be more consistently immunoreactive with antibodies directed against tau protein.[48] Direct peptide sequencing of the NFT confirmed that they were indeed composed of tau.[49] However, the early success in characterizing the NFT was tempered by the identification of tau containing NFTs in a wide range of neurodegenerative conditions, which seemed to indicate that tau deposition was not an early or specific event in the pathogenesis of AD. The amyloid plaques of AD were similarly characterized by direct peptide sequencing, and this led to the identification of the constituent Aβ peptide and the cloning of the full length amyloid precursor protein (APP) gene.[50] Subsequently, the seminal identification of APP mutations in autosomal dominant AD demonstrated that genetic mutations could cause a familial version of a common neurodegenerative disease, and they emphasized the role of one of the principal pathological

components.[13] *In vitro* techniques were then used to explore the biology of APP and the effect of APP mutations, and these data were correlated with further examination of AD brain. The APP mutations have a major effect on its proteolytic cleavage. α-, β- and γ- secretase are responsible for the normal processing in two major pathways: cleavage of APP by α- and γ-secretase leads to the production of a p3 APP fragment not associated with AD, whereas cleavage by β- and γ-secretase leads to the production of the 40 or 42 aa Aβ fragment deposited in AD.[51] Aβ 42 possesses two additional hydrophobic residues which lead to increased insolubility. All of the APP mutations described to date are clustered around secretase recognition sites and they all either increase the total production of Aβ or increase the relative amount of Aβ 42.[52] The description of these mutations was extended to explain the pathogenesis of sporadic AD and was formulated into the "amyloid cascade" hypothesis of AD, that is, that primary abnormalities in the processing or production of APP, at any one of a series of steps, led to clinical disease. The role of APP processing was further reinforced by the description of mutations in presenilin-1 and presenilin-2 in further AD families.[52] Although the function of the presenilins is not fully defined, mutations in presenilin 1 and 2 also seem to alter secretase related APP processing and increase the production of Aβ 42.[52] It has been suggested that presenilins 1 and 2 are γ-secretase, although this is debated.[53] The use of specific antibodies to Aβ 40 and Aβ 42 was then used to re-examine tissue from sporadic and familial AD cases and the ratio between Aβ 40 and Aβ 42 amyloid isoforms. This work has confirmed that an alteration in the Aβ 40/42 ratio is an early event in the pathogenesis of AD.[54, 55] This work on AD demonstrates how the description of mutations in genes which cause AD can generate testable theories for the disease pathogenesis.

**Parkinson's disease**

In contrast to AD, molecular evaluation of the hallmark lesion of PD, the Lewy body, followed directly from the identification of the first gene which causes autosomal dominant PD. Up until 1997, Lewy bodies were identified most readily using immunocytochemistry against ubiquitin.[56] Ubiquitin tags

misfolded or damaged proteins for degradation and is present in a wide range of neurodegenerative diseases, and the presence of ubiquitin immunoreactivity in the Lewy body is not a specific finding.[57] In 1997 Polymeropoulos and colleagues described the first gene responsible for familial PD, α-synuclein, which codes for a protein which had previously been identified as a component of amyloid plaques in AD.[16, 58] Following the description of the Ala53Thr mutation in one family with autosomal dominant PD, α-synuclein immunohistochemistry showed that α-synuclein was deposited in Lewy bodies, in both sporadic and familial PD.[59] Immunocytochemical studies of brain stem and cortex that α-synuclein is the most sensitive marker of Lewy bodies in these areas. Although a further mutation, Ala30Pro has been described in another family with autosomal dominant PD,[17] α-synuclein mutations do not seem to be a common cause of familial or sporadic PD.[60, 61] Furthermore, despite the apparent importance of α-synuclein deposition in sporadic PD, polymorphic variation in the α-synuclein gene itself does not seem to be an important predisposing factor to the development of sporadic PD.[62] However, the finding that the Ala53Thr and Ala30Pro mutations both accelerate the rate of α-synuclein filament formation *in vitro* suggests that this process of abnormal filament formation may be an early stage in the pathogenesis of familial PD, and similar mechanisms may be important in sporadic PD.[63]

PD and AD illustrate how genetic and pathological studies of neurodegeneration compliment each other and lead to powerful hypotheses of disease pathogenesis. Defining the gene mutations for the familial diseases explains their pathogenesis. Although this research also generates well supported ideas on the development of the sporadic disease, in which pathogenic disease gene mutations do not occur, there is a leap of faith in extrapolating the results of studies of the familial disease to sporadic disease. Few genes studied in familial neurodegenerative disease can be shown to be directly involved in sporadic disease. The precise aetiology of these sporadic disorders, which may depend on a combination of several genetic and environmental factors has not yet been established.

## 1.7 Neurodegeneration in an evolutionary context

A recent trend has been to evaluate disease in evolutionary terms. For example, the prevalence of the homozygous lethal sickle cell haemoglobin variant (HbS) can be explained by the selective pressure on HbS due to the benefits conferred by HbS heterozygosity in resistance to malarial infection. Similarly the 1 in 20 population frequency of cystic fibrosis mutant alleles in Caucasian populations may relate to a protective effect on the mutant allele against the effects of cholera toxin. Is there any selective pressure that could explain the widespread emergence of genetic factors which favour the development of disease like AD or PD? It has been suggested that ageing and death may be genetically predetermined aspects of life, which may have been selected for because of the overall species advantage in diverting resources to the younger, most reproductively fit members of the species. However, as Kirkwood has argued, this seems unlikely for two main reasons.[4] Firstly, the evolutionary background for humans is the predator rich, accident and infection filled environment of several thousand years ago, in which it would have been unusual for any individual to survive past the age of 50; secondly, any mutation which perturbed a programmed organism death gene would lead to a selective advantage. Alternative viewpoints were proposed by Kirkwood and Medawar.[64] Medawar suggested that there had been no selective pressure against mutations which affected survival after the normal age of death i.e. 40-50 years, and therefore the ageing process was due to the effect of the cumulative effect of those random mutations which had been exposed by improvements in organism survival. [65] Kirkwood described an alternative "disposable soma" theory, in which he envisaged a metabolic "trade off", under the evolutionary pressure of scarce environmental resources, between the metabolic demands of cellular repair and the metabolic demands of procreation and germ cell production. The most efficient amount of cellular repair would therefore be that which maintained the organism up to the normal lifespan determined by extrinsic factors. An excessively stringent cellular maintenance and repair mechanism would be energetically wasteful and divert energy from reproductive capacity. Thus, the likely prospect of death by the age of 40 would mean that it would not be

necessary to have cellular mechanisms which would allow the maintenance of a perfect soma beyond that age. This evolutionary interpretation of ageing may have very important implications for theories of neurodegeneration, particularly as the brain is a post-mitotic organ which cannot regenerate by cell division. It may have been evolutionarily undesirable to maintain efficient cellular waste disposal mechanisms which provide good neuronal maintenance beyond the age of 40 or 50, and this economy may cause problems which are exposed with increasingly longevity. In other words, ageing may be due to the accumulation of cellular waste products, and the excessive accumulation of specific waste products in specific cell groups may lead to diseases such as AD. This may help to explain how, in general terms, age is a risk factor for all neurodegenerative diseases, but in some circumstances genetically predisposed individuals may develop young onset disease.

## 1.8 Conclusions

The progress in our understanding of neurodegeneration follows the development of new investigative techniques, in particular silver staining, electron microscopy, and histochemical and immunocytochemical analysis. A combination of clinical study, molecular genetics and pathology provides a powerful approach with which to study neurodegeneration. Genetics has provided firm evidence for aetiological factors which relate to hallmark pathological lesions, and aetiological theories which can be tested with pathological examination. Evolutionary theories of neurodegeneration support the role of protein accumulation, due to excess protein deposition or inefficient clearance, as an ageing related phenomenon. Major challenges remain in understanding the relationship between sporadic and familial neurodegeneration and in developing a theory of the relationship between normal ageing and neurodegeneration. This thesis focuses on genetic and pathological aspects of tau related neurodegeneration in FTD, PSP and PDC.

# CHAPTER 2 Tau

## 2.1 Summary

Tau is an alternatively spliced protein which binds to microtubules and regulates microtubule assembly, axonal outgrowth and helps to determine neuronal polarity. The evidence implicating tau in Alzheimer's disease (AD) and its biochemical changes is reviewed. The deposition of tau in a range of other neurodegenerative conditions, and the core clinical, biochemical and pathological features of these conditions are described. The historical, clinical and pathological features of progressive supranuclear palsy (PSP), which forms a main focus of this thesis, is described in greater detail.

## 2.2 The tau gene and protein structure

Tau is one of a family of microtubule associated proteins (MAPs), which bind to microtubules and modulate microtubule function. MAP-1 and 2 have molecular weights of 330 kDa and 300 kDa, whereas tau is much smaller with a molecular weight of 50-70 kDa. The *tau* gene is encoded on chromosome 17q21.[66] It consists of 16 exons, 3 of which (exons 4a, 6 and 8) are not expressed in human brain, 1 of which (exon –1) is part of the promoter and transcribed but not translated, and three of which (exons 2, 3 and 10) are alternatively spliced to produce six different tau isoforms in adult human brain.[67] These isoforms ranging in length from 352 to 441 amino acids.[67, 68]

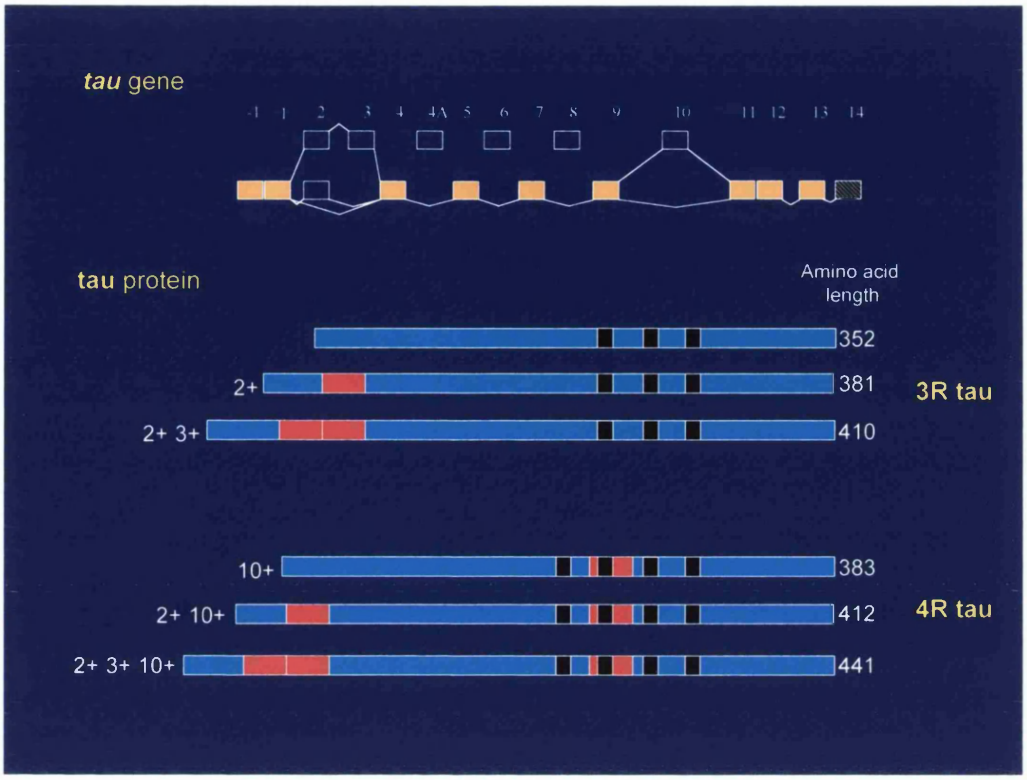



**Figure 2-1 Alternative splicing of the microtubule associated protein tau**

**352-441 amino acid**

**Boxes in gene - exons, hollow boxes - alternatively spliced exons, red boxes – alternative protein domains, black boxes – microtubule binding domains. Adapted from figure provided by Prof. J. Hardy.[67]**

The alternative splicing of tau is developmentally regulated, foetal brain containing only the smallest 352 amino acid three repeat tau without an amino terminus (exon 2/3), or carboxy terminus exon 10 insert.(Figure 2-1) Peripheral sensory and sympathetic neurons contain a "big" tau with an additional amino terminus insert of 254 amino acids.[69] The gene itself contains two CpG islands, which may act as transcription control elements – one upstream of the transcription start (exon-1) and one internal CpG island (exon 9) associated with a repetitive Alu I sequence.[67] The DNA sequence downstream of the second CpG island, which contains the imperfectly repeated microtubule binding domains encoded by exons 9,10,11 and 12, show considerable homology with MAP-2, but upstream the sequences diverge widely.[67, 70] These sequence differences may contribute to the correct cellular localization of the two

molecules, and perhaps also to the role of tau, but not MAP-2 in neurological disease. The tau promoter is a nerve growth factor responsive neuron specific 355 base pair GC rich region upstream of tau exon –1, which lacks a TATA box.[71] This absence of a TATA box is thought to enable the generation of multiple transcription start sites.[72] The interaction between tau and microtubules is mediated by a repeated 18 amino acid sequence encoded by exons 9,10,11 and 12, separated by an imperfectly repeating 13 or 14 amino acid sequence, via a flexible array of distributed weak sites.[73, 74] Tau isoforms containing exon 10 are referred to as four repeat (4R) tau and those lacking exon 10 are referred to as three repeat (3R) tau.(Figure 2-1) The ratio between 4R and 3R tau is normally tightly regulated and in normal human adult brain the ratio is usually estimated to be around 0.8, i.e. 4R tau is produced at slightly lower levels than 3R tau.[75]

In neurons, immunostaining shows that MAP-1 is present throughout the cell, in axons, cell bodies and dendrites. However, MAP-2 and tau have largely complementary cellular distributions. MAP-2 is found in the cell body and dendrites whereas tau is enriched in axons.[76] Although tau was initially reported to be a neuronal protein confined to the axonal compartment,[76] subsequent reports have described the presence of low levels of tau in the cell body,[77] in oligodendrocytes,[78] and in astrocytes.[77] These discrepancies may reflect the conformational heterogeneity of tau and the variable recognition of tau by different antibodies. The primary peptide structure of tau contains a large number of Pro-Gly repeats, and a number of Ser/Thr-Pro motifs which act as targets for protein kinases; and both of these factors are likely to lead to conformational and immunogenic variation.[79, 80]

Microtubules are polymers made from monomeric tubulin whose functions include maintaining cellular stability, axoplasmic transport, stabilizing mitotic processes and establishing neurite outgrowth and cellular polarity.[81] These functions are clearly central to maintaining normal cellular function and disturbance of this normal microtubule function is likely to have detrimental effects.[82] The role of tau in normal microtubule function is supported by evidence in cell free systems that tau promotes the polymerisation of tubulin into microtubules, and that in cell culture it similarly promotes the assembly of

microtubules and stabilizes them against nocadazole mediated depolymerisation.[83] Tau is implicated in the regulation of microtubule function by the observations that in rat phaeochromocytoma derived PC-12 cells the levels of tau protein correlates with the extent of microtubule formation and neurite outgrowth,[84] that induction of the expression of tau mRNA in differentiating cells occurs at the onset of axonal outgrowth,[84] and that suppression of normal tau expression by the administration of tau antisense oligonucleotides inhibits normal axonal outgrowth and the establishment of neural polarity.[85] However, surprisingly ***tau*** knockout mice appear to develop normally and have only subtle abnormalities in the microtubules of a sub-population of neurons.[86] This suggests that under some circumstances, probably when the defect occurs early in development, other MAPs may be able to take on the normal role of tau. Tau may have a number of roles in addition to its interaction with microtubules, but these remain relatively poorly understood. The amino terminus of tau interacts with cytoskeletal and plasma membrane components, and the carboxy terminus binds to protein phosphatase 2A and presenilin-1.[87]

## 2.3 Abnormalities of tau in Alzheimer's disease

Interest in tau in neurodegeneration has centred on its role in the pathogenesis of AD. As described in chapter 1, tau was identified as the major protein constituent of NFTs in the 1980s. However the description of amyloid processing and amyloid precursor mutations in FAD focused attention on amyloid dysfunction as a primary cause of AD and seemingly identified tau NFT deposition as a secondary "downstream" phenomenon. However, a number of lines of evidence suggest that tau may have a more central role in the neurodegenerative process. The pattern of tau deposition closely corresponds to the pattern of cell loss in Alzheimer's disease, whereas amyloid deposition can occur in many cortical areas without cell loss.[5, 88, 89] The extent of tau deposition more closely follows the clinical disease course than does amyloid deposition. The Braak hypothesis of AD, based on detailed autopsy examination of patients at different disease stages, is that disease progression corresponds closely with

the extent of tau deposition, whereas the extent of amyloid deposition does not relate to the clinical stage.[5, 89] Specifically, Braak and Braak hypothesize that the disease process begins in areas such as the trans-entorhinal cortex, spreads to involve the hippocampi, other mesial temporal areas and then to involve the neocortex. The relationship between tau deposition and both cell loss and clinical disease stage suggests that NFT deposition is an important cause of neuronal damage. Tau is deposited predominantly as intracellular NFTs which are visualized as paired helical filaments (PHFs) with the electron microscope. The PHFs of AD consist of intertwined fibres with a regular repeating diameter variation from 8 to 20 nm.[68] Biochemical analysis of AD tau indicates that PHF-tau is deposited in a sarkosyl insoluble form, and that the sarkosyl insoluble tau fraction runs as a large major triplet of tau bands at 55, 64 and 68 kDa on protein immuno-electrophoresis (Western blotting), together with a minor band at 72 kDa.[90] These tau protein bands are hyperphosphorylated since, following alkaline phosphatase treatment the abnormal tau bands resolve to form the 6 tau isoforms that can be identified in normal brain.[91] In addition, PHF-tau is recognised by a range of phosphorylation dependent antibodies which do not recognise soluble, dephosphorylated tau. The phosphorylation of tau occurs at the Ser/Thr–Pro sites clustered around the microtubule binding domains.[68] This phosphorylation is mediated by a number of protein kinases which specifically recognise the serine/threonine-proline motif - most importantly mitogen-activated protein kinase and glycogen synthase kinase-3.[92] The phosphorylation state of tau depends on a balance between kinase and phosphatase activity, and the most important protein phosphatase is thought to be protein phosphatase 2A.[93] Phosphorylated tau has impaired microtubule binding properties. The tau phosphorylation hypothesis of AD states that phosphorylation of tau is an early event which inhibits tau-microtubule interactions and leads to the formation of a cytoplasmic pool of phosphorylated tau, which then aggregates to form PHFs. However, some evidence from post mortem flash frozen tissue suggests that the extent of tau phosphorylation *in vivo* may be limited.[94] Possibly, the increased phosphorylation seen in AD tau is a post mortem artefact, related to persistent protein kinase activity in AD brain in the absence of phosphatase activity, or to conformational changes in AD tau. Interestingly, it has been shown that tau from

quickly processed biopsy-derived, but not autopsy-derived, normal brain tissue shares many of the phosphorylation epitopes with AD PHF-tau.[94] Some *in vitro* studies suggest that tau forms into PHF-tau regardless of the phosphorylation state, and foetal tau is highly phosphorylated without adverse cellular effects.[95] However, more recently, hyperphosphorylated tau from AD brain has been shown to aggregate abnormally under reducing conditions and this seems to relate to neutralisation of basic charged residues surrounding the flanking regions.[96] Although the evidence for tau phosphorylation as an early event in AD is equivocal, the biochemical studies of tau have paved the way for a classification of tau deposition disorders.

## 2.4 Tau deposition in other neurodegenerative disorders

A number of other conditions are characterized by the deposition of tau containing NFTs: progressive supranuclear palsy (PSP-Steele Richardson Olszewski disease), corticobasal degeneration (CBD), Pick's disease (PiD), frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), post-encephalitic parkinsonism (PEP), the Parkinsonism dementia complex of Guam (PDC; bodig), Niemann-Pick disease type C, subacute sclerosing panencephalitis (SSPE) and post-traumatic parkinsonism (PTP;"dementia pugilistica").[97] In addition, mutations in the parkin gene, responsible for autosomal recessive juvenile parkinsonism are also sometimes associated with NFT pathology in a PD like distribution, suggesting that dysfunction of parkin may also lead to tau deposition.[98, 99] These diverse tau deposition conditions have been grouped together as "tauopathies". The non-amyloid tauopathies are often considered together as a group. The ultrastructure and protein chemistry of NFTs have been studied in detail for AD and the different tauopathies may be subdivided according to these characteristics (Table 2-1). In contrast to AD, in PSP the NFTs are straight filaments which are composed of only two of the major triplet of bands at 64 and 68 kDa.[100, 101] Dephosphorylation analysis indicates that this PSP tau protein is made up of only a subset of the alternatively spliced tau isoforms, the majority of which contains exon 10 derived amino acids (4R tau)(Figure 2-1).[100] In PiD the tau protein is made up of only the smallest

two bands at 55 and 64 kDa, which are composed of a separate tau isoform sub-population consisting of 3R tau.[100, 102, 103] *In vitro* experiments confirm that four repeat tau preferentially forms into straight filament NFTs. [95] Thus, tauopathies can be classified according to the tau isoforms deposited (Table 2-1). It is possible that these differences in tau protein deposition reflect a topographically restricted pattern of ***tau*** expression, for example cortical neurons which happen to be damaged in AD may express all six isoforms of the tau gene whereas substantia nigra neurons may preferentially express 4R tau. Alternatively, the tau protein expression may reflect a more fundamental aspect of the pathogenesis of the disease. Evidence from the study of the autosomal dominant kindreds suggests that the latter possibility is the case in the genetically mediated forms of these diseases.

**Table 2-1 Biochemical classification of tauopathies**

| Tau isoforms | 3R + 4R tau | | 4R tau | | 3R tau | |
|---|---|---|---|---|---|---|
| Western blot | Triplet (59,64,68 kDa) | | Doublet (64,68 kDa) | | Doublet (59,64 kDa) | |
| Inheritance | Familial | Sporadic | Familial | Sporadic | Familial | Sporadic |
| Filament ultrastructure | Paired helical | | | Straight | | |
| Diseases | AD | AD | | PSP | Myotonic dystrophy | PiD |
| | | PDC | | CBD | | |
| | | PEP | | | | |

## 2.5 Pathological and clinical features of tauopathies

PSP is the most extensively studied of the non-amyloid tauopathies, and has a distinctive topographical and molecular pathology. The cellular hallmark of the disease is neurofibrillary degeneration; and the distribution and intensity of damage to the brain stem and basal ganglia is used to pathologically define the syndrome.[104] The main lesions are in the substantia nigra pars compacta and reticulata, the globus pallidus, the subthalamic nucleus, and the midbrain and pontine reticular formation. This destruction of midbrain and pontine structures leads to identifiable changes of brainstem atrophy on neuroimaging with

dilatation of the cerebral aqueduct, thinning of the midbrain tegmentum and dilatation of the fourth ventricle.[105] A recent blinded study reviewing differentiation of parkinsonian syndromes using MRI indicates that dilatation of the third ventricle and a midbrain diameter of less than 17 mm are useful in distinguishing PSP from other atypical parkinsonian syndromes.[106] The brainstem reticular pathology in PSP includes the midbrain and pontine nuclei involved in the supranuclear control of gaze: the rostral interstitial nucleus of the medial longitudinal fasciculus, the interstitial nucleus of Cajal, the nucleus of Darkschewitsch and the raphé nucleus interpositus.[107, 108] Additionally, the cholinergic pedunculo-pontine nucleus is damaged which is thought to have a role in the control of sleep and balance.[109] Contrary to the earliest reports, cortical pathology does occur in PSP and is most marked in the deepest cortical layers of the pre-central gyrus, occurring to a lesser extent in pre-frontal areas.[110] However, clinical and functional imaging data indicate that the major cognitive deficit in PSP is in frontal function and, given the distribution of cortical NFT formation, this presumably relates in part to a disturbance of reciprocal thalamo-cortical connections.[111, 112]

CBD, PEP, PDC and to a lesser extent PiD all share a similar propensity to damage the globus pallidus and substantia nigra.[113-115]. Similarly, pathological studies of Niemann-Pick disease type C, which often presents with dystonia, tremor and eye movement disorders, show that NFT formation in this condition may preferentially affect the basal ganglia and brainstem.[116] Although brainstem and basal ganglia NFTs in SSPE and PTP are less well documented, the involvement of the brainstem in SSPE and the substantia nigra in PTP is recognized.[117, 118] The clinical observation that many of these conditions affect the supranuclear control of gaze further suggests that these diseases, all of which involve tau protein deposition, share similarities in their topographic pathology.[114, 119-121]

Cortical damage and glial pathology is more variable and allows clinical and pathological distinction to be made between these conditions. CBD and PiD are distinctive for their asymmetric fronto-parietal and fronto-temporal atrophy respectively, whereas more mild involvement of the deepest cortical layers,

particularly the pre-central gyrus is seen in PSP. [114, 122] Amnesia may be a prominent feature in PTP and PDC with mesial temporal pathology involving the hippocampus, entorhinal and trans-entorhinal cortex, whereas these areas are relatively spared in PSP and CBD.[118, 123] Distinctive glial inclusions have been reported to be relatively specific for some diseases and these seem to relate to compartmentalization of tau within glial cells. In PSP tau accumulates in the cell body producing the tufted astrocyte appearance; in CBD tau accumulates at the distal astrocytic processes producing astrocytic plaques; and in PiD tau is distributed more diffusely through the cell soma.[124] Although distinctions can be made between these diseases, pathological and clinical similarities suggest that cell groups in the basal ganglia and brainstem share a common mode of cell damage, with abnormal tau accumulation. These cell groups may respond in a standard way to diverse underlying primary pathogenic processes or be linked by a similar aetiology. The background and clinical features of PSP will be described in further detail.

PSP: Historical aspects

PSP was first described as a distinct clinico-pathological entity by John Steele, J Clifford Richardson and Jerzy Olszewski in 1963 following Richardson's clinical observations on several patients in Toronto in the late 1950s.[119, 125] Although experienced neurologists at that time were unable to categorize the syndrome, a number of reports from the early twentieth century indicate that it is not a new disorder.[126, 127] An early photograph showing the typical posture of PSP has been identified,[128] and review of the film archives of Denny-Brown have shown a number of cases which can be identified to have been PSP.[129] The clinical acumen of Steele and Richardson together with the expertise of Olszewski in delineating brain stem anatomy allowed this "new" clinico-pathological entity to be described and their seminal report was followed by many case reports and case series from around the world.[130] The documentation of these individual cases and case series through the 1960s, 1970s and 1980s have been followed more recently by epidemiological studies.[131, 132]

PSP: Clinical diagnosis

PSP is frequently misdiagnosed, most commonly as PD, but when PSP is considered, its distinctive features usually allow a confident diagnosis. Most patients present with gait disturbance and unsteadiness with a tendency to fall backwards. The gait has a characteristic reeling or staggering quality, due to the stiff posture of the trunk and neck with irregular large steps forward, which allow a distinction to be made from the veering broad based gait of cerebellar ataxia.

Some patients present with early complaints of visual disturbance which are related to fixation instability and disruption of the control of saccadic eye movements. Unfortunately, as visual acuity itself is not affected by PSP, these symptoms may be initially thought to be psychogenic in origin until the typical disturbance of downward gaze emerges. The neuro-ophthalmological features of patients with established PSP are usually clear cut.[133] Frontalis overactivity and a diminution of the blink rate to less than 4/minute lead to a "surprised" facial appearance. Eye opening may be impaired either by active involuntary contraction of orbicularis oculi (blepharospasm) or by inability to voluntarily open the eyes ("apraxia of eyelid opening").[134] Fixation on a stationary object may be interrupted by visible constant velocity saccadic intrusions in which the gaze is diverted briefly away and then back to the target, described as square wave jerks.[135] In the earliest stages of the disease there may be slowness of vertical saccadic eye movements which progresses to limitation of downwards vertical saccadic eye movements and then to a complete vertical gaze palsy.[133] The doll's head manoeuvre may be used to generate a normal vertical vestibular-ocular response demonstrating the integrity of the third nerve nuclei and confirming that the eye movement disorder is supranuclear. Some limitation of upgaze is a frequent accompaniment of normal ageing and may be seen in PD, and so limitation of downgaze is a much more specific finding suggestive of PSP. In the late stages of the disease, involvement of the horizontal eye movement system may lead to a complete supranuclear gaze palsy.[133]

In established classical cases the extrapyramidal features of PSP differ from those seen in PD. Lack of spontaneous and associative movements, dysarthria

and facial immobility may suggest the diagnosis of PD during conversation and history taking. However, careful observation and examination often reveals a taut spastic face, a growling dysarthria, neck held in extension with axial rigidity, and a symmetrical, relatively mild distal bradykinesia often in the presence of normal muscle tone and in the absence of rest tremor. PSP rarely responds to L-DOPA therapy and it should be considered in the differential diagnosis of L-DOPA unresponsive Parkinsonism.

While amnesia resulting from mesial temporal damage is not a feature of PSP, history from relatives and carers and specific bedside tests may reveal cognitive impairment. Functional imaging and clinical psychological studies show frontal hypometabolism and a weakness in frontal lobe tasks respectively.[112] There may be a prodromal history of personality change or difficulty in carrying out day to day tasks which reflect frontal/subcortical disinhibition, apathy, irritability or difficulties in planning or judgment. Emotional lability with aggressive outbursts are common. Bedside testing may reveal difficulty in performing a three stage command and markedly impaired verbal fluency in initial or category naming tests. Neuropsychological testing is often characterised by profound slowing of responses, with correct replies eventually being produced when sufficient time is allowed (bradyphrenia).[111]

In the later stages of PSP increasingly severe dysarthria and dysphagia occurs. Although these features are usually described as being part of a pseudo-bulbar palsy and brisk jaw and facial jerks may be present, the aetiology of these bulbar features is probably multifactorial with a contribution from damage to extra-pyramidal, pyramidal and brainstem reticular structures.

PSP: Differential diagnosis

Aside from PD a number of other conditions may be misdiagnosed as PSP, usually on the basis of a parkinsonian syndrome with gaze abnormalities. These conditions include CBD, MSA, progressive subcortical gliosis, some forms of autosomal dominant cerebellar ataxia (particularly spinocerebellar ataxia (SCA)-7 and SCA-2), and vascular pseudo-parkinsonism. Whipple's disease is important to consider since, although rare, it is a treatable cause of progressive

neurological disease with a gaze palsy and may be diagnosed by small bowel biopsy or positive cerebrospinal fluid polymerase chain reaction (PCR) for *Tropheryma whippeli,* in the appropriate clinical context. In younger patients Niemann-Pick disease type C and occasionally other storage disorders may present in a similar way to PSP. In the elderly population vascular pseudo-parkinsonism is common. Vascular pseudo-parkinsonism and communicating hydrocephalus may be identified by a shuffling small stepped gait with a good arm swing ("lower body parkinsonism"; "marche à petit pas"),[136] and relative sparing of axial and upper limb function. Rarely, neurosyphilis and compressive midbrain lesions may produce a midbrain neuro-ophthalmologic disorder and these conditions need to be excluded with syphilis serology and neuro-imaging.[137]

National Institute for Neurological Disease (NINDS) operational criteria for the research diagnosis of PSP have recently been formulated.[138] These criteria focus on postural instability and evidence of damage to the vertical gaze system as the two core features of the disease together with the absence of clinical or investigative features suggesting an alternative diagnosis. These criteria have been reported to have an 80% sensitivity and specificity but potentially may exclude patients with PSP who develop behavioural or personality change significantly before the occurrence of a gait disorder and those who have atypical presentations without a supranuclear gaze palsy.[139, 140]

PSP: Epidemiology

The first epidemiological study of PSP reported a prevalence of 1.4/100,000 in a New Jersey based study, but this is likely to be an underestimate because of the exclusion of "atypical parkinsonism", misdiagnosed as PD. Additionally this study relied on active notification of cases from family practioners, nursing homes and neurologists.[131, 141] Population based studies, investigating the prevalence of PSP within a large London general practice have revealed a prevalence rate for PSP of 6.4/100,000.[142] A more recent general practice based study in Newcastle has confirmed that the prevalence of PSP in the UK urban population lies at around 6/100,000.[143] The median life expectancy from

symptom onset to death is nine years.[131] The 1991 UK Parkinson's disease society brain bank study showed that 25% of clinically diagnosed PD cases, had alternative pathological diagnoses, and PSP was the commonest misdiagnosis accounting for around 6% of the cases in this series.[141] Studies of this type suggest that PSP may be substantially under diagnosed and that the true population prevalence of PSP may be much greater than has been reported. Case control studies have not demonstrated any definite risk factors for the disease.[144, 145]

PSP: Functional anatomy

The renaissance of neurosurgical approaches for PD and the study of primate models of extrapyramidal dysfunction have led to reconsideration of the functional pathology of the basal ganglia. Current models are inconsistent in their explanation of the symptomatology of potentially the "simplest" disease, PD,[146] and the functional anatomy of PSP is even more complex and poorly understood. PSP involves damage to both the putaminal and caudate striatal projections of the substantia nigra pars compacta (SNpc), the ventro-lateral and dorso-medial areas respectively.[9] This homogeneous depletion of the SNpc is in contrast to the ventro-lateral nigral selectivity of PD, which preferentially damages the putaminal nigro-striatal projection. This anatomical difference can be visualized in vivo with PET imaging of loss of 18-F-DOPA uptake by both caudate and putaminal nigro-striatal nerve terminals in PSP as opposed to preferential loss of putaminal uptake in PD.[147] The treatment of PD with L-DOPA is thought to lead to a restoration of normal output from the basal ganglia with attenuation of pallido-thalamic GABAergic inhibition, but in PSP the major output centres of the basal ganglia, the substantia nigra pars reticulata and the globus pallidus internus, are already severely damaged by the underlying disease process.[148] The subthalamic nucleus has been shown to be overactive in Parkinson's disease and lesioning or high frequency stimulation (producing suppression) of the subthalamic nucleus is proving to be a highly successful manoeuvre in ameliorating the tremor, bradykinesia and rigidity of Parkinson's disease.[149] The subthalamic nucleus is also damaged in patients with PSP.[150] This destruction of the major basal ganglia output areas and the subthalamic

nucleus, which are intact and overactive in PD, presumably contributes to the clinical differences between PSP and PD.

PSP: Treatment

Currently, supportive treatment is the mainstay of management. Explanation of the diagnosis and contact with patient support groups may benefit patients, particularly as they may have been misdiagnosed as having PD or other disorders earlier in their illness. Physiotherapy and occupational therapy are of importance in helping with aids for balance and avoidance of falls. The early identification of problems with swallowing is important which should prompt referral to speech therapy services for swallowing assessment and advice on appropriate measures to avoid the complications of aspiration.[151] Some patients and their families benefit from the insertion of a percutaneous gastrostomy (PEG) tube. Additional communication aids such as light-writers are usually not of benefit because of the concurrent eye movement disorder.

Patients usually do not derive great benefit from dopaminergic medication due to widespread damage to structures in the basal ganglia, but an eight week trial of high dose L-DOPA should always be tried.[152] Early reports suggested that amantadine may be of more benefit in improving the motor deficits in PSP, but this has not been subject to a formal randomised trial and the response is at best modest. Most interest in PSP has centred on the use of cholinergic treatments, particularly because of the suggestion that cholinergic nuclei may be responsible for the problems with balance.[153] Although oral physostigmine and cholinergic agonists do not produce a useful symptomatic benefit in PSP,[154, 155] intravenous physostigime has been shown to improve cerebral metabolism,[156] and some measures of neuropsychometric and oculomotor performance.[157] These data suggest that if significant enhancement of central nervous system cholinergic transmission can be achieved then some symptomatic improvement might be attained. However, a recent trial of donepezil in PSP has not demonstrated a useful functional improvement in response to donepezil treatment.[158] Adrenergic agents have also been used in PSP because of the adrenergic deficit resulting in part from damage to the locus coeruleus.[159] Although these agents were initially

thought to improve motor performance, this has not been replicated and their use has been limited by the occurrence of cardiovascular side effects.[159, 160]

## 2.6 Conclusions

PSP and AD are the best studied of a range of conditions which involve tau deposition. Biochemical analysis of tau deposition reinforces the differences between the tauopathies. Combined with the evidence from clinico-pathological correlation in AD, this suggests that tau may have a central role in the pathogenesis of a range of neurodegenerative conditions. Consideration of the treatment of PSP emphasises the ineffectiveness of symptomatic treatments for neurodegenerative diseases and the pressing need to develop new disease modifying treatments. The examples of AD and PD suggest that molecular pathology and genetics are the best ways to approach developing theories of disease pathogenesis and new therapies. The remainder of this thesis describes the application of molecular genetic techniques to the investigation of tau related neurodegeneration, correlated with pathological and clinical data.

# CHAPTER 3 Methods

## 3.1 DNA extraction

DNA was extracted from frozen brain and blood using a proteinase K/phenol-chloroform extraction method. In our laboratory this protocol has provided higher DNA yields with less protein contamination than methods using commercially available kits.

### Blood

Ten mls. of EDTA stored venous blood was diluted to 50 mls. with reagent A and spun at 10,000 rpm for 15 mins. The supernatant was removed, the white cell pellet retained and the reagent A wash step repeated. The white cell pellet was incubated with 2.5 mls Lysis Buffer (Applied Biosystems) and Proteinase K (Sigma) overnight at 37°C. following vortex mixing.

### Frozen brain

DNA was extracted from frozen brain using an overnight protein digestion step. 100-200 mg brain was finely cut and mixed with 500 µl brain buffer, 25 µl 10% Sodium Dodecyl Sulphate (SDS) and Proteinase K. It was incubated overnight at 37°C following vortex mixing.

The following day 2.5 mls (blood) or 500 µl (brain) Phenol/Chloroform/Isoamyl alcohol 25:24:1 (Applied Biosystems) was added to the lysed pellet mix, mixed by hand for 5 minutes and spun at 5000 rpm for 10 minutes. The supernatant was removed, taking care not to remove the protein containing interface, and the phenol step repeated. Finally, the supernatant was mixed with 2.5mls (blood) or 500 µl(brain) of chloroform and spun at 5000 rpm, in order to removed the phenol. The aqueous phase supernatant was added to 1.5mls of ethanol containing 250 µl 7.5 M Ammonium Acetate, pre chilled to -70°C. The visible

DNA pellet was hooked out using a plastic pipette and resuspended in 400 µl Tris-EDTA.

**DNA quantification**

DNA was quantified by mixing DNA stock solution with water and quantifying the dilution with UV spectrophotometer absorption. The absorption at 260 nm indicates the concentration of DNA in the sample, whereas the concentration at 280 nm indicates the (contaminating) protein concentration. The ratio of absorption at 260 and 280 nm provides an index of the purity of the DNA sample, ratios of >1.5 indicating relatively pure DNA. For a dilution of 5 µl stock DNA in 300 ul of water, 3000 x absorption at 260 nm = DNA concentration in ng/µl.

## 3.2 PCR

The polymerase chain reaction (PCR) is a DNA amplification method developed by Mullis which allows the amplification of specific DNA sequences from a starting DNA template, such as genomic DNA to allow further analysis and manipulation.[161] It relies on the properties of DNA polymerase in synthesising a complementary DNA strand to DNA template starting at a bound complementary DNA oligonucleotide primer. Specificity for the reaction is provided by the forward (anti-sense) binding and reverse (sense) binding primers. Forward and reverse primers of sequence length greater than 18 nucleotides are normally sufficient to ensure specificity for a unique DNA segment within the genome. The PCR reaction involves a denaturing step at 95°C, an annealing step usually at 40-66 °C which allows primer binding, and an elongation step at 72°C for complementary strand synthesis. The reaction mix is cycled through the described temperature changes which leads to an exponential growth of PCR product, until the enzyme or deoxyribonucleoside triphosphates (dNTPs) are exhausted. The reaction can be automated with the use of thermostable ***Taq DNA polymerase*** which is active at 72°C and not substantially degraded by exposure to 95°C. The reaction mix includes a standard PCR buffer at a final

concentration of 50 mM KCl, 10 mM Tris Cl and 1.5 mM $MgCl_2$, and dNTPs at a concentration of 200 μM which provide both energy to drive the PCR reaction and nucleotides for sequence extension. The PCR reaction is usually started with a standard denaturing period of 5 min. at 95°C and concluded with a 10 min. final extension period at 72°C. The main variables which can be altered are the specific annealing temperature for the primer pair, the number of PCR cycles, the length of the PCR cycle extension phase and the magnesium concentration. A one minute extension time is allowed per kb of DNA amplified. Primer sequences are designed so that forward and reverse sequences have similar G+C content, minimal secondary structure and a low level of self complimentarity. Ideally, long runs of single bases are avoided and the 3' end of the primer contains a G or C. The annealing temperature of the primer can be estimated from Tm = 4(A+T) + 2(G+C).

Three types of proprietary Taq DNA polymerase were used for standard PCR reactions: Promega Red Taq (Promega), Perkin Elmer AmpliTaq Gold (Applied Biosystems) and Qiagen Taq with Q solution (Qiagen). Of these three types of Taq, Qiagen Taq polymerase was found to give the most reliable and reproducible results and was used as the standard Taq for routine laboratory work. Q solution is a proprietary Qiagen chemical which works in a similar way to dimethyl sulphoxide (DMSO) in enhancing PCR efficiency by weakening disulphide bonds and DNA secondary structure.

An adaptation of the standard PCR protocol was found to be particularly helpful in rapidly optimising PCR reactions – "touchdown" PCR. This involves altering the primer annealing temperature systematically through the PCR process. The standard PCR reaction used was touchdown 60-50. In this reaction the initial annealing temperature is set at 60°C and in each subsequent PCR cycle the annealing temperature decreases by 0.5°C over 20 cycles to 50°C. A final rounding off series of cycles is made (usually 12) with the annealing temperature set at 50°C. The advantage of this PCR method is that the initial PCR cycles take place with a high annealing temperature with assures high specificity binding to the genomic DNA template. Later in the PCR sequence the annealing

temperature is lower, but the DNA template is predominantly newly synthesised PCR product so false binding to similar but incorrect template sequences is not likely to be a problem. The PCR touchdown technique was found to be highly effective. Primers and standard conditions for PCR reactions are given in Table 3-2.

**Table 3-1 Standard PCR mix**

| | Microsatellite markers | | Sequencing |
|---|---|---|---|
| | Perkin Elmer | Qiagen | Qiagen |
| 10x buffer | 0.75 µl | 1 µl | 5 µl |
| Mg | 0.75 µl | - | - |
| DNTPs | 0.75 µl | 1 µl | 5 µl |
| Q sln | - | 2 µl | 10 µl |
| PrimerA | 0.5 µl | 0.3 µl | 2 µl |
| PrimerB | - | 0.3 µl | 2 µl |
| DNA | 4 µl (25 ng) | 4 µl | 1 µl (50 ng) |
| Taq | 0.07 µl | 0.05 µl | 0.25 µl |
| Water | 0.68 µl | 1.53 µl | 24.75 µl |
| Total Volume | 7.5 µl | 10µl | 50 µl |

Primers kept as working dilutions of 20 µM (pM/ul), Qiagen 10x buffer contains $MgCl_2$ at 1.5 mM.

**Table 3-2 Primers and PCR conditions**

| PCR product | Forward primer | Reverse primer | Size bp | TD |
|---|---|---|---|---|
| Tau exon 1 | CAA CAC TCC TCA GAA CTT ATC | CAG TGA TCT GGG CCT GCT GTG | 229 | 60-50 |
| Tau exon 2 | CAG CTC CAC AGG ACA CTG CTC | GGA GTG AGC ACA TCT CTC AG | 298 | 60-50 |
| Tau exon 3 | GGG CTG CTT TCT GGC ATA TG | CCT CAC TTC TGT CAC AGG TC | 297 | 60-50 |
| Tau exon 9 | CGAGTCCTGGCTTCACTCC | CTTCCAGGCACAGCCATACC | 370 | 60-50 |
| Tau exon 10 | GGT GGC GTG TCA CTC ATC C | GTA CGA CTC ACA CCA CTT CC | 200 | 60-50 |
| Tau exon 11 | GCT CAT TCT CTC TCC TCC TC | GCA GTT CCA GCC TCA CCA GG | 188 | 60-50 |
| Tau exon 11 mismatch | GCT CAT TCT CTC TCC TCC TC | CCA GGA CTC CTC CAC CCC ATG CAG C | 173 | 60-50 |
| Tau exon 12 | GTC CTG TCA TTG TCT TCT TC | ACC CAC TGG ATG CTG CTG AG | 437 | 60-50 |
| Tau exon 13 | CTT TCT CTG GCA CTT CAT CTC | CCT CTC CAC AAT TAT TGA CCG | 299 | 60-50 |
| Tau exon 13 long | ACTTCATCTCACCCTCCCTC | CCTCTCCTTCTCCCTCTTCTAC | 597 | 60-50 |
| Tau deletion | GAAGACGTTCTCACTGATCTG | GGTTTGAGAAGCCCTGACC | 420/181 | 60-50 |
| NACP Pro | Hex GCAATAGAGTAGACAAAAGGATGG | CTACATGACTGGCCCAAGATTAA | 257-261 | 50-45 |
| NACP Int | Fam ATTCTTCTCACCTCTGGTATC | TTAAAGGTGAATAACACTTTGGC | 265-366 | 50-45 |
| ApoE | TCCAAGGAGCTGCAGGCGGCGCA | ACAGAATTCGCCCCGGCCTGGTACACTGCCA | | 60-50 |
| GFAP x5 | CATATGTGTCCCCCACCTAG | GCCCTGGCCCGCGCTCA | 163 | 50-45 |
| D17S800 | Hex GGT CTC ATC CAT CAG GTT TT | ATA GAC TGT GTA CTG GGC ATT GA | 168-178 | 60-50 |

| PCR product | Forward primer | Reverse primer | Size bp | TD |
|---|---|---|---|---|
| D17S1793 | Fam CCAGCCCAAGGTTTACATC | CACCAGTTCCCTGCGAG | 193-203 | 65-55 |
| Tau intronic polymorphism | Fam GCC TCG CAA ATT GCT GGG AT | AGG TGA CTG GGT AGA GAC AGA GC | 143-151 | 60-50 |
| D17S1868 | PE Linkage Set 2 | PE Linkage Set 2 | 300-324 | PE |
| D17S902 | Ned GAGGTTGCAGTGAGTTGAGA | GGA ACA TCC TCC TTC ACT CTT | 155-165 | 60-50 |
| D17s931 | Ned AACATTTGCTTGGGGCT | GGT GAG TAG TGC TCT GGG AA | 228-238 | 60-50 |

TD – Touchdown PCR protocol, PE – Perkin Elmer

## 3.3 Genotyping

Genotyping of sequence variation was performed by restriction enzyme digestion of PCR products, in which a variable site introduced a change in a restriction enzyme recognition site. The suppliers buffers and reaction conditions were used and the reaction products were visualised on a 3.5% agarose gel containing ethidium bromide. The ApoE digestion products were visualised on a polyacrylamide gel (BioRad). Expected digest patterns were evaluated using the WebCutter program available at: http://www.ccsi.com/firstmarket/firstmarket/cutter/cut2.html.

**Table 3-3 Polymorphism Analysis**

| | Polymorphism | Enzyme | A allele/Wild type | B allele/Mutant |
|---|---|---|---|---|
| Tau | | | | |
| Exon 1 | A→G 13 bp before start Met | Alu I | 229 bp | 183, 46 bp |
| Exon 2 | C→T 18bp after 3' end | Bsa HI | 201, 97 bp | 298 bp |
| Exon 3 | A→G 9 bp after 3' end | Ban II | 165, 132 bp | 165, 68, 64 bp |
| Exon 9ii | T→C nucleotide 765 (Asn255Asn) | Tai I | 370 bp | 127, 243 bp |
| Exon 9ii | G→A nucleotide 810 (Pro270Pro) | BstN I | 257, 54, 26, 20, 6 bp | 201 ,56, 54, 26, 20 bp |
| Exon 11 | G→A 34 bp after 3' end | Alu I | 173 bp | 149, 24 bp |
| Exon 13 long | T→C nucleotide 290 of exon 13 | Tsp501 I | 276, 94, 78, 72, 62, 15 bp | 370, 78, 72, 62, 15 bp |
| P301S | C →T nucleotide 116 of exon 10 | Sma 1 | 118, 82 bp | 200 bp |
| Exon 10+16 | C →T nucleotide 148 of exon 10 | Nsp1 | 200 bp | 148, 52 bp |
| Exon 10 +14 | C →T nucleotide 146 of exon 10 | Afl III | 110,56,34 bp | 110, 90 bp |
| GFAP exon 5 | G→A nucleotide 858 GFAP gene | Cfo I | 100,39,24 bp | 139,24 bp |

**Table 3-4 ApoE Genotypes, Cfo I digest**

| Genotype | Fragment sizes (bp) |
|---|---|
| Uncut | 227 |
| 2/2 | 91, 81 |
| 2/3 | 91,81,48 |
| 2/4 | 91,81,72,48 |
| 3/3 | 91,48 |
| 3/4 | 91,72,48 |
| 4/4 | 72,48 |

Genotyping of microsatellite polymorphisms was performed using PCR with fluorescent labelled primers (NED, HEX, FAM) followed by electrophoresis of the PCR product on the Applied Biosystems 377 DNA sequencer. Genotyping gels were run with a 4% urea/polyacrylamide gel. The fluorescent dyes are chosen to be compatible with the ABI filter set fluorescence system. Selection of PCR products which either are labelled with one of three available different dyes or which are non overlapping in size allows the analysis of up to 8-10 different PCR products in each lane. Each genotyping lane is run with an internal size standard, labelled with either the TAMRA or the ROX dye. Gels are scanned by a vertically fixed laser at the bottom of the gel plate and all the PCR products up to 500 bp in length run through the laser and into the bottom buffer chamber. Gels are then graphically represented as if they were run on a conventional run and stop electrophoresis system with the smallest PCR products at the bottom of the gel picture. Gels were analysed with Genescan software (Applied Biosystems) which allows manual tracking of the PCR products lane and then extracts the lane dye fluorescence data together with the size standard data to generate base pair sizes for each fluorescent band. Genotyping was performed using the semiautomated Genotyper software (Applied Biosystems) which "searches" for

the expected allele sizes base on entered maker data. Macros for genotyping, developed by Richard Crook, Mayo Clinic Jacksonville were used. Genotyping of SNPs and microsatellite markers was performed blinded to control/case status.

### 3.4 DNA sequencing

DNA sequencing was performed using the BigDye Terminator (Applied Biosystems) sequencing reaction kit and analysed on the ABI 377 sequencer. This uses a version of the Sanger sequencing method. The PCR product of interest is purified and passed through a modified PCR sequencing reaction in which fixed proportion of the dNTPs are dye terminators which do not permit further product extension and are labelled with a different fluorescent dye for each base. The result of the sequencing PCR reaction is to produce an array of PCR products of different length each one being coloured according to the terminal nucleotide base. Electrophoresis which is able to resolve the sequence products by one base pair will be able to generate a readable trace which indicates the DNA sequence.

Protocol

PCR products were purified with Qiaquick spin columns (Qiagen). 1μl sequencing primer (3.2 pM/μl) and 4 μl BigDye Sequencing reaction mix (PE-ABI) and 11 ul PCR product cycled using the manufacturers programme on either an ABI 9700 PCR machine or Techne PCR machine. Sequencing products were then precipitated by taking the 16 μl of sequence reaction and incubating with 80 μl 60% ethanol for at least 1 hour. The alcohol/sequence product mix was then spun for 45 minutes at 14,000 rpm. The ethanol was removed leaving adherent sequence product at bottom of tube. The wash step was repeated with 80μl 70% ethanol for 30 minutes then re-spun at 14,000 rpm. The alcohol was removed by and the tubes were then dried in a heating block for seconds at 95°C. The sequence products were dissolved with 4μl loading mix (formamide: loading blue, 5:1), prior to loading on the sequencer. Sequencing runs were performed on 4% urea/polyacrylamide gel, the 377 sequencer was run with the dRhod Big Dye

setting, 36E 1200; to run for 7hours <500bp, 10 hours >500bp. Around 1/3 of the sequencing reported in this thesis was processed from purified PCR product, at the core sequencing facilities at Mayo Clinic Jacksonville or Imperial College Applied Biotechnology Centre, and sequence traces returned for analysis. The remaining 2/3 was processed at Neurogenetics, Institute of Neurology. Sequence traces were processed using Factura software and aligned and compared for heterozygote/deletion detection using AutoAssembler software. Sequence traces were also viewed on PCs using GeneStudio software.

## 3.5 Genome wide search

Peripheral blood leukocytes from Chamorro cases and controls were immortalised in the laboratory of Prof. Teepu Siddique to provide a robust DNA source. Using purified DNA extracted from these cell lines, 834 fluorescent microsatellite markers were genotyped from the standard Perkin-Elmer Version 1 and Version 2 linkage mapping sets (ABI) and from the Research Genetics MapPairs Version 8 set. The DNA was diluted and set up in one half of a 96 well plate to facilitate the addition of DNA to the PCR reaction. Water controls and DNA from anonymous individuals of known genotype from the CEPH genotyping panels were included. Cases and controls were randomly distributed to allow blinded genotyping. 96 or 384 well plates were used for high throughput PCR. Perkin-Elmer and Research Genetics markers microsatellite markers were amplified according to the manufacturers instructions as indicated above and analysed using the ABI 377 DNA sequencer and software described above .

## 3.6 Statistical analysis

Power calculations were performed using the online proportion difference power/sample size calculator (http://members.aol.com/johnp71/proppowr.html). This calculator specifies the sample size needed to achieve a significance level ($\alpha$), with a given power (certainty) given the proportions in the two groups under comparison and the relative sample sizes. Comparison of bi-allelic

polymorphisms was made using the $\chi^2$ test, with Yates correction for expected values $\leq 5$. The analysis of multi-allelic microsatellite markers is more difficult using the $\chi^2$ test since sparsely filled tables with infrequent alleles reduce the power of the test because of the increasing degrees of freedom. In the initial analysis of the *tau* intronic microsatellite marker "clumping" was used, that is allele A0 and genotype A0A0 were analysed against all others. In this instance there was a prior expectation that the A0 allele and/or A0A0 genotype would be positively associated with PSP. For the determination of Hardy Weinberg equilibrium the observed vs. expected genotypes, based on the allele frequencies and the Hardy-Weinberg formula, were compared using the $\chi^2$ test. For the analysis of multi-allelic markers with no prior expectation of association the Likelihood Ratio Test developed by Terwilliger was used.[162] This can be used for single markers as the *dislamb* programme or for multiple markers in multipoint analysis using *dismult*. This program generates a likelihood ratio test $\chi^2$ (LRT) value, a p value normally taken to be significant and to indicate association if $p<0.0001$ and a $\lambda$ value indicating the strength of the association. These programs are both available as part of the *diseq* allelic association analysis package, freely available at www.mrc.hgmp.ac.uk.[162]

### 3.7 Reagents/Standard mixes

**Brain buffer** (0.05 M EDTA, 0.075 M Na Cl adjusted to pH 8.0):

***For 500 mls:*** 2.19 g NaCl, 50 ml. 0.5 M EDTA

**Reagent A:**

***For 1 L.:*** 320 mM sucrose, 5 mM Mg Cl2, 10 mM Tris-HCl, 1% Triton X1001L: 109.4 g sucrose, 1.02g MgCL2.6H20, 10 mls. 1M Tris, 10mls. Triton X 100, Autoclaved

**4% Acrylamide gel for sequencing:**

***For 50 mls.:*** Urea 18g, Nanopure water 27.5 mls., 40% acrylamide, 19:1 (Biorad), 5.3 ml, Amberlite resin (Sigma), 0.5 g

**10x TBE for sequencing:**

***For 1 L.:*** Tris 0.89 M (pH 8.3) 108 g., Boric acid 0.89 M., 55.0g, EDTA 0.02M 8.3 g

**10x TE:**

***For 1 L:*** 1.21g Tris, 0.37g EDTA, adjusted to pH 8.0

## 3.8 Suppliers

**ANACHEM Ltd.**
Anachem House
Charles St.
Luton
Bedfordshire
LU2 0EB

**BIO-RAD Laboratories**
Bio-Rad House
Maylands Avenue
Herts
HP2 7TD

**NEW ENGLAND BIOLABS (UK)**
73 Knowl Piece
Wilbury Way
Hitchin, Hertfordshire
SG4 0TY

**PE BIOSYSTEMS**
Kelvin Close
Birchwood Science Park North
Warrington

Cheshire
WA3 7PB

**PROMEGA**
Delta House
Chilworth Research Centre
Southampton SO16 7NS

**QIAGEN Ltd.**
Boundary Court
Gatwick Road
Crawley
West Sussex, RH10 9AX

**SIGMA-ALDRICH COMPANY Ltd.**
Fancy Road
Poole
Dorset
BH12 4QH

### 3.9 Ethical approval

Cases were referred from the dementia research group and movement disorders clinicians at the National Hospital and nationally following contact made to clinicians via the Association of British Neurologists.

Blood for DNA analysis was taken following informed consent and the projects were approved by the Ethics Committee of the National Hospital and the Institutional Review Board, Mayo Clinic Jacksonville.

# CHAPTER 4 Allelic association studies

## 4.1 Summary

This chapter is divided into five sections. A positive association between the A0 tau polymorphism and PSP is described. There is no association between A0 and pathologically diagnosed PD, or clinically diagnosed CBD and FTD. The relationship between the A0 polymorphism and other polymorphisms within *tau* were analysed. This variability across *tau* can be assigned to two common haplotypes, H1 and H2. These haplotypes indicate that linkage disequilibrium across extends for at least 100 kb in the *tau* region. Flanking markers around *tau* were analysed and provide no evidence for more extensive linkage disequilibrium or for a rare H1* haplotype associated with PSP. Pathologically diagnosed PSP cases were divided into clinically typical and atypical forms and the relationship between clinical presentation, tau western blot and clinical presentation is discussed. There was no association between α-synuclein, ApoE and PSP or MSA. No effect of *tau* or ApoE on the age of onset of PSP and MSA was found.

## 4.2 Introduction

Association studies describe the relationship between a disease and a polymorphic allele. By definition, polymorphisms are genetic mutations in which the commonest allele in the general population occurs at a frequency of 0.99 or less, i.e. there are at least two common variants in the general population, each of which occurs at a frequency of 1% or greater.[163] Association studies attempt to define situations in which an allele occurring in the general population is over-represented in the disease group. In this situation the disease associated allele does not cause a disease in the same way that a Mendelian high penetrance mutation does, but increases susceptibility to the disease, in conjunction with environmental or other genetic factors. Positive association studies, in which there is a significant difference between disease and control allele frequencies may occur, because: (i.) the disease associated allele has a direct effect on the

disease pathogenesis (or the non-associated allele has a protective effect) – a "true" association, (ii.) there is population stratification, that is there are underlying differences between the control and disease populations, most commonly ethnic, which lead to a spurious difference in allele frequencies in the two groups, (iii.) there is linkage disequilibrium between the disease associated allele and an adjacent functionally important allele.[164] Linkage disequilibrium arises in a population as a function of the relatedness of the population and is the frequent inheritance of a genetic region which has not been split by meiotic recombination, which may contain a number of linked polymorphisms. The average linkage disequilibrium within the outbred Caucasian population is estimated to extend between 6 and several hundred kb of DNA, although estimates of this vary.[165, 166] Population stratification can be difficult to detect but can be guarded against by ensuring that the case and control population are of the same race, and by ensuring that the groups studied are in Hardy-Weinberg equilibrium. Hardy-Weinberg equilibrium relates the genotype frequencies directly to the allele frequencies within the groups studied, and essentially assumes homogeneity between parental and offspring populations.[167] For a bi-allelic polymorphism with alleles **A** and **B** whose allele frequencies are $a$ and $b$ the genotypes **AA**, **AB** and **BB** would be expected to occur with a frequency of $a^2$, $2ab$ and $b^2$. The expected genotype frequency based on the Hardy-Weinberg equation can be compared with the observed genotype frequency using a $\chi^2$ test on the 2x3 table. The $\chi^2$ test in this instance has one degree of freedom since the allele frequency $a$ determines all expected genotype frequencies ($b = 1 - a$). A number of criteria have to be met in order that a population studied is within Hardy-Weinberg equilibrium: the absence of assortative mating; lack of migration into or out of the population of a second group with differing allele frequencies; no selection exerted on any of the genotypes. Less importantly, within the size of populations used for association studies no new mutations should enter the population and genetic drift should not alter genotype frequencies between generations.[167] A further possible confounding factor in the interpretation of association studies is the problem of multiple testing within and between studies, and publication bias.[164] Frequently a significance level of $p<0.05$, or a 1 in 20 probability of a result occurring by chance alone, is set as the level which

indicates a significant deviation from the null hypothesis, no difference between the control and disease groups. In a study which looks at five separate polymorphisms because of multiple testing the chance of any one of them reaching $p<0.05$ is 1 in 4. Conventionally, the significance level should then be made more stringent using the Bonferroni correction with the value set at $p<0.05/5 = 0.01$. This is not always done. Further problems occur when different groups analyse the same polymorphism. If twenty groups analyse a polymorphism which is not associated with a disease then by chance alone one group would be expected to detect an apparently significant difference at the 5% level. This creates significant problems in interpreting the literature, particularly when negative data is not published.

In 1997 Conrad and colleagues described the first evidence of a genetic link between ***tau*** and neurodegeneration.[168] Using Southern hybridisation of a $(CA)_{12}$ repeat they identified a complimentary TG repeat within the *tau* cosmid clone k6, which spans exons 4 to 9.[67] The long range PCR product size implied that this repeat sequence lay about 7 kbp downstream of exon 9 and 5 kbp upstream of exon 10. Variability at this polymorphism was analysed in normal controls, Alzheimer disease patients and 22 pathologically diagnosed PSP patients. They reported five alleles: A0 with 11 TG repeats and A1, A2, A3 and A4 defined by the presence of 12, 13, 14 or 15 TG repeats. Both the A0 allele and the A0/A0 genotype were positively associated with PSP, in comparison with normal controls and patients with Alzheimer's disease.[168] In control subjects 57.4% of genotypes were A0/A0 and 74.6% of alleles were A0, compared with 95.5% of PSP genotypes and 97.7% of PSP alleles, giving a reported odds ratio of 15.6 (Table 4-2). Following this publication, this finding was replicated in a further small North American series,[169] but there were no further insights into the function of this polymorphism or reports of its role in other tauopathies.

## 4.3 Analysis of the *Tau* A0 allele in a UK population

The *tau* A0 allele was analysed in a UK patient series. Patients with PSP were recruited from the PDS Brain Research Centre, London, through the PSP (Europe)

Association, and from movement disorder hospital specialists. Normal controls and controls with other neurodegenerative diseases were also studied. The study group consisted of 53 Caucasian PSP patients (25 pathologically diagnosed, 28 clinically diagnosed), 13 clinically diagnosed CBD patients, 32 clinically diagnosed FTD patients, 50 pathologically diagnosed PD patients and 75 pathologically confirmed European controls without neurodegenerative disease (Table 4-1). Clinically diagnosed patients were diagnosed using modified NINDS clinical criteria for clinically probable or possible PSP.[138] The NINDS PSP diagnostic criteria include a large number of exclusion points which may not be fully documented in clinical records, therefore, exclusion criteria were applied as available in case notes. This represents a modification of the application of the criteria, which are probably most accurately used in prospective studies. The pathologically diagnosed patients met the preliminary NINDS pathological criteria for the diagnosis of PSP, although in some cases these individuals had atypical clinical presentations or incomplete clinical details. The CBD patients had asymmetrical cortical syndromes (apraxia or cortical sensory loss) with parkinsonism. FTD patients met Lund-Manchester criteria for the diagnosis of FTD.[170] The control subjects were white brain bank control subjects, obtained from the UK PDS brain research centre, without neurological disease and with an average age at death of 72. These controls were also used in the subsequent case control studies described.

To investigate the proposed effect of the A0 polymorphism the frequencies of the A0 allele and A0A0 genotypes were determined in the control, PSP, PD, FTD and CBD groups. "Clumping" was used for the statistical analysis to avoid the loss of power resulting from infrequently occurring alleles and genotypes. Hardy-Weinberg equilibrium was calculated for the observed genotypes as compared to the expected genotypes derived from allele frequencies. The expected genotype occurrence was adjusted up to 1 for rare genotype frequencies. As there was a prior expectation that the A0 allele/A0A0 genotype would be associated with PSP comparisons were made of the A0 allele vs. all other alleles and the A0A0 genotype as opposed to all other genotypes (Table 4-1).

## Results

The A0 allele was over-represented in the PSP population as compared to the control population (0.91 vs. 0.73, $\chi^2$ 14.3, d.f.=1, p<0.001) and the A0A0 genotype was also significantly more common (0.84 vs. 0.53, $\chi^2$12.3, d.f. =1, p<0.01) (Table 4-1). This effect was less in the pathologically diagnosed group of PSP patients (A0A0 genotype frequency 0.72) and this was largely accounted for by the clinically atypical/incompletely documented group (A0A0 frequency in clinically typical group 0.85; clinically atypical 0.66). The polymorphism was in Hardy-Weinberg equilibrium ($\chi^2$ 0.92, d.f.=3, p>0.05). The other tauopathies studied, including CBD, showed no association effect for either the A0 allele ($\chi^2$ <2, d.f. =1, p>0.1) or the A0A0 genotype ($\chi^2$ <1, d.f. =2, p>0.5) in all comparisons. The odds ratio for the effect of A0A0 genotype on the development of PSP is 4.3 (95% confidence interval, 3.6-10.1). Because of the low prevalence of PSP in the general population the positive predictive value for the development of PSP in individual with an A0/A0 genotype is less than 1%.

**Table 4-1 Tau alleles and genotypes at the A0 polymorphism.**

| | Control | PD | PSP | FTD | CBD |
|---|---|---|---|---|---|
| N | 75 | 50 | 53 | 32 | 13 |
| Alleles | | | | | |
| A0 | 109 (0.73) | 75 (0.75) | 96 (0.91) | 51 (0.80) | 20 (0.77) |
| A1 | 12 (0.08) | 6 (0.06) | 4 (0.038) | 1 (0.016) | 0 (0) |
| A2 | 2 (0.013) | 1 (0.01) | 0 (0) | 2 (0.031) | 0 (0) |
| A3 | 27 (0.18) | 18 (0.18) | 6 (0.057) | 9 (0.14) | 6 (0.23) |
| A4 | 0 (0) | 0 (0) | 0 (0) | 1 (0.016) | 0 (0) |
| Genotypes | | | | | |
| A0A0 | 40 (0.53) | 29 (0.58) | 44 (0.84) | 20 (0.63) | 8 (0.62) |
| A0A1 | 9 (0.12) | 4 (0.08) | 4 (0.07) | 1 (0.031) | 0 (0) |
| A0A2 | 0 (0) | 1 (0.02) | 0 (0) | 2 (0.063) | 0 (0) |
| A0A3 | 20 (0.27) | 12 (0.24) | 4 (0.07) | 8 (0.25) | 4 (0.31) |
| A1A3 | 3 (0.04) | 2 (0.04) | 0 (0) | 0 (0) | 0 (0) |
| A2A2 | 1 (0.013) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| A3A3 | 2 (0.027) | 2 (0.04) | 1 (0.019) | 0 (0) | 1 (0.077) |
| A3A4 | 0 (0) | 0 (0) | 0 (0) | 1 (0.031) | 0 (0) |

This data was subsequently reanalysed using the LRT package used in later studies. This provides a basis for analysing the association of PSP with all alleles

of *tau* rather than a single allele. This confirmed the evidence for a significant association at the 1% level with LRT $\chi^2$ 12.8, p=0.00017, $\lambda$ 0.65.

## Discussion

This study confirms a positive association between the *tau* A0 allele and A0/A0 genotype and the development of PSP. Taken together with the other positive studies using different PSP and control patient groups,(Table 4-2) this association strongly supports the concept that the most common population wide variant at the *tau* intronic dinucleotide polymorphism, designated A0, is positively associated with PSP. The replication of this study by seven geographically separate studies reduces the risk that this positive result is due to population stratification, although it is possible that PSP patients represent a genetic subset of all Caucasians which may generate false positive associations because of genetic homogeneity. Statistically, the finding of a similar result in many separate studies greatly reinforces the evidence that there is a genuine allelic association between A0 and PSP. While the chance of making a type II error in a series of different studies is quite high, the chance of making a Type 1 error (rejecting the null hypothesis when it is true) in seven separate patient samples is exceptionally small.

**Table 4-2 PSP/tau association studies**

| Study | Ref | Ethnic group | Control/ PSP N | PSP/Control A0 allele frequency | PSP/Control A0A0 genotype frequency |
|---|---|---|---|---|---|
| Morris (this work) | [171] | Caucasian | 75/53 | 0.91/0.73 | 0.84/0.53 |
| Bennett | [172] | Caucasian | 36/30 | 0.93/0.76 | 0.87/0.61 |
| Conrad 97 | [168] | Caucasian | 61/22 | 0.98/0.75 | 0.96/0.57 |
| Conrad 97 | [168] | Chamorro controls | 39 | -/0.85 | -/0.75 |
| Conrad 98 | [173] | Japanese | 67/31 | 1.00/0.99 | 1.0/0.97 |
| Higgins | [169] | Caucasian | 86/24 | 0.92/0.76 | 0.83/0.53 |

| Study | Ref | Ethnic group | Control/ PSP N | PSP/Control A0 allele frequency | PSP/Control A0A0 genotype frequency |
|---|---|---|---|---|---|
| Lazzarini | [174] | Caucasian | 46/16 | 0.94/0.70 | 0.88/0.50 |
| Oliva | [175] | Caucasian | 50/30 | 0.93/0.73 | 0.87/0.52 |
| Okuizumi | [176] | Japanese | 54/13 | 1.00/1.00 | 1.00/1.00 |
| Hoenicka | [177] | Caucasian | 79/16 | 0.97/0.69 | 0.94/0.47 |
| Baker | [178] | Caucasian | 139/64 | 0.90/0.71 | 0.80/0.51 |

In contrast to the studies of Caucasian groups similar case control studies in the Japanese populations have not demonstrated a *tau* – PSP association. Two studies reported no significant difference between the PSP and control A0 allele frequency in Japanese groups (Table 4-2).[173, 176] This lack of association has been interpreted as suggesting either that there is a significant linkage disequilibrium effect in the Caucasian population, not present in the Japanese, between the A0 allele and an adjacent functionally important variable site, or that there is a difference in the role of *tau* in the development of PSP in Caucasian and Japanese populations.[173, 176] These Japanese studies highlight the importance of the background allele frequency in genetic association studies. The A0 allele is virtually ubiquitous within the Japanese population, and therefore genetic association studies would not be expected to establish allelic association even if there was a significant biological effect. The background frequency problem can be illustrated by the analogy of an island in which smoking was virtually ubiquitous. In this instance the association between smoking and lung cancer would be very difficult to determine in case control studies.

*Tau* is an excellent candidate gene for determining susceptibility to PSP as tau is the major constituent of neurofibrillary tangles in PSP, and subsequent work showing that mutations in *tau* are responsible for FTDP-17 reinforces the likely primary role of *tau* in neurodegeneration. Given the strength of *tau* as a candidate

gene for PSP the more likely explanation for the apparent inconsistency between the Japanese and Caucasian studies would be that the biological effect of tau predisposition to PSP is identical, but that this effect is not detectable against the background Japanese allele distribution. A better test of a possible biological role for the A0 allele/*tau* in the Japanese population would be an epidemiological study which could accurately compare the prevalence of PSP among the Japanese to the prevalence of PSP among Caucasians, and to relate these difference to the population genetic differences. The working hypothesis would be that the population genetic differences between the Caucasian and Japanese populations lead to an approximately doubling of the prevalence of PSP among Japanese population. Clearly, epidemiological work of this type presents formidable methodological problems, and to date the population prevalence of PSP in Japan has not been reported.

This study was the first to investigate the A0 polymorphism in FTD and CBD, in a small number of clinically defined cases, and shows no association between this allele and the development of these diseases. At the time that this study was carried out it was felt that CBD was more likely be pathologically homogenous than FTD. The lack of association between CBD and A0 was somewhat surprising since CBD is pathologically very similar to PSP. Both of these conditions involve the deposition of four repeat tau (Table 2-1). However, recent studies have described a number of different pathological substrates which can underlie the CBD phenotype, including PiD which involves a different type of tau deposition.[179, 180] A follow up study to this one was performed by Houlden and colleagues, crucially including only pathologically defined cases of CBD, and including three pathologically confirmed cases from the current work.[181] The rarity of CBD entailed using an international consortium to ascertain 57 pathologically diagnosed CBD cases and showed that pathologically defined CBD is indeed positively associated with the A0 allele of *tau.* This important work implies that CBD and PSP share some common pathogenic processes and also suggests that that the CBD cases described in this initial UK clinically defined CBD study were pathologically heterogeneous. The CBD-A0 association was confirmed in a study including a smaller number of pathologically diagnosed

cases.[182] While CBD is by definition a tau deposition disorder FTD is considerably more heterogeneous and may include a number of different pathological entities.[183] The lack of association between A0 and FTD may be accounted for by different types of pathologies (i.e. tau and non-tau) as well as by different pathological processes leading to tau deposition (i.e. PiD and non-PiD).[100]

How can the association between one allele of a microsatellite marker within *tau* be explained and further explored? Microsatellites are a type of repetitive DNA within the genome. Microsatellite polymorphisms are thought to arise through slippage events during meiosis and lead to variability in the length of genomic di-, tri- and tetra-nucleotide repeats in the general population. They are not generally known to have functional effects, although their polymorphism has been invaluable in family based genetic linkage studies. The *tau* A0 polymorphism lies deep within an intron and is distant from known transcription or splicing control mechanisms, so initially it seemed unlikely that it could have a direct role in disease pathogenesis.[168] However, the position of the intron containing the A0 allele suggested a potential role. The polymorphism lies 5' of the alternatively spliced exon 10. Potentially this area could directly govern alternative splicing of *tau* exon 10, or confer susceptibility to some *cis* acting factor. This is a plausible aetiological mechanism, since as previously described, in PSP NFTs consist predominantly of four-repeat tau protein,[100] and therefore a change in *tau* splicing could drive the pathogenesis of PSP. However, although intronic splice enhancers, consisting of repeated elements have been described, these usually occur much closer to the alternatively spliced exon.[184]

An alternative possibility is that the A0 polymorphism is in linkage disequilibrium with an adjacent element which confers susceptibility to PSP. Linkage disequilibrium is the process by which genomic polymorphisms which are physically close are inherited together in apparently unrelated individuals in a general population. As discussed, estimates of the amount of linkage disequilibrium vary between different populations and in different parts of the genome.[185] If a linkage disequilibrium effect is responsible for the A0 allelic association with PSP then there are two possible explanations. Firstly, that a rare

mutation has occurred on a A0 chromosome which is geographically widely distributed in the worldwide population, as a subset of A0 chromosomes, and leads to PSP. In this circumstance it will be possible to identify a rare sequence change on the A0-PSP chromosome (designated H1*) which leads to the disease and which distinguishes the H1* chromosome from other A0 chromosomes (designated H1). Additionally, because of linkage disequilibrium effects reflecting the more recent occurrence of the PSP-tau mutation, polymorphisms on the H1* chromosomes will be separable from the population wide, more common non-pathogenic H1 chromosomes.

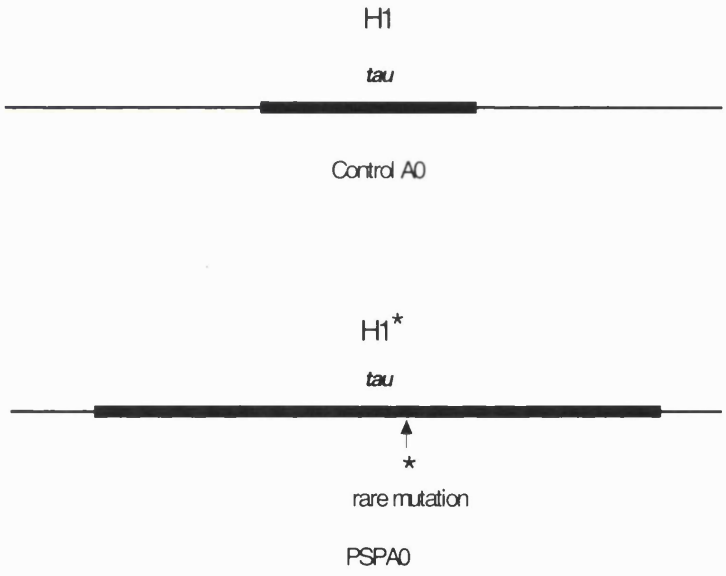



**Figure 4-1 Rare mutation vs. population wide susceptibility to PSP**

**Bold bar indicates shared chromosomal segment of linkage disequilibrium, which extends over a further distance in the H1* chromosome, as the rare mutation has arisen more recently on a subset of H1 or A0 chromosomes.**

The second possibility is that the susceptibility to PSP is conferred by the common population wide H1, which because of the low prevalence of PSP, can only contribute in a small way to the aetiology of PSP. Considering this from the perspective of diagnostic tests under the first possibility some H1* marker alleles, will be less frequent, and have a higher positive predictive value for the

development of PSP than H1 marker alleles. The pathogenic gene mutation will be a relatively highly penetrant mutation. Under the second possibility, the H1 chromosome will again be associated with a raft of DNA variability but these alleles will be approximately equally prevalent in the general population H1 chromosomes, and each of them will have a low positive predictive value for the development of PSP. These will be sequence changes of low penetrance which require a number of other genetic or environmental factors to determine the development of disease. These two possibilities should be separable on genetic grounds, by further analysis of the *tau* region.

Finally, the possibility that PSP is a recessive condition needs to be considered.[169] In Mendelian genetics a recessive disease is one in which two disease alleles are necessary for its expression. This would seem unlikely for PSP as some individuals with PSP are heterozygote for A0, although the A0A0 genotype is significantly over-represented. This may depend on the number of pathologically verified cases included in the sample. Presumably, a single A0 allele does confer some additional risk of PSP, since a case-control study of a large number of A0 heterozygotes vs. non A0 controls would be expected to show an excess of PSP cases in the A0 heterozygote group.

This study, in conjunction with other studies of the A0-PSP association, is a starting point in understanding the aetiology of PSP. The next step should be directed towards understanding linkage disequilibrium around A0 in both PSP cases and controls and identifying functionally important variable sites within *tau.* For the other tauopathies, FTD and CBD it will be important to concentrate on pathologically defined subgroups. This has already proved to be effective in demonstrating a *tau* – CBD link and prompted a more detailed study of PiD.

## *4.4* Analysis of the association between Pick's disease and *tau*

PiD is a pathological entity characterised by neuronal loss with the deposition of spherical tau containing neuronal inclusions (Pick bodies), particularly in the dentate fascia of the hippocampus and the superficial layers of the frontal and temporal neocortex. As described, (2.5) the development of tau protein analysis,

specific immunocytochemical markers has allowed an accurate subdivision of tau deposition disorders based on their genetic and molecular pathological features.[186]

PiD has distinctive molecular pathological features, in contrast to PSP and CBD, involving the predominant deposition of three repeat tau protein.[100] Furthermore, PiD can be distinguished immuno-histochemically from other diseases which involve the deposition of abnormally hyperphosphorylated tau, by the absence of phosphorylation of tau Ser262,which is specifically recognised by the anti-tau antibody 12E8.[187] Therefore, Pick bodies do not stain with antibody 12-E8.

Given the relationship between A0, PSP and CBD, it is hypothesised that A0 relates directly to the deposition of 4R tau. As PiD is a 3R tau disease the A3 *tau* allele may be associated with 3R tau deposition in PiD. In order to generate a sufficient number of cases for an association study, neuropathologically diagnosed cases of PiD were identified following neuropathological examination at six centres in the United States, Canada and the United Kingdom. These cases were not known to have originated from a common geographical area. PiD was diagnosed on the basis of frontotemporal lobar atrophy with the formation of ballooned neurons (Pick cells) and Pick bodies in the superficial layers of the frontal and temporal neocortex and the dentate fascia of the hippocampus. The clinical and pathological features of one case has been reported in detail.[188] One case was diagnosed following non-dominant frontal biopsy, and in this case only frontal tissue was available. One of the cases had a positive family history of dementia, shown histopathologically to be Alzheimer's disease, but the remainder of the cases had no known family history of dementia. Thirty-four cases of PiD were compared with 215 controls. The genetic analysis of 11 cases of PiD was performed in London as part of this thesis, these data were combined with genetic data from other centres to provide a sufficiently powerful series. Power analysis indicates that this sample has an 80% chance of detecting a five-fold increase in H2H2 genotype frequency from 0.05 to 0.25 and a 70% chance of detecting a doubling of the H2 allele frequency from 0.22 to 0.44. Standard pathological examination was performed. Immunocytochemistry was performed using antibodies to *tau* (AT-8) and ubiquitin. Where available, immunocytochemistry was also performed using antibody 12-E8 (1:25 000, Elan Pharmaceuticals).

Controls were identified from a European Caucasian brain bank as described and an American Caucasian control series (US controls, n=145, mean age 63).

## Results

Thirty-four cases with typical PiD pathology were identified. The exon 1, 3, 9 and tau deletion polymorphisms were determined in 22 cases and in each case inferred to be inherited in linkage disequilibrium. These polymorphisms determined tau H1 and H2 haplotypes, as will be described (4.5).[178] The A0 allele is equivalent to the H1 haplotype. The PiD genotype frequencies were in Hardy Weinberg equilibrium ($\chi^2$ =0.02, df=2, p>0.05). There was no difference between the H2 haplotype frequency in controls and PiD cases ($\chi^2$ =1.9, df=1, p>0.05), or the H1H1 ($\chi^2$ = 1.5, df=1, p>0.05) or H2H2 ($\chi^2$ = 0.1, df=1, p>0.05) genotype frequencies between controls and PiD cases.

**Table 4-3 Comparison of *tau* genotypes in neuropathologically diagnosed Pick's disease cases and normal controls.**

| | PiD | US control | UK control |
|---|---|---|---|
| n | 34 | 145 | 70 |
| Haplotypes | | | |
| H1 | 48 (0.70) | 228 (0.78) | 111(0.79) |
| H2 | 20 (0.29) | 62 (0.22) | 29 (0.21) |
| Genotypes | | | |
| H1H1 | 17 (0.5) | 91 (0.63) | 44 (0.63) |
| H1H2 | 14 (0.41) | 45 (0.31) | 23 (0.33) |
| H2H2 | 3 (0.09) | 9 (0.06) | 3 (0.04) |

The majority of PiD cases examined did not have 12-E8 immunoreactive inclusions (9/10 cases). The case with 12-E8 positive inclusions reported earlier by Lieberman and colleagues showed 12-E8 immunoreactivity in Pick bodies of the dentate gyrus, albeit to a lesser extent than that seen with other antibodies to phosphorylation dependant epitopes in tau (see Fig. 2 B [188]).

## Discussion

PSP and CBD are clearly associated with the A0/H1, and both conditions involve the deposition of four repeat tau. PiD involves the deposition of three repeat tau and therefore it seems reasonable to hypothesise that PiD might be associated

with a lower frequency of H1 than normal. Although there is a suggestion that the *tau* H2 haplotype frequency is higher in patients with PiD this difference was not significant in this sample. PiD involves the deposition of three repeat tau, but does not involve an association with H1 or H2. This finding is concordant with the finding of Russ and colleagues, who in a smaller series did not find PiD to be associated with the tau H1 or H2 haplotypes.[189] There may however, be further tau polymorphisms outside the standard sequenced regions, which may have a role in the pathogenesis of PiD.[190] The relationship between the type of tau protein deposition and the *tau* haplotype deserves investigation over a wide range of pathologically identified cases in different diseases and aged controls.

## 4.5 Further analysis of the Tau region

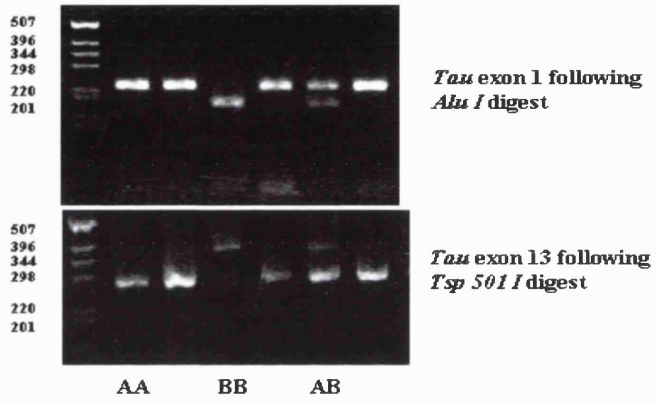
In early 1999 Baker and colleagues described a series of bi-allelic SNPs in *tau,* between exons 1 and 13.[178] These polymorphisms were inferred to be inherited together. Each polymorphism occurs as a common and less common allele in a roughly 0.7/0.3 ratio. At any given polymorphism individuals who were homozygous for the common allele i.e. genotype AA were also homozygous at each of the other polymorphisms analysed. Conversely, individuals with AB or BB genotypes were consistently AB or BB through *tau.* These common A polymorphisms were described as lying on a *tau* H1 haplotype, which extended over 100 kb.[178] The *tau* H1 haplotype was associated with PSP and these data suggested that the functionally important part of *tau* in relation to the development of PSP could lie anywhere within the 100 kb region of the gene. In order to investigate his further and to explore the two main possibilities for the linkage disequilibrium effect in the *tau* /PSP association a series of SNPs within *tau* and adjacent genetic markers were analysed. In view of the possible heterogeneity within the PSP series identified in the initial PSP-A0 association study a smaller group of 31 clinically typical (12 pathologically confirmed) PSP patients and 31 pathologically confirmed normal cases were studied. They were genotyped for SNPs in exons 1, 2, 3, 9, 11 and 13 of *tau* (3.3). Additionally, an SNP in exon 5 of the adjacent glial fibrillary acidic protein gene (GFAP) was analysed.[178, 191] Microsatellite markers adjacent to *tau* were selected from the

Marshfield integrated genetic map, to span a 4.8 cM region surrounding tau, and analysed using fluorescent labelled primer PCR, followed by genotyping on ABI 377 DNA sequencer. The markers analysed were, (Kosambi sex averaged centimorgans in brackets): D17S800 (62.01cM), D17S1793 (63.09 cM), Tau intronic polymorphism (64.16 cM), D17S1868 (64.16 cM), D17S902 (64.16 cM), D17S931 (66.85 cM).

The SNP genotypes were analysed with a chi-squared statistic and microsatellite genotypes were analysed using Terwilliger's likelihood ratio test.[162]

**Results**

The A allele signifies the most common allele at each polymorphism. The common allele (designated allele A) of each SNP at sites 1, 2, 3, 9ii,11 and 13 was positively associated with the development of PSP, and AA homozygotes are significantly over-represented in the PSP population as compared with the control groups.(Table 4-4). As described by Baker and colleagues each individual has a consistent AA, AB or BB genotype at each of these sites and this finding helps to confirm that these polymorphisms are inherited together in linkage disequilibrium. The common A alleles are described as lying on a tau H1 haplotype and the rarer B alleles lie on a H2 tau haplotype. The H1 haplotype accounts for 67% of control *tau* chromosomes and 100% of PSP *tau* chromosomes. Importantly, the A0 and the A1 alleles lie on the H1 haplotype and the A3 and A4 alleles lie on the H2 haplotype.




**Genotyping of PSP and control cases control lanes 1 AA, lane 3 BB, lane 5 AB, PSP lanes 2,4,6 BB. The B allele is distinguished by a 183 bp band in the exon 1 digest and a 370 bp band in the exon 13 digest. The genotypes are consistent at exons 1 and 13 of *tau*.**

**Figure 4-2 SNP genotyping digest of *tau* exons 1 and 13**

The rarer intragenic *tau* 9iii SNP, which is only present on a fraction of H1 chromosomes is not associated with PSP. (Figure 4-3) The extragenic GFAP exon 5 SNP was not associated with the development of PSP. The flanking microsatellite markers, were also not associated with PSP.

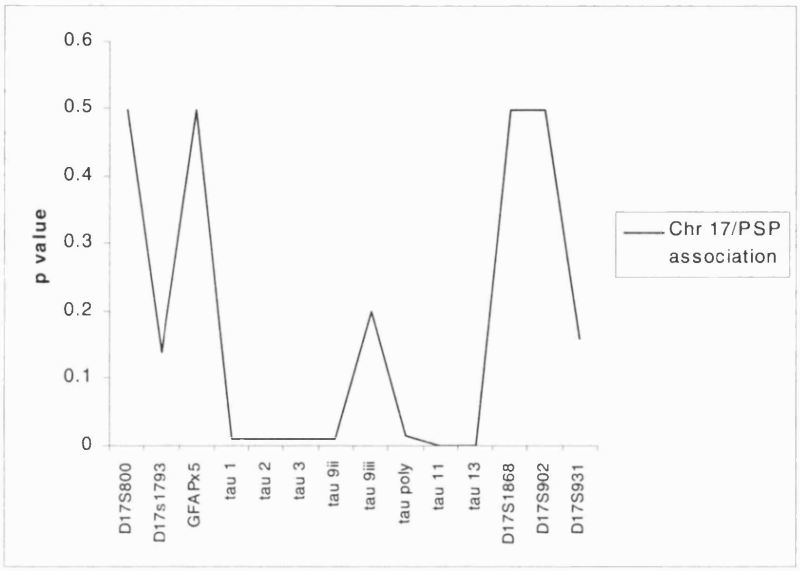



**Figure 4-3 Analysis of association between *tau* intragenic and flanking markers and PSP**

**Table 4-4 SNP analysis in and around *tau***

| | PSP | | | Control | | | Comparison* |
|---|---|---|---|---|---|---|---|
| | AA | AB | BB | AA | AB | BB | |
| SNP | | | | | | | |
| Tau ex 1 | 27/27 | 0/27 | 0/27 | 20/30 | 8/30 | 2/30 | $\chi^2$ 10.9, p<0.001 |
| Tau ex 2 | 30/30 | 0/30 | 0/30 | 19/29 | 8/29 | 2/29 | $\chi^2$ 12.5, p<0.001 |
| Tau ex 3 | 23/23 | 0/23 | 0/23 | 18/26 | 6/26 | 2/26 | $\chi^2$ 8.5, p<0.01 |
| Tau ex 9ii | 25/25 | 0/25 | 0/25 | 20/29 | 7/29 | 2/29 | $\chi^2$ 9.3, p<0.01 |
| Tau ex 11 | 30/30 | 0/30 | 0/30 | 20/30 | 8/30 | 2/30 | $\chi^2$ 12.0, p<0.001 |
| Tau ex 13 | 30/30 | 0/30 | 0/30 | 16/26 | 8/26 | 2/26 | $\chi^2$ 14.0, p<0.001 |
| Tau ex 9 iii | 26/28 | 2/28 | 0/28 | 27/27 | 0/27 | 0/27 | $\chi^2$ 2.0,p>0.1 |
| GFAP ex 5 | 17/26 | 6/26 | 3/26 | 24/30 | 5/30 | 1/30 | $\chi^2$ 0.7, p>0.1 |

**Table 4-5 Control and PSP microsatellite marker allele comparison**

| | n | LRT | p |
|---|---|---|---|
| D17S800 | 56 | 0 | 0.5 |
| D17S1793 | 61 | 1.17 | 0.14 |
| Tau intronic polymorphism* | 61 | 8.9 | 0.014 |
| D17S1868 | 53 | 0 | 0.5 |
| D17S902 | 45 | 0 | 0.5 |
| D17s931 | 61 | 1.0 | 0.16 |

***significant at the 5% level; analysis of PSP vs. control alleles using the Likelihood ratio test: likelihood ratio test (LRT) and probability of the null hypothesis, no difference between PSP and control allele frequencies.**

## Discussion

This study confirms that the common alleles at the *tau* exon 1, 2, 3, 9ii, 11 and 13 SNPs are each associated with the development of PSP, and that these SNPs, spanning around 100kb of *tau,* appear to be inherited together in unrelated individuals in linkage disequilibrium.[178] Furthermore, it illustrates that the microsatellite alleles A0 and A1 both lie on the H1 haplotype and A3 and A4 lie on the H2 haplotype. It is more likely that the *tau* – PSP predisposition effect depends on a bi-allelic polymorphism at some point in the *tau* region as bi-allelic polymorphisms encode amino acid polymorphisms and promoter variability. Microsatellite polymorphisms are not known to have a functional role and may give excess information in relation to the biologically relevant sequence variation. This study suggests that the area which might contain the biologically relevant change for the PSP predisposition effect could lie anywhere within this 100 kb region. The Baker study did not set an outer limit to the association and this is explored in the current study. Neither the flanking microsatellite markers, nor the intragenic or flanking SNP are associated with PSP. If a H1* haplotype, containing a rare pathogenic mutation, existed one might expect that the association effect might extend further than the 100 kb span of the *tau* gene. By analogy, linkage disequilibrium occurs around the common Caucasian cystic fibrosis mutation ΔF508, which means that allelic association effects can be detected up to 1 Mb away from the pathogenic deletion.[192] The absence of a

detectable association beyond tau suggests that the association is due to population wide variability rather than a rare mutation tau H1* haplotype. However, this study relied on markers placed on an approximate genetic map rather than using markers with a known physical relationship to *tau*.

The publication of the draft sequence of the human genome and the bacterial artificial chromosome (BAC) map of chromosome 17 allows a more precise indication of the relative position of the markers studied. The assembly of the contig of BACs surrounding *tau* and localization of markers and genes within this contig relies on US National Center for Biotechnology (NCBI) database searches. Entrez nucleotide searches identify the accession number and full sequence of markers and gene exons (http://www.ncbi.nlm.nih.gov/entrez/). BLAST searches (http://www.ncbi.nlm.nih.gov/blastest/blast.cgi) identify BACs which contain the sequence of interest. Searching the high throughput genome sequence database allows identification of BACs which contain unordered sequence pieces. Analysis of the GenBank sequence of tiled BAC order produces a much more accurate estimation of the marker separation and distance from *tau* despite the fact that the sequence of some of the BACs had not been fully completed.

**Table 4-6 Physical and genetic map positions of markers flanking *tau***

| Marker | Marshfield genetic map position | BAC accession number | GenBank BAC position |
|---|---|---|---|
| D17S1793 | 63.09 cM | AC019085.2 | 43.5 Mb |
| D17S800 | 62.01 cM | AC004231.1 | 46.4 Mb |
| D17S902 | 64.16 cM | AC068675.2 | 49.2 Mb |
| GFAP SNP | - | AC015936.2 | 51.4 Mb |
| D17S931 | 66.85 cM | AC015855.4 | 52.5 Mb |
| Tau | 64.16 cM | AC010792.4 | 53.6 Mb |
| D17S1868 | 64.16 cM | AC069454.2 | 57.7 Mb |

This information illustrates the discrepancy between the genetic and physical map positions. Although in general terms 1 cM is said to approximate to 1 Mb, this varies between the sexes and in different areas of the genome. In this physical/genetic comparison markers with a genetic difference of 2 cM are separated by up to 11 Mb, illustrating the unreliability of genetic techniques for the fine mapping of genomic areas. The closest physical markers to tau analysed in this study are 1.1 Mb centromeric and 4.1 Mb telomeric to *tau*. The publication

of the BAC order and the localisation of SNP and microsatellite markers on this map will clearly enable the fine mapping of the *tau* region and a precise measure of the extent of the PSP-chromosome 17 association and the linkage disequilibrium around *tau*. A number of other groups have analysed other polymorphisms flanking tau.[172, 190, 193, 194] Bonifati and colleagues have described a rare polymorphism which lies on a minority of PSP-tau chromosomes in Italian PSP patients but may occur with a greater frequency than in Italian controls.[195] Higgins describes single nucleotide polymorphisms in exons 1, 4A and 8 of tau.[193] Initially these were reported to be absent in age matched controls, but a subsequent paper described the disease associated haplotype to be present in cases (98%) and controls (33%).[190] Interestingly, these polymorphisms were not analysed in relation to the SNPs described here or in the Baker paper.[190] Preliminary analysis suggests that these SNPs are additional polymorphisms which lie on the H1/2 haplotype division and do not provide any additional information as to the aetiology of PSP (M.Huttton, M Ozansoy personal communications). Probably more relevant is the recent observation that the H1 haplotype includes SNPs in the *tau* promoter region which might be important in governing basal tau transcription or alteration in tau transcription in response to modulating factors.[194, 196] Similar approaches to linkage disequilibrium mapping have recently been taken in mapping the extent of allelic association with AD around the ApoE locus.[197]

This study confirmed the extent of linkage disequilibrium around *tau* in control individuals and opened up the area in which the biologically relevant sequence variation may lie. However, no evidence has emerged in this study or others that there is a H1* susceptibility haplotype which occurs with a frequency of less than 0.7, implying that other factors must be important in the pathogenesis of PSP.

## 4.6 Pathological, clinical and genetic heterogeneity in PSP

The observation that pathologically diagnosed PSP cases had a lower occurrence of the A0 allele warranted further investigation, particularly in view of the reports of clinically atypical PSP cases.[139] Typically, PSP presents with progressive gait

instability and also involves parkinsonism, a vertical supranuclear gaze palsy, frontal dysfunction, axial rigidity and a pseudo-bulbar palsy.[138] This clinical presentation corresponds to the classical pathological description. However, a number of authors have reported different clinical syndromes in patients with pathologically diagnosed PSP. Davis and colleagues reported four patients with atypical presentations of PSP.[140] Two of these patients had prominent cortical dysfunction and two had normal eye movements. A further series of patients with pathologically diagnosed PSP, and normal eye movements was reported in 1995,[139] raising the possibility of a "clinically atypical PSP" subgroup, with a more benign prognosis. Further detailed analysis of the neuropathology of this group has demonstrated that the patients without a supranuclear gaze palsy may have less damage to the pontine nucleus raphé interpositus.[108]

The features of the pathologically diagnosed PSP series were evaluated and compared with the *tau* haplotypes and tau protein profiles in an attempt to further define the nosology of typical/atypical PSP. This study may also provide further evidence for the possible role of the tau H1 haplotype. The pathological analysis was performed by Drs. Revesz and Daniel, and data on the tau western blot profiles was provided by Dr. Graham Gibb, Institute of Psychiatry, London.

The tau haplotype was assigned by analysis of the tau intronic microsatellite polymorphism and the exon 9ii single nucleotide polymorphism as previously described.[178] The globus pallidus or putamen was selected for protein analysis in each case. In four cases the pons was also examined.

**Clinical Features**

Among 26 pathologically diagnosed cases of PSP 15 clinically atypical and 11 clinically typical cases were identified.(Table 4-7) The clinical features were identified on the basis of retrospective notes review and the cases were referred from a variety of sources between 1987 and 1998. In some case records there was incomplete recording of eye movement examination, presenting symptoms and the time of onset of balance disturbance. The clinically typical cases all met the Tolosa criteria for PSP with slow vertical saccades or a more severe vertical eye movement disorder, falls/postural instability and additional supportive features.[198]

Five of these cases did not have falls in the first year following symptom onset, and in one case the presence or absence of falls was not recorded, so only five of these clinically typical cases met the NINDS criteria for the diagnosis of PSP.[138] The atypical group exhibited a variety of clinical syndromes. Four clinically atypical cases presented with asymmetrical bradykinesia and a good response to L-DOPA treatment. Four had L-DOPA unresponsive Parkinsonism and three of these four cases had normal eye movements. Two cases had an eye movement disorder with relatively preserved balance and some response to L-DOPA. A further patient had had a corticobasal degeneration syndrome with asymmetrical dystonia and apraxia. The other four had insufficient clinical documentation to make a confident clinical diagnosis, but three of these had significant postural instability raising the possibility that they had an unreported eye movement disorder and were in fact typical PSP cases.

**Table 4-7 Pathologically diagnosed PSP cases: clinical diagnosis, tau haplotype and tau protein pattern**

| Case | Clinical PSP status | Clinical diagnosis | Age at onset | Age at death | Tau haplotype | Tau protein pattern | Plaque formation |
|---|---|---|---|---|---|---|---|
| 1 | Atypical | PD | 70 | 77 | H1H1 | Doublet | ++ |
| 2 | Atypical | PD | 74 | 82 | H1H1 | Non-doublet | + |
| 3 | Atypical | PD | 76 | 91 | H1H1 | Non-doublet | ++ |
| 4 | Atypical | PD | 53 | 70 | H2H2 | Non-doublet | - |
| 5 | Atypical | PD | 72 | 79 | H1H1 | Doublet | - |
| 6 | Atypical | AD | 77 | 80 | H1H2 | Doublet | ++ |
| 7 | Atypical | PD | 56 | 77 | H1H2 | Non-doublet | + |
| 8 | Atypical | PD | 56 | 61 | H1H2 | Non-doublet | +/- |
| 9 | Atypical | PEP | 58 | 68 | H1H1 | Non-doublet | +/- |
| 10 | Atypical | Atypical PD | 77 | 84 | H1H1 | Non-doublet | +/- |
| 11 | Typical | PSP | 66 | 68 | H1H1 | Doublet | ++ |
| 12 | Atypical | PD | 59 | 72 | H1H1 | Doublet | - |
| 13 | Typical | PSP/CJD | 63 | 66 | H1H1 | Non-doublet | +/- |
| 14 | Atypical | PD | 76 | 86 | H1H1 | Non-doublet | ++ |
| 15 | Typical | PSP | 74 | 79 | H1H1 | Doublet | ++ |
| 16 | Typical | PSP | 68 | 72 | H1H1 | Doublet | +/- |
| 17 | Typical | PSP | 65 | 73 | H1H1 | Non-doublet | - |
| 18 | Typical | PSP | 64 | 72 | H1H1 | Doublet | +/- |
| 19 | Typical | PSP | 65 | 75 | H1H1 | Non-doublet | +/- |
| 20 | Typical | PSP | 64 | 69 | H1H1 | Doublet | +/- |
| 21 | Typical | PSP | 64 | 72 | H1H1 | Doublet | - |

| Case | Clinical PSP status | Clinical diagnosis | Age at onset | Age at death | Tau haplotype | Tau protein pattern | Plaque formation |
|---|---|---|---|---|---|---|---|
| 22 | Typical | PSP | 67 | 72 | H1H1 | Doublet | +/- |
| 23 | Atypical | PSP | 62 | 69 | H1H1 | Non-doublet | - |
| 24 | Typical | PSP | 66 | 67 | H1H1 | Doublet | +/- |
| 25 | Atypical | CBD | 55 | 64 | H1H1 | Doublet | - |
| 26 | Atypical | PD | 74 | 85 | H1H1 | Non-doublet | +/- |

**Key: - No amyloid pathology, +/- some non-neuritic plaques, + some neuritic plaques, confined to the mesial temporal area, ++ some neocortical neuritic plaques**

The majority of the clinically typical PSP cases had the characteristic PSP tau protein electrophoretic pattern as compared with around one third of the atypical cases (73% vs. 33%)(Table 4-8Table 4-7). The typical PSP tau profile contains the upper two bands of PHF-tau plus an additional faint larger protein band (Figure 4-4, lanes1 and 2). Atypical PSP tau protein profiles could be divided into two types of pattern; the first banding pattern was very similar to PHF-tau in that there were three major protein species that aligned with the three major tau bands in PHF-tau (Figure 4-4, lanes 1 and 3), as well as the faint fourth protein species which is present in both PHF-tau. The second atypical PSP tau protein array demonstrated 6-8 protein bands which migrated faster than the majority of PHF-tau, typical PSP and atypical PSP pattern 1 tau bands respectively (Figure 4-4, lane 4).

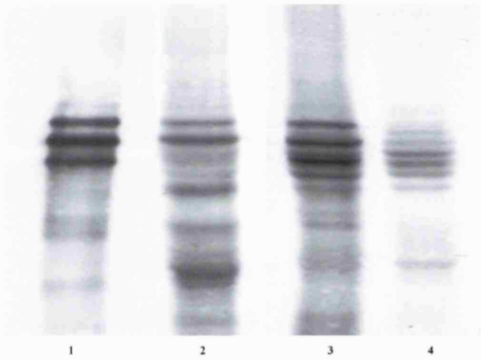



**Figure 4-4 Typical and atypical insoluble tau electrophoretic patterns.**

**Western blots stained with antibody TP70. Lane 1 AD pattern, lane 2 typical PSP, lane 3 atypical PSP pattern 1, lane 4 atypical PSP pattern 2. Courtesy of Dr. Graham Gibb, Institute of Psychiatry, London**

All clinically typical, pathologically typical PSP cases were homozygous for the PSP susceptibility genotype, H1H1 as compared with 73% of the clinically atypical cases. Conversely, the majority of all cases with the deposition of PSP-type tau possessed the PSP susceptibility genotype (12/13, 92%).

**Table 4-8 Comparison of clinically typical and atypical PSP groups**

| | n | Mean age at onset | Mean age at death | Typical PSP genotype | Typical PSP protein pattern | Extra hippocampal neuritic plaques |
|---|---|---|---|---|---|---|
| Clinically atypical | 15 | 66.3 | 76.3 | 73.3% | 33.3% | 26.7% |
| Clinically typical | 11 | 66.0 | 71.4 | 100.0% | 72.7% | 18.2% |

In order to determine whether concurrent Alzheimer type pathology could account for the differences in the tau western blot pattern, tissue was specifically examined for Alzheimer type pathology. Although some cases had mesial temporal and neocortical senile and neuritic plaques none of the cases met the CERAD criteria for the diagnosis of Alzheimer's disease.[199] The prevalence of extra-hippocampal neuritic plaque formation was similar in the two groups. However the average age at death of cases with extra-hippocampal neuritic plaque formation cases was greater (80 vs. 72 years).

## Discussion

This series suggests that there may be an "atypical PSP" subgroup in which the classical distribution of PSP pathology occurs, but with the deposition of AD-type tau protein rather than PSP-type tau in at least 2/3 of the cases. The atypical PSP subgroup, which is defined on the basis of an atypical clinical presentation, is less likely to have the PSP susceptibility genotype and more likely to have an AD tau deposition pattern. The differences in tau protein deposition in this series cannot be explained by regional differences in tau pathology, since the same brain areas were studied, and is not explained by concurrent Alzheimer type pathology. This combination of molecular and topographic pathology in these cases is most similar to the pathology seen in the parkinsonism dementia complex of Guam (PDC), the FTDP-17 family with the tau R406W mutation and post-encephalitic parkinsonism (PEP).[113, 200] In these conditions subcortical deposition of AD type tau occurs.[200-202] However, mesial temporal involvement may be more pronounced than is seen in PSP, and this is usually accompanied by clinical amnesia. There are three further conditions in which subcortical tau neurofibrillary tangle deposition occurs with parkinsonism. These are some

cases of parkinsonism associated with parkin mutations,[99] neurofibrillary tangle parkinsonism as described by Rajput and colleagues,[203] and AD associated parkinsonism.[204] Detailed molecular studies of these conditions are not available and it is not clear how closely they resemble the atypical PSP cases described in this series. However, although the pathology of parkin mutations may include neurofibrillary tangle deposition, our atypical PSP cases have an older age of onset than is usual for these cases.[205] Neurofibrillary tangle parkinsonism as described by Rajput and colleagues predominantly involves the substantia nigra and locus coeruleus but not other subcortical sites such as the subthalamic nucleus, which are characteristically involved in PSP. The atypical PSP cases described in this series had a more widespread subcortical neurofibrillary tangle deposition. Unlike the atypical PSP cases in this study the AD associated parkinsonism cases were associated with sufficient amyloid plaque formation to be classified as AD.[204] Our data indicate that when the strictest criteria for PSP diagnosis are used, and this includes both pathological and clinical information, then the associated H1/H1 genotype frequency is 100%.

Although only a small number of cases have been studied in this series, the fact that there is an association between the *tau* H1H1 genotype and the deposition of PSP-type tau may provide further indication of the role of the H1 haplotype in the pathogenesis of PSP. The strength of the association between the H1 haplotype and typical PSP raises the possibility that this association is directly linked to the process which leads to the deposition of four repeat tau protein isoforms. By analogy with FTDP-17 this may involve a change in the splicing of exon 10 or a change in the microtubule binding or self-aggregating properties of exon 10.

The molecular pathological data in this series argues against a region specified tau deposition response, as has been suggested in Pick's disease.[103] Tissue from the same area in patients with otherwise similar pathology may contain either AD type tau or PSP type tau, and in cases where both the brain stem and basal ganglia has been examined the tau profile has been found to be consistent. This argues that the type of tau deposited is disease rather than region specific. However, since the protein analysis is performed on homogenized brain areas we

cannot exclude the involvement of different cellular populations within these areas, and this can only properly be examined by techniques such as *in situ* mRNA hybridisation.

Pathologically diagnosed PSP may be a heterogeneous clinical and aetiological entity. The presence of typical PSP-tau deposition is correlated with a typical clinical presentation and the presence of the PSP susceptibility genotype. This study reinforces neurofibrillary tangle diseases as an occasional pathological basis for a clinical Parkinson's disease like syndrome and suggests possible pathways by which the susceptibility genotype may increase the likelihood of the development of PSP.

## 4.7 Analysis of ApoE and α-synuclein

Given the population prevalence of the PSP associated allele/genotype (~0.7/0.5) and the overall prevalence of PSP (0.000064) other factors must play a role in the disease pathogenesis. These factors may be genetic or environmental but to date, case control studies have not produced convincing evidence of additional environmental risk factors.[144, 145] Two additional genes, implicated in neurodegeneration, were studied. The ApoE ε4 allele is a risk factor for AD, with estimates of an ε4 allele frequency in AD of 0.50 as compared with a frequency of ~0.18 in control populations. Further analysis suggests that the ApoE ε4 allele accelerates the age of onset of AD in some, but not all, AD patients.[206] The functional role of the ε4 allele is uncertain but it is has been suggested that it could influence either tau deposition and/or amyloid deposition.[207] The normal function of ApoE is to mediate the uptake of very low density lipoprotein (VLDL) via interaction with the cell surface low density lipoprotein (LDL) receptor.[207] In the brain the LDL receptor related protein (LRP) acts as the major receptor for ApoE and α-2 macroglobulin. LRP is also involved in interacting with the amyloid precursor protein, and ApoE itself can directly interact with both APP and tau. It has been suggested that ApoE may exert an effect on the development of AD either by accelerating the formation of amyloid plaques or of neurofibrillary tangles containing tau.[207] Thus the

demonstration of a possible role for ApoE in relatively rare tau neurofibrillary tangle-only conditions such as FTDP-17 or PSP may be important in understanding the relative importance of ApoE in tau and amyloid deposition.

As described earlier,(1.6) mutations in the α-synuclein gene have been reported in rare families with autosomal dominant PD.[16, 17] α-synuclein is deposited in an abnormally aggregated form in the Lewy bodies of PD brain in both sporadic and familial PD cases.[208] α-synuclein may have a role in synaptic vesicle turnover, regulating apoptosis, and regulating phospholipase D activity and so potentially could influence neurodegeneration in a number of different ways.[209] It is an additional candidate gene whose variability may exert an influence on sporadic neurodegenerative disease Variability in *ApoE* and *α-syn* was investigated in PSP.

Multiple system atrophy (MSA) is a sporadic disease, characterised by the formation of α-synuclein inclusions. It is one of the commoner neurodegenerative causes of L-DOPA unresponsive parkinsonism, and analysis of variability of ApoE and α-syn in MSA has therefore been included in this study. MSA is characterised by varying combinations of parkinsonism, cerebellar ataxia, autonomic failure or pyramidal signs,[210] and has an age adjusted prevalence of 4.4/100,000.[142] It causes degeneration of the nigro-striatal and olivo-ponto-cerebellar tracts, with variable involvement of the intermediolateral cell columns.[211] Cerebellar (olivopontocerebellar degeneration), parkinsonian (striatonigral degeneration) and autonomic (Shy-Drager) subtypes of MSA have been unified by the description of characteristic argyrophilic neuronal and glial inclusions at autopsy, most specifically by the formation of the oligodendroglial deposits known as glial cytoplasmic inclusions, which contain α-synuclein.[212] MSA has a relatively early age of onset, with an average of 54 years.[211] Familial occurrence of MSA has not been described. Its aetiology remains obscure, and genetic susceptibility studies investigating a possible effect of variability in Apolipoprotein E and cytochrome P450 –II D6 (CYP-IID6) have not shown an association effect.[213, 214] Similarly, a study of the SCA-1 and SCA-3 gene expansions in MSA has not provided any evidence that

MSA represents a sporadic variant of these autosomal dominant diseases.[215] Variability in α-synuclein and ApoE was investigated in MSA.

The study population consisted of 72 controls, 50 PSP patients and 47 MSA patients. The 50 PSP patients included 13 pathologically confirmed clinically typical PSP cases and met modified NINDS possible or probable criteria as previously described in this thesis.[171] Clinically atypical/pathologically typical PSP cases were not included. The 47 MSA cases were either pathologically diagnosed (22 cases) or met the Quinn clinical criteria for probable MSA.[216] Intronic and promoter polymorphisms in *α-syn* were analysed together with the ApoE genotype. Statistical significance was set at $p<0.008$ (0.05/6) using the Bonferroni correction for three polymorphisms analysed in two diseases. The power of this study to detect a doubling of the second commonest allele in frequency was calculated to be 0.7 for the α-syn promoter, 0.5 for ApoE and 0.6 for the α-syn intronic polymorphism. The allele frequency distribution in cases and controls were compared with the likelihood ratio test (LRT) $\chi^2$ analysis.

**Results**

The genotypes in the control group for each polymorphism were in Hardy-Weinberg equilibrium ($\chi^2$ test, $p>0.05$). Variability in *apo E* or *α-syn* was not associated with MSA using either allele (Table 4-9), or genotype (Table 4-10) frequencies. Although there was a suggestion that the α-syn intronic polymorphism allele B was less frequent in MSA (0.18 vs. 0.32), this did not reach statistical significance. Neither *apo E* nor *α-syn* were associated with the development of PSP.

**Table 4-9 Allele frequencies for ApoE, α-synuclein promoter and intronic polymorphisms in controls, MSA and PSP patients**

| | Control | PSP | MSA | | Control | PSP | MSA |
|---|---|---|---|---|---|---|---|
| NACP Promoter | | | | Apo E | | | |
| 257 | 32(0.22) | 29(0.31) | 21(0.23) | 2 | 6(0.05) | 6(0.06) | 7(0.07) |
| 259 | 101(0.70) | 63(0.67) | 63(0.68) | 3 | 104(0.80) | 81(0.84) | 76(0.81) |
| 261 | 10(0.07) | 2(0.02) | 8(0.09) | 4 | 20(0.15) | 9(0.09) | 11(0.12) |
| 263 | 1(0.01) | 0(0.00) | 0(0.00) | n | 65 | 48 | 47 |

| | Control | PSP | MSA | | Control | PSP | MSA |
|---|---|---|---|---|---|---|---|
| N | 72 | 47 | 46 | | | | |
| NACP Intron | | | | | | | |
| A0 | 56(0.39) | 51(0.51) | 48(0.55) | | | | |
| A1 | 31(0.22) | 19(0.19) | 15(0.17) | | | | |
| A2 | 10(0.07) | 3(0.03) | 9(0.10) | | | | |
| B | 45(0.32) | 27(0.27) | 16(0.18) | | | | |
| N | 71 | 50 | 44 | | | | |

All case control comparisons not significant, LRT $\chi^2$ <2, p>0.1.

**Table 4-10 Genotype frequencies for ApoE, α-synuclein promoter and intronic polymorphisms in controls, MSA and PSP patients**

| | Control | PSP | MSA | | Control | PSP | MSA |
|---|---|---|---|---|---|---|---|
| α-synuclein promoter | | | | Apo E | | | |
| 257 257 | 3(0.04) | 7(0.15) | 3(0.07) | 2 2 | 0(0.00) | 0(0.00) | 0(0.00) |
| 257 259 | 24(0.33) | 15(0.32) | 13(0.28) | 2 3 | 4(0.06) | 6(0.13) | 3(0.10) |
| 257 261 | 2(0.03) | 0(0.00) | 2(0.04) | 2 4 | 2(0.03) | 0(0.00) | 4(0.03) |
| 259 259 | 36(0.50) | 23(0.49) | 22(0.48) | 3 3 | 43(0.66) | 33(0.69) | 33(0.57) |
| 259 261 | 4(0.06) | 2(0.04) | 6(0.13) | 3 4 | 14(0.22) | 9(0.19) | 7(0.23) |
| 261 261 | 2(0.03) | 0(0.00) | 0(0.00) | 4 4 | 2(0.03) | 0(0.00) | 0(0.07) |
| 259 263 | 1(0.01) | 0(0.00) | 0(0.00) | | | | |
| N | 72(1.00) | 47(1.00) | 46(1.00) | n | 65(1.00) | 48(1.00) | 47(1.00) |
| α-synuclein intron | | | | | | | |
| A0 A0 | 12(0.17) | 15(0.30) | 11(0.25) | | | | |
| A0 A1 | 7(0.10) | 6(0.12) | 10(0.23) | | | | |
| A0 A2 | 4(0.06) | 1(0.02) | 6(0.14) | | | | |
| A0 B | 21(0.30) | 14(0.28) | 10(0.23) | | | | |
| A1 A2 | 3(0.04) | 2(0.04) | 0(0.00) | | | | |
| A1 A1 | 4(0.06) | 2(0.04) | 2(0.05) | | | | |
| A1 B | 13(0.18) | 7(0.14) | 1(0.02) | | | | |
| A2 A2 | 1(0.01) | 0(0.00) | 0(0.00) | | | | |
| A2 B | 1(0.01) | 0(0.00) | 3(0.07) | | | | |
| B B | 5(0.07) | 3(0.06) | 1(0.02) | | | | |
| N | 71(1.00) | 50(1.00) | 44(1.00) | | | | |

## Discussion

The possible role of ApoE in PSP has been investigated by three other groups.[217-219] In common with the current study a link between the ApoE ε4 allele and PSP

was not established. A Japanese study of ApoE and PSP suggested that the ApoE ε2 allele might be present at an increased frequency in Japanese PSP patients.[218] This finding has not been replicated in the current study of Caucasian patients, which is the largest study described to date. One other group has not found an association between *α-syn* and PSP.[169]

Comparisons between MSA and PSP are particularly interesting since both are sporadic neurodegenerative parkinsonian conditions, involving the deposition of a protein implicated in familial neurodegenerative disease. The identification of α-synuclein as a component of the neuronal and glial deposits of MSA is likely to have been an important step in understanding its pathogenesis. This followed the description of *α-syn* mutations (Ala53Thr and Ala30Pro) in rare families with autosomal dominant PD and the subsequent demonstration of α-synuclein deposition in the Lewy body inclusions of PD.[220] Immuno-labelled electron microscopic studies suggest that Lewy body filaments are composed of α-synuclein, and the described pathogenic *α-syn* mutations in some families with autosomal dominant PD accelerate filament formation *in vitro*.[221] Similar filamentous deposits are found in MSA, and the diameter of the filaments that are seen in MSA GCIs matches that of *in vitro* α-synuclein aggregates. Protein analysis of α-synuclein in MSA shows that there is increased insoluble fraction α-synuclein present in MSA brain,[222] and that additional high molecular weight α-synuclein species are present which may represent abnormally aggregated α-synuclein.[221] Furthermore, these biochemical changes in α-synuclein may be present in areas with a low GCI density, suggesting that changes in α-synuclein are a widespread feature in MSA brain, and precede the development of GCIs and neuronal loss. These data suggest that pathological aggregation of α-synuclein in MSA may have an important role in the disease process.

Although variability in *α-syn* has not been considered to be a risk factor for sporadic PD,[62, 223] the different α-synuclein pathology in MSA suggested that genetic variability in *α-syn* could be a possible contributory factor to the development of MSA. In this study, analysis of two *α-syn* microsatellite markers has not provided support for population wide *α-syn* variability as a susceptibility

factor in MSA, and these markers will also reflect variability in non-coding regions surrounding *α-syn.* In addition, *ApoE* is not associated with MSA. Despite the pathological and familial evidence regarding the role of α-synuclein in neurodegeneration, the primary basis of MSA, like sporadic PD remains obscure.

## 4.8 Age at onset analysis

Although the initial genetic investigation of ApoE in AD showed that the ApoE ε4 allele was positively associated with AD, recently emphasis has been placed on the role of ApoE in modulating the age of onset. Analysis of age of onset in sporadic and familial cases of AD suggested that individuals with one ε4 allele had an earlier age of onset of AD by around 6-8 years in comparison with individuals with the ε3ε3 genotype.[224] Furthermore, the ε2 allele seems to exert a protective effect against the development of AD, delaying the age of onset by 6-8 years. These age of onset analyses provide evidence for the modulatory effect of a disease susceptibility allele, presumably acting in conjunction with other environmental or genetic factors. A number of studies, including the one reported in this thesis, have failed to demonstrate an association between PSP and ApoE, but none have separately considered modulation of age of onset.[217, 218]

PSP is strongly associated with the A0 allele and its associated H1 haplotype of the tau gene.[168, 171, 178] One recent study of 26 patients with PSP suggested that individuals with an A0/A0 genotype had a significantly earlier age of onset than individuals with non A0/A0 genotypes, although this latter group comprised only six patients.[225] The ApoE ε4 allele has also been related to a range of neurological diseases and adverse outcomes including response to chronic and acute head injury, response to intracerebral haemorrhage and recovery from cardiopulmonary bypass. Furthermore, it is has recently been suggested that young onset PD may be associated with the ApoE ε4 allele.[226]

The effect of ApoE and tau on the age of onset of PSP and MSA was evaluated. The subjects and methods are as described in Section 4.7 and Chapter 3. The tau

H1/H2 haplotype was inferred from the tau intronic insertion/deletion polymorphism which is in complete disequilibrium with the H2 haplotype in Caucasian populations. The mean and median age of onset for the PSP group was 62.9 and 63 years respectively, and for the MSA group was 54.1 and 55 years. Young onset cases were defined as cases with an age of onset less than or equal to the median age of onset. The frequencies of the ApoE ε4 allele and the tau H1 haplotype were compared in young and late onset cases using a chi squared test. The power of the study to detect a difference is dependant on the frequency of the ε4 and H1 alleles. Using a significance level of $p<0.05$ the ApoE ε4 study has a 60% probability of detecting an increase in the ε4 frequency in young onset cases to from 0.1 to 0.45, whereas the tau H1 study has a 40% probability of detecting an increase in the H1 frequency from 0.8 to 1.0 in young onset MSA cases, and only a 10% probability of detecting and increase in the young onset PSP H1 frequency from 0.9 to 1.0.

## Results

There was no significant difference between the young and late onset PSP and MSA tau H1 or ApoE ε4 allele frequencies ($\chi^2 <2$, $p>0.05$).

**Table 4-11 Age at onset analysis of tau and ApoE PSP and MSA**

| | Young onset PSP* | Late onset PSP | Young onset MSA+ | Late onset MSA |
|---|---|---|---|---|
| e4 | 3(0.07) | 4(0.1) | 4(0.09) | 7(0.19) |
| e2 or e3 | 39(0.93) | 38(0.90) | 40(0.91) | 29(0.81) |
| n | 21 | 21 | 22 | 18 |
| | | | | |
| H1 | 42(1.0) | 37(0.97) | 36(0.81) | 27(0.79) |
| H2 | 0 | 1(0.03) | 8(0.18) | 7(0.21) |
| n | 21 | 19 | 22 | 17 |

*** Young onset PSP defined as age at onset less than or equal to 63 years, + Young onset MSA defined as age at onset less than or equal to 55 years.**

## Discussion

We have not detected an effect of ApoE on the age of onset of either the sporadic neurofibrillary tangle disorder, PSP, or the sporadic α-synuclein inclusion

disorder, MSA. This is in keeping with a recent report that ApoE status does not modify the age of onset within frontotemporal dementia families with tau mutations, and adds to the data suggesting that, among neurodegenerative conditions, ApoE specifically modulates only amyloid deposition disorders.[227]

Although tau is associated with PSP, we have not demonstrated an association between tau and the age of onset of either PSP or MSA. Since clinico-pathological studies suggest that clinically and pathologically typical PSP is nearly always associated with the *tau* H1/H1 genotype it is likely that the demonstration of an effect on age of onset will require a large number of PSP cases, given the high background H1 frequency. This effect of the H1 background is reflected in the power analysis of this study. Presumably other genetic or environmental factors are predominantly responsible for variation in the age of onset of these neurodegenerative conditions.

## 4.9 Conclusions

*Tau* is robustly associated with PSP, and to date the *tau* H1-PSP association described in this chapter together with the ApoE ε4 –AD association are the most secure allelic associations described in neurodegeneration. *Tau* genotyping may be useful in the clinical diagnosis of PSP. Diagnostic tests depend on the pre test diagnostic situation.[228] In practical terms, the test would be used as an adjunct to clinical diagnosis based on clinical diagnostic criteria, applicable in the clinic, and compared with pathological diagnosis as the gold standard. The ApoE genotype has been suggested to be a useful ancillary test for patients with possible AD, but in fact an ApoE genotype result only has a small effect on the probability of a correct diagnosis of AD, based on clinical criteria and then confirmed pathologically (pre-test probability 0.85-post-test probability 0.94) (Table 4-12). The usefulness of an H1H1 genotype result in the diagnosis of PSP can be considered in three situations – among all patients with a diagnosis of parkinsonism including PD, among patients with non L-DOPA responsive parkinsonism and among patients with NINDS clinical diagnostic criteria probable PSP. As an adjunctive test it would appear to be most useful among

patients with non-L-DOPA responsive parkinsonism, doubling the probability that a patient will have PSP (Table 4-12). However, among NINDS diagnostic criteria positive patients, an H1H1 haplotype result only produces a modest increase in the likelihood that a patient has PSP, and given that the diagnostic criteria can be applied in clinic without recourse to specialized investigations, it seems unlikely that *tau* genotyping will have widespread clinical usefulness.

**Table 4-12 Effect of the H1H1 genotype on the diagnosis of PSP**

| Study population | All parkinsonism | Non-L DOPA responsive parkinsonism | NINDS clinically probable PSP | AD – effect of ε4 on clinical diagnosis |
|---|---|---|---|---|
| Sensitivity | 1.00 | 1.00 | 1.00 | 0.75 |
| Specificity | 0.50 | 0.50 | 0.50 | 0.71 |
| Prevalence[138, 141] | 0.10 | 0.33 | 0.80 | 0.85 |
| Pre test probability | 0.10 | 0.33 | 0.80 | 0.85 |
| Positive predictive value | 0.18 | 0.50 | 0.89 | 1.00 |
| Negative predictive value | 1.00 | 1.00 | 1.00 | 0.42 |
| Likelihood ratio for positive test | 2.00 | 2.00 | 2.00 | 2.64 |
| Likelihood ratio for negative test | 0.00 | 0.00 | 0.00 | 0.34 |
| Pre test odds | 0.11 | 0.50 | 4.00 | 5.70 |
| Post-test odds | 0.22 | 1.00 | 8.00 | 15.05 |
| Post test probability | 0.18 | 0.50 | 0.89 | 0.94 |

The demonstration of an allelic association between *tau* and PSP has not, as yet, led to major advances in our understanding of the pathogenesis of PSP. Further work which might illuminate this can be split into three areas: i.) refinement of

the allelic association, ii.) exploration of hypotheses based on post mortem material and iii.) the search for additional genetic and environmental aetiological factors.

**Allelic association**

This chapter contains an analysis of the *tau* region and confirms the 100 kb linkage disequilibrium around the *tau* locus and fails to show allelic association at makers initially estimated to be within 4 Mb of *tau* but subsequently shown to span a larger genomic area. The publication of the draft sequence of the human genome allows a more systematic approach to mapping this area. The most logical approach would be to map out the association between *tau* flanking SNPs and PSP, progressing outwards from the defined association in the *tau* promoter and exon 13 of *tau*. The analysis of bi-allelic and multi-allelic markers within *tau* suggests that common SNPs provide a more secure and straightforward analysis of allelic association. The multi-allelic markers provide a "loss of information" as compared with bi-allelic markers and functionally important changes are likely to be bi-allelic changes i.e. single base pair changes in protein coding, splice-site or promoter regions. It is anticipated that the PSP –chr17 association will decay with greater physical distance from *tau*. When this association decay has been mapped, linkage disequilibrium around *tau* can be mapped. In effect, this would plot association between the *tau* H1/H2 haplotypes and the surrounding SNPs. If the decay matches the PSP association decay then it can be assumed that the control *tau* H1 haplotype is the disease susceptibility haplotype but has a very low penetrance. If the control linkage disequilibrium decays much more rapidly, then it can be inferred that PSP patients are more closely related to one another than the general population and that the *tau* region is likely to contain a rare, functionally important sequence change. This mapping work is important to understanding the susceptibility conferred by the *tau* haplotype and is needed to absolutely exclude the unlikely possibility that a sequence change in a flanking gene is important in conferring susceptibility to PSP.

**Exploration of aetiological hypotheses**

A hypothesis for the link between H1 and PSP can be proposed based on the findings described in this chapter. CBD and PSP, both four repeat tau diseases are predisposed to by the *tau* H1 haplotype whereas PiD, which involved the deposition of three repeat tau is not. Similarly, pathologically typical PSP, involving the deposition of 4R tau, is more strongly associated with H1 than variants of PSP involving the deposition of 3R and 4R tau. This suggests that the susceptibility to PSP could depend on a change in the alternative splicing of *tau*. This could be either a change in the basal ratios of 4R:3R RNA transcription or a change in splicing in response to an external factor. This possibility could be explored using rt-PCR analysis of RNA from post mortem tissue from patients with PSP and normal controls with both the H1 and H2 haplotypes. Two studies have so far reported on this issue. Chambers and colleagues report that there is a change in the 4R:3R ratio in PSP brain but only in the brainstem, and not in other areas known to be affected by the disease process.[229] In contrast, Hoenicka and colleagues report no difference between 4R:3R rations in PSP and control cases.[177] Post mortem studies of this type have to be qualified by a number of potential difficulties. Firstly, good quality RNA preparations may be difficult to obtain in human post-mortem tissue. Secondly, post-mortem examinations are usually performed after a disease duration of several years at which point the cellular changes which were present at the start of the disease may no longer occur. Thirdly, the most severely affected neurones may have died, so if an increased 4R:3R ratio is highly toxic then it may be difficult to detect after substantial neurodegenerative cell loss. The main alternative hypothesis is that the H1 haplotype involves some fundamental difference in *tau* promoter function which alters disease susceptibility. Determining relative levels of transcriptional activity in human autopsy or biopsy material is particularly problematic, making this difficult to prove. Conventionally, a "neutral" internal gene control is used such as β-actin or a glycolytic pathway enzyme. This may be valid in healthy tissue, but in diseased cells the basal synthesis of these internal controls may be changed. Furthermore, altered glial activity in disease areas, which are often characterised by extensive gliosis may not be separable in homogenised tissue preparations. A potential solution to these difficulties may be found in *in situ* hybridisation techniques which may allow quantification of relative RNA

amounts in single cells. Further support for aetiological hypotheses of the relation between the *tau* H1 allele and PSP may come from cell culture and transgenic animal models.

**Additional genetic and environmental factors**

*Tau* was suggested as a candidate gene for PSP because of the pathological features of the disease. Two further candidate genes, selected because of their role in other neurodegenerative conditions have been examined and not found to be associated with PSP. To date there have been no systematic investigations of the sibling recurrence risk for PSP and no reports of the occurrence of PSP in mono- or di- zygotic twins. This lack of genetic epidemiological data makes it difficult to make any predictions of the number of additional genes which remain to be identified in PSP. By comparison, examination of the epidemiology of autism and comparison of the high concurrence rate in monozygotic twins and the less frequent concurrence in siblings suggests that autism may be due to the interacting effect of at least five separate genes, and a situation like this may apply to PSP. Determining which genes should be investigated, for genetic multifactorial diseases is a difficult task. However, the genetic approach seems to offer the strongest chance of success.

# CHAPTER 5 Sequence analysis of *tau*

## 5.1 Summary

FTDP-17 is due to mutations in ***tau***. ***Tau*** was sequenced in 22 British families with FTD of whom 11 had mutations: most commonly exon 10 +16 mutations were seen, but the exon 10 +14 and P301S mutations were also identified. Pathologically, familial FTD can be divided into FTD with tau inclusions, FTD with ubiquitin inclusions and FTD lacking distinctive histopathology. The presence of ***tau*** mutations correlated with the presence of tau pathology. In general, pathologically defined PiD cases did not have ***tau*** mutations, however two individuals with the G398R mutation were identified. Both of these cases had atypical immunohistochemical characteristics. The majority of PSP cases sequenced did not have ***tau*** mutations although one young onset individual clinically diagnosed to have PSP was identified to have a ***tau*** exon 10 +16 mutation, illustrating the clinical overlap between these conditions.

## 5.2 Introduction

In 1998 it was first demonstrated that mutations in ***tau*** were pathogenic.[75, 230, 231] This discovery opened up some important areas in the investigation of neurodegeneration, including: i. the mechanisms of tau related neurotoxicity, ii. the clinical/genetic/pathological classification of tau related neurodegeneration, and iii. the reappraisal of the role of tau in other neurodegenerative diseases. In addition, comparison between diseases involving tau, α-synuclein, prion protein and polyglutamine repeat deposition suggests a common theme, involving abnormal and accelerated insoluble protein deposition. The description of the ***tau*** mutations was the culmination of an international effort in the clinical and genetic analysis of a series of families with FTD. Identification of linkage to chromosome 17 in these families provided the common ground for the identification of the clinical and pathological similarities between these diseases.

## 5.3 FTDP-17

The identification of linkage to chromosome 17q21 in the dementia-disinhibition-parkinsonism-amyotrophy complex family (DDPAC, family Mo) led to a series of reports of further families with linkage in this region, summarized in a 1996 workshop report on FTDP-17.[232, 233] The original report of the DDPAC family described a clinical syndrome in which insidious personality change with disinhibition began at an average age of 45. This was accompanied by hypersexuality and hyperphagia. Patients with DDPAC cases progressively became become increasingly withdrawn, apathetic and emotionally blunted.[234] L-DOPA un-responsive parkinsonism was an associated feature and one case (III-39) also developed clinical amyotrophy with EMG evidence of widespread denervation. Anterior horn cell disease was not present in other members of this kindred and this included normal EMG examination in two further individuals. Although amyotrophy seems to have been an unusual feature it was incorporated in the name given to this disease. Further heterogeneity was apparent in the presence of a saccadic gaze disorder in 3/7 individuals. Features suggestive of parietal cortical involvement/CBD, such as apraxia, alien limb phenomena and myoclonus were not described.[234] Subsequent reports of the clinical features of chromosome 17-linked families described broadly similar syndromes although each was given a distinct name. These included pallido-pontine-nigral degeneration (PPND),[235] progressive subcortical gliosis (PSG), [236] and familial multiple system tauopathy with presenile dementia (FMSTD).[237] In addition, three Dutch families were described with hereditary frontotemporal dementia linked to chromosome 17 (HFTD 1,2 and 3),[238] although linkage in these families was based on the additive lod scores among all three families rather than individual definitive linkage in any one family. These families had similar clinical features with disease onset in the 40s-50s, early personality change with disinhibition, and later withdrawal and apathy, with extrapyramidal features. Amyotrophy was not a common feature in other FDTP-17 families.

The pathological topography of these families was similar. Frontal and temporal atrophy was a consistent finding, together with involvement of the substantia

nigra, amygdala and caudate/putamen/globus pallidus.[233] There was rather more uncertainty in the description of the immunocytochemical pathology. The two Dutch families HFTD-1and HFTD-2 were reported to have no tau deposition following immuno-histochemistry with both tau and paired helical filament specific antibodies,[238] and yet were subsequently shown to involve both mutations in tau and extensive tau deposition.[239] Similarly the report of the PSG-1 family's linkage to chromosome 17 emphasized the presence of prion protein deposition and did not describe tau pathology despite the use of tau immunocytochemistry,[236] although subsequently this family was shown to have tau deposition.[240] The report of the 1996 workshop concluded that the FTDP-17 families could be pathologically divided into four groups: i. tau pathology with ballooned neurons, ii. tau pathology without ballooned neurons, iii. ballooned neurons alone, and iv. dementia without specific pathological features.[233] The majority of these families have subsequently been shown to have significant tau deposition pathology.[241]

A number of candidate genes, located in the 17q21 critical region which spanned an estimated 2 cM, were sequenced and found to be normal in FTDP-17 before the identification of *tau* mutations. These genes included glial fibrillary acidic protein (GFAP), dual specific phosphatase (VHR), human homologue 3 of *Drosophila* light discs protein (DLG-3), RAPIP-8 and FTD-CG2.[191, 242] If the pathology-chromosome 17 linkage relationship had been clearer in 1996 then it is likely that tau would have been identified as the causative gene for FTDP-17 at an earlier stage. However, many groups reported normal tau sequence in families subsequently shown to have tau mutations, including the group which ultimately identified the majority of mutations.[243] Additional factors that delayed the identification of *tau* mutations included the frequent occurrence of intronic mutations which may have been thought to be non-pathogenic, the uncertain position of *tau* on the physical map of chromosome 17q21 and the apparent exclusion of *tau* by one recombination event.[242]

## 5.4 *Tau* mutations in FTDP-17

*Tau* was the primary candidate gene for FTDP-17 and three reports in 1998 defined pathogenic *tau* mutations. The first tentative report of the possibility of *tau* as the pathogenic gene in FTDP-17 was made by Poorkaj and co-workers who described a coding change, V337M in exon 12 of *tau* in Seattle family A.[230] This sequence change was reported together with a number of polymorphic variants within *tau.* Three supportive criteria for the definition of a pathogenic mutation were met: i. co-segregation of the mutant allele with the disease phenotype, ii. evolutionary conservation of the wild-type residue, iii. absence of the mutant allele in a series of control chromosomes. However, this report was qualified by the absence of *tau* mutations in other FTDP-17 families and the lack of any functional explanation for the effects of the sequence change. In June of 1998 Hutton and co-workers were able to conclusively demonstrate that *tau* was the causative gene for FTDP-17 by describing six separate coding and intronic mutations in *tau* and providing an explanation for the mechanism of action of the intronic mutations.[75] The coding mutations described were G272V and P301L which disrupted a conserved PGGG motif in the microtubule binding domains of exons 9 and 10 respectively, and the exon 13 mutation R406W which occurred adjacent to two serine residues, Ser396 and Ser404, which may act as sites for phosphorylation by Ser/Thr-Pro directed protein kinases.[75] The splicing mutations exon 10 +13, +14 and +16 were thought to alter the splicing of exon 10 and both *in vitro* evidence, using an exon trapping system, and *in vivo* evidence, using RNA analysis from post-mortem tissue, was presented which supported the role of *tau* splicing mutations in FTDP-17.[75] The original chromosome 17 linked family DDPAC was shown to have a *tau* exon 10 +14 mutation. The description of these mutations was closely followed by description of a further *tau* exon 10 splice site mutation, exon 10 +3, in the best characterized of the FTDP-17 families, FMSTD, by Spillantini and co-workers.[231] The *tau* splicing hypothesis was supported by protein analysis of tau in the family and demonstration of an abnormal tau protein isoform ratio.[231]

The description of ***tau*** mutations in FTDP-17 clearly offers insights into the pathogenesis of FTDP-17 and AD. This work also raises questions concerning: i. the prevalence of tau mutations among familial and sporadic FTD cases, ii. the relationship between FTDP-17 and other familial and sporadic tau deposition conditions, iii. the genetic and pathological classification of FTD, and iv. the relationship between the tau H1 haplotype association demonstrated in PSP and FTDP-17. This chapter involves the sequence investigation of *tau* in familial and sporadic FTD series, familial and sporadic PSP, Pick's disease and post-encephalitic parkinsonism.

## 5.5 Familial FTD

The prevalence of *tau* mutations and the clinico-pathological correlation in a series of British families with FTD was investigated in collaboration with the Dementia Research Group (DRG), Institute of Neurology and the Neuropathology Departments, Institute of Psychiatry and Institute of Neurology. The pathological findings are described in detail as they are particularly useful in interpreting the genetic work which forms the substantive data in this thesis. Twenty two families with autosomal dominant familial FTD were identified and recruited. Affected members of these families met clinical diagnostic criteria for fronto-temporal dementia.[170] The clinical features of these families are summarized based on case material prepared by the DRG (Table 5-1). Nearly all of these families had a clinically apparent frontal dementia syndrome with progressive behavioural and personality change. Additional features present included parkinsonism, in some cases related to anti-psychotic drug use, and dysphasia. The clinical diagnoses originally given to members of these families included PiD, frontal dementia and FTD. Historically, PiD was a frequently used clinical diagnostic label for this syndrome, more recently, the terms fronto-temporal dementia or frontal dementia have been used.

**Table 5-1 Sequence analysis of *tau* in familial FTD**

| Family # | AAO | Clinical features | Pathological diagnosis | Tau sequencing |
|---|---|---|---|---|
| 1 | 55 | Frontal syndrome | FTD-NLS [✦] | N |
| 2 | 57 | Frontal syndrome, Amnesia, Non-fluent dysphasia | FTD-NLS | N |
| 3 | 51 | Frontal syndrome, Amnesia, Gait apraxia | FTD-tau | Exon 10 +16 |
| 4 | 48 | Frontal syndrome, Non-fluent dysphasia, Parkinsonism | FTD-tau | Exon 10 +16 |
| 5 | 56 | Nominal dysphasia, Amnesia | PiD/AD | N |
| 6 | 46 | Frontal syndrome, Amnesia, Non-fluent dysphasia | FTD-tau | Exon 10 +16 |
| 7 | 57 | Frontal syndrome, Amnesia, Non-fluent dysphasia, Anti-psychotic induced parkinsonism | FTD-tau | Exon 10 +16 |
| 8 | 59 | Frontal syndrome, Dressing apraxia, Parkinsonism, Alien limb syndrome | FTD-NLS | N |
| 9 | 54 | Personality change with semantic dysphasia, Parkinsonism and Dressing apraxia | FTD-tau | Exon 10 +16 |
| 10 | 56 | Frontal syndrome, Dressing Apraxia, Anti-psychotic induced parkinsonism | FTD-Ub | N |
| 11 | 48 | Frontal syndrome, Amnesia, Non-fluent dysphasia | FTD-tau | Exon 10 +16 |
| 12 | 76 | Amnesia, Frontal syndrome, Parkinsonism | - | N |
| 13 | 66 | Social disinhibition, Dysphasia | - | N |
| 14 | 53 | Frontal syndrome, Cognitive slowing | FTD-tau | Exon 10 +16 |
| 15 | 44 | Frontal syndrome, Anti-psychotic induced parkinsonism, Nominal dysphasia | FTD-tau | Exon 10 +16 |
| 16 | 53 | Frontal syndrome | - | N |
| 17 | 60 | Frontal syndrome, Amnesia, Nominal dysphasia | FTD-Ub | N |
| 18 | 43 | Frontal syndrome, Nominal dysphasia, parkinsonism | FTD-tau | Exon 10 +16 |
| 19 | 51 | Frontal syndrome with disinhibition | FTD-Ub | N |
| 20 | 34 | Frontal syndrome, dysphasia, poor balance | - | Exon 10 P301S |
| 21 | 52 | Frontal syndrome, Left sided pyramidal weakness, Fasiculations, Dysphasia | FTD-NLS[*] | N |

| Family # | AAO | Clinical features | Pathological diagnosis | Tau sequencing |
|---|---|---|---|---|
| 22 | 45 | Frontal syndrome with dyspraxia | - | Exon 10 +14 |

**AAO – Average age at onset, N – Normal, PiD – Pick's disease, AD – Alzheimer's disease,[+] no immuno-histochemistry available, [*] No ubiquitin positive inclusions, but some sparse granular ubiquitin immuno-reactivity, FTD-Ub Frontotemporal dementia with Ubiquitin positive tau negative inclusions, FTD-tau Frontotemporal dementia with tau positive inclusions, FTD-NLS Frontotemporal dementia without characteristic inclusions**

Members of 17 of these families underwent autopsy examination. Each individual studied had broadly similar pathological features involving fronto-temporal atrophy and superficial laminar neuronal loss with spongiosis. Involvement of the basal ganglia was common, and often identifiable macroscopically with flattening and atrophy of the head of the caudate. These features led to some cases having originally been thought to be Huntington's disease. The use of immunocytochemistry allowed the FTD families identified to be subdivided into three main types. Individuals from ten families were identified who had tau inclusion pathology (FTD-tau), three families were identified which had tau negative, ubiquitin positive inclusions (FTD-Ub) and four families were identified which had no characteristic inclusions but prominent neuronal loss with superficial spongiosis (FTD-NLS) (Figure 5-1). The ubiquitin inclusions in the FTD-Ub families were thread-like or dot-like inclusions, and were present in superficial neocortical layers and the dentate fascia of the hippocampus (Figure 5-1). FTD-NLS families had a similar pattern of macroscopic change and fronto-temporal cell loss but without distinctive intracellular inclusions. The clinical presentations of all three pathological subtypes were similar. Parkinsonism was present in some cases of each pathological sub-type, and so does not appear to be a useful distinguishing clinical feature. CBD-like features were present in both FTD-NLS and FTD-tau families. Only one family in this series was identified to have clinical motor neuron involvement (family 21), and this family did not have typical neuronal ubiquitin inclusions, although some granular neuronal ubiquitin immunoreactivity was identified.

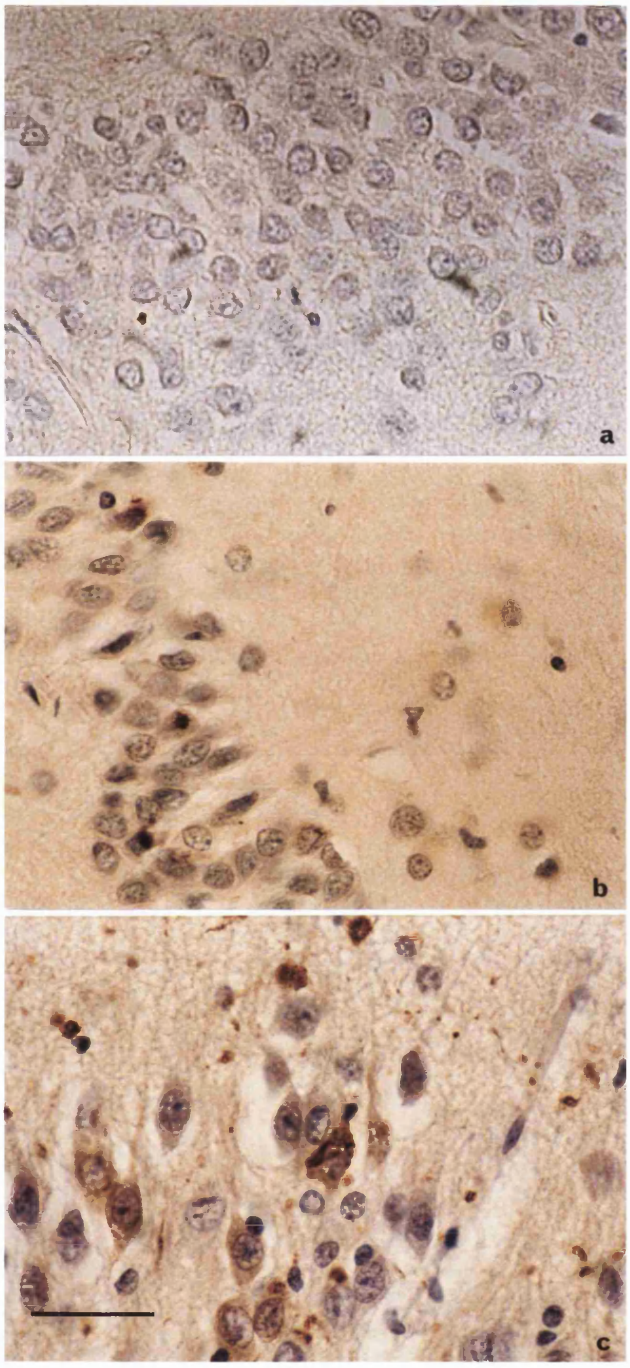



**Figure 5-1 Three pathological subtypes of FTD**

**High power view of dentate gyrus showing lack of ubiquitin immunoreactivity in FTD-NLS (a), dense ubiquitin inclusions in FTD-Ub. (anti-ubiquitin antibodies, polyclonal rabbit, 1:500, Dako).(b) and dense and granular tau immunoreactivity in FTD-tau (anti-tau antibodies AT8, monoclonal mouse 1:200, Innogenetics), in a tau exon 10 +16 mutation individual (c). Scale bar=50μm. Courtesy of Mr. N Khan and Prof. P Lantos, Department of Neuropathology, Institute of Psychiatry**

Nine of the ten FTD-tau families identified had a very similar pathological picture, with extensive neuronal and glial tau deposition. The neuronal inclusions included globose and flame shaped neurofibrillary tangles together with dense round inclusions reminiscent of Pick bodies, while the glial pathology involved coiled comma shaped oligodendroglial inclusions in white matter and tufted astrocytes with tau deposition in the proximal astrocytic processes.(Figure 5-2) In the remaining tau FTD family, the index case had typical pathological features of Pick's disease with numerous Pick bodies in both neocortex and the mesial temporal cortex including the dentate gyrus. Subsequently a pathological report was obtained on another individual from family 5 which showed the typical changes of AD, suggesting that this was not a concordant FTD family. No other family with the pathological changes of Pick's disease was identified in this series.

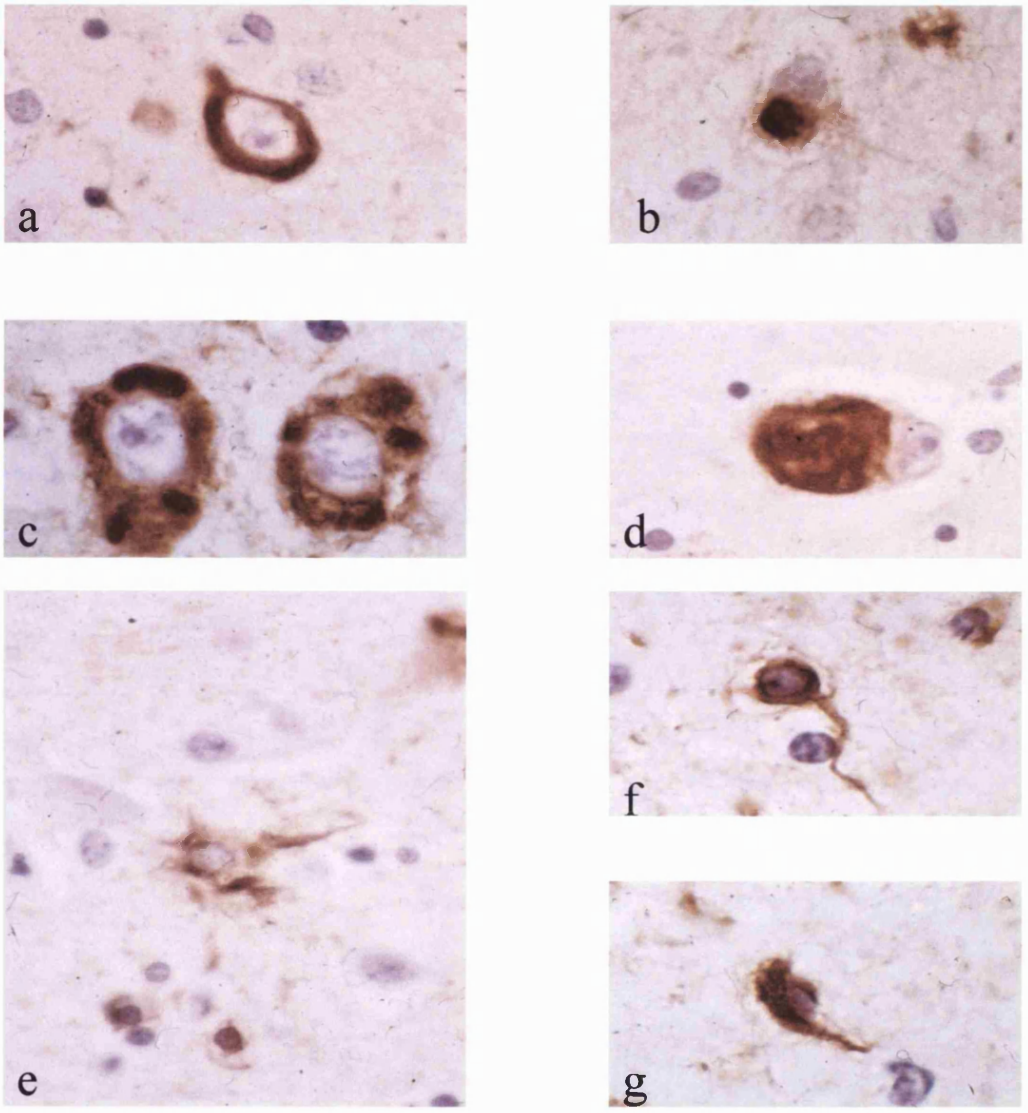



**Figure 5-2 Neuronal and glial tau deposition patterns in FTD-tau**

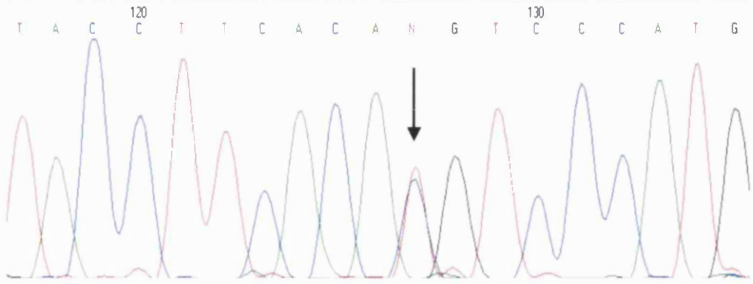
**High power views stained with antibody AT-8: a-d neuronal tau deposition showing: a) coiled, b) dense Pick body-like, c) diffuse granular with dense deposits and d) globose neuronal inclusions; and e-g glial tau deposition showing e) tufted astrocyte and f),g) coiled oligodendroglial inclusions. Courtesy of Dr. Tamas Revesz, Institute of Neurology**

Following this clinical and pathological analysis DNA from an affected individual in each family was obtained from a stored blood sample, or from frozen brain, and exons 9-13 of *tau* were sequenced.

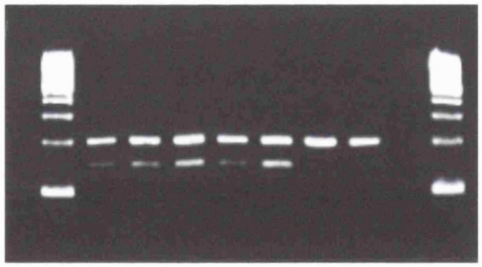
**Results**

Half (11/22) of the families were identified to have *tau* mutations(Table 5-1). Nine families had the tau exon 10 +16 mutation (Figure 5-3), one family had the tau exon 10 +14 mutation (Figure 5-5) and one family with the exon 10 P301S coding mutation was identified (Figure 5-4). The average age at onset for the P301S mutation family was 34 years as compared with an average age at onset for the +16 mutation families of 49 years. Each mutation was identified by direct sequencing of the forward and reverse DNA strands and by confirmatory restriction fragment length polymorphism.


Mutant base pair change at ***tau*** exon 10 splice site +16 position

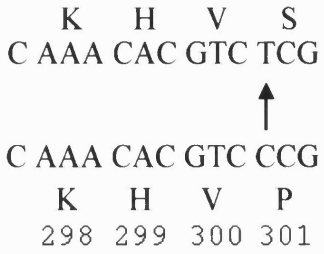



ABI electropherogram showing C/T heterozygote at ***tau*** exon 10 +16 position

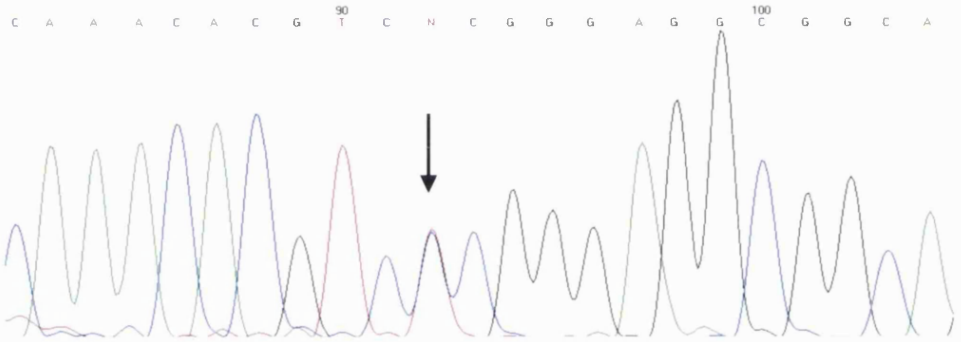


***Nsp I*** digest exon 10 PCR product. +16 C-T mutant (lanes1-5), DNA control (lanes 6,7), water control (lane 8). 100 bp size standard. Mutant allele introduces novel restriction site to 200 bp PCR product yielding 148 and 52 bp fragments.

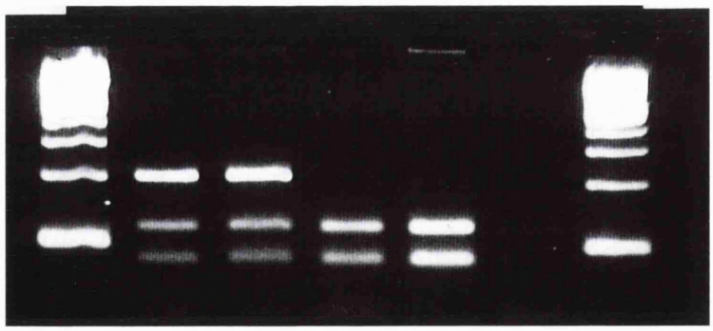
**Figure 5-3 Exon 10 + 16 mutation**




Mutant base pair change at ***tau*** exon 10 codon 301



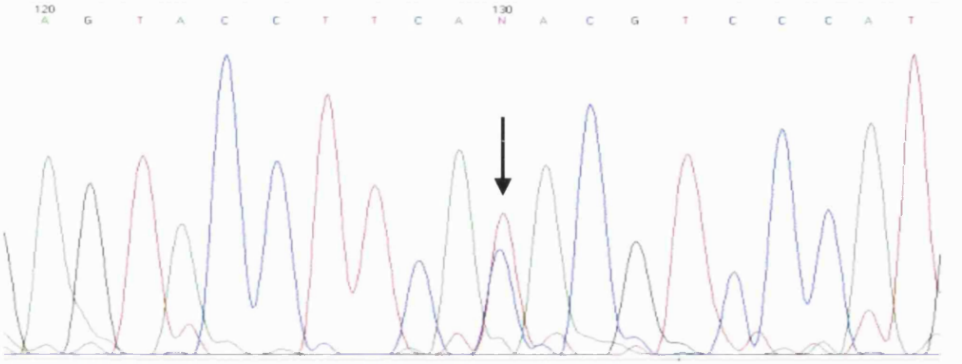
ABI electropherogram showing C/T heterozygote at
***tau*** exon 10 codon 301



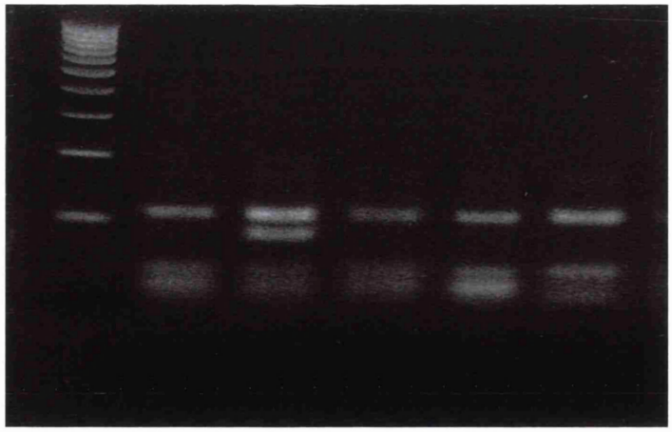
***Sma I*** digest exon 10 PCR product. P301S mutant (lanes1,2), wild type control (lanes 3,4), water control (lane 5). 100 bp size standard, mutant allele 200 bp, wild type allele 118, 82 bp.

**Figure 5-4 Exon 10 P301S mutation**


Mutant base pair change at ***tau*** exon 10 splice site
+14 position



ABI electropherogram showing C/T heterozygote at
***tau*** exon 10 +14 position



***Afl III*** digest exon 10 PCR product. +14 C-T mutant (lanes 2),
DNA control (lanes 1,3-5). 100 bp size standard. Mutant allele
removes restriction site: wild type 110, 54, 36 bp., mutant 110, 90 bp.

**Figure 5-5 *Tau* exon 10 +14 mutation**

## Discussion

Three separate mutations in *tau* were identified in this series of British families with FTD. Each has been previously reported in other families with FTDP-17. The exon 10 +16 mutation was reported in the first detailed description of mutations in FTD, in four separate families.[75] Two of these families were British originating from the Manchester series of FTD families, accounting for 2/3 of the mutations described in British families, in this series. The identification of the mutation in nine further apparently separate British families supports the fact that this is the most common mutation found among British FTD families. It is likely that these families all originated from a single founder individual in the UK and has become widely dispersed. The identification of this number of separate families with +16 mutations allows genetic investigation of the founder effect and analysis of disease mutation penetrance. Analysis of microsatellite markers surrounding *tau* in an extended family with FTDP-17 will indicate the disease chromosome haplotype and further apparently separate families can be genotyped to see if they share the disease chromosome haplotype and therefore a common founder. DNA samples were not available from sufficient FTD family members to enable reconstruction of disease chromosome haplotypes or analysis of disease mutation penetrance. The *tau* exon 10 +16 mutation is absent from over 150 Caucasian controls. As discussed, i*n vivo* and *in vitro* work by others has established the mechanism of action of the *tau* +16 mutation. Analysis of post-mortem tissue from individuals with exon 10 +16 mutations demonstrates that these cases have an increased 4R:3R *tau* RNA ratio and an increased 4R:3R protein ratio.[75] *In vitro* analysis of tau splicing in an exon trapping system also confirms that this mutation alters the alternative splicing of tau and serves to drive the inclusion of exon 10 in the final RNA transcript. It is hypothesised that this is due to destabilisation of an exon 10 exon-intron stem loop structure which normally sequesters the exon 10 splice site. Destabilisation of this structure opens up the splice site, allows binding of the U1 snRNP component of the spliceosome and promotes the inclusion of exon 10.[244]

The exon 10 +14 mutation was described in the original family with DDPAC and another family with this mutation is described in this study. The presence of Irish ancestry in the individual identified in this series supports the idea that this individual shares common ancestry with the DDPAC kindred. This mutation is also absent from Caucasian controls. Similarly to exon 10 +16, there is convincing *in vivo* and *in vitro* evidence which supports the ideas that this mutation exerts its effect via disruption of the normal alternative splicing of exon 10. *In vitro* experiments by Grover and colleagues suggests that the +14 mutation has a more profound effect on exon 10 splicing and a greater effect on the amount of 4R *tau* RNA produced.[244] However, in the families presented in this thesis the age at onset of the +14 family overlaps with that of the +16 families. In contrast, the P301S family identified in this study has a relatively young age at onset. The P301S mutation has also been described by Sperfeld and colleagues, who confirm an exceptionally early age of onset, in the third decade.[245] Proline residue 301 is highly conserved and mutations at this residue have not been demonstrated in control subjects. Interestingly, this mutation has a particularly marked effect on the acceleration of filament formation, in comparison with the later onset FTDP-17 mutations such as P301L and G272V, but it also has a destabilising effect on microtubule assembly.[246]

This study also illustrates the genetic and pathological heterogeneity of familial FTD. In this series 11/22 (50%) of familial FTD cases had *tau* mutations. Three other groups have studied the prevalence of *tau* mutations in familial FTD.[247-249] Rizzu and colleagues report that 47% of patients with FTD and a positive family history have mutations in *tau* whereas Houlden and colleagues suggests that only 11% (6/54) of FTD families have identifiable *tau* mutations. However, neither of these studies provided a pathological analysis of the families analysed. More recently, Poorkaj and colleagues have studied a large series of familial FTD cases and identified tau mutations in 10.5% of the familial cases and only 33% of the familial cases with tau pathology. In contrast, our study suggests that the presence of tau pathology in a familial FTD case strongly predicts the presence of a *tau* mutation, whereas the presence of FTD-NLS or FTD-Ub pathology effectively excludes a mutation in *tau*. Possible reasons for the discrepancies

between these studies include the age at onset of families studied, the strength of evidence of a concordant family history and a founder effect within European populations leading to a higher *tau* mutation prevalence. Our results correlate well with analysis of pathology in the unequivocally chromosome 17q21 linked FTD families, in which practically all affected members had significant tau deposition and concomitant mutations in the tau gene.[241] Although familial tau deposition neurodegeneration without *tau* mutations can occur in clinically diagnosed progressive supranuclear palsy families,[177] this has not been commonly described in families with pathologically diagnosed tau deposition FTD with multiple affected members. One exception may be the hereditary dysphasic disinhibition family (HDD2), linked to the *tau* region with a maximum lod score of 3.68. Tau immunocytochemistry has identified variable tau deposition in this family, although a recent report describes depletion of tau on Western blot analysis.[250, 251] A mutation in *tau* has not been reported in HDD2.

This series suggests that there are no clinical features which can reliably distinguish these three familial FTD subtypes, and signs of motor neuron disease with frontotemporal dementia were uncommon. FTD-Ub pathology has been identified in 3/17 (18%) of the pathologically diagnosed FTD families. These are similar to the ubiquitin inclusion family described by Kertesz and colleagues,[252] the sporadic cases with semantic dementia identified by Rossor and colleagues,[253] and the sporadic and familial cases identified by Jackson and colleagues and described as motor neuron disease inclusion dementia.[254] The pathological reports confirm that superficial neocortical cell loss with vacuolation and ubiquitin inclusions in the dentate gyrus of the hippocampus are core features of this disease. Ubiquitination is a frequent finding in a wide range of neurodegenerative disease processes and may be a marker for the deposition of a number of different abnormal proteins. However, given the clinical and pathological similarity between the families with FTD-Ub it is likely that these families will have a common genetic basis. A recent report has described the linkage of familial ALS to chromosome 9 and although the pathology of these families have not been reported it is possible that this corresponds to the FTD-Ub families, with negative ***tau*** sequencing, described in this thesis.[255]

We have also identified 4/17 (24%) families with FTD-NLS. The relationship between the FTD-NLS reported here and other reported FTD subtypes is harder to establish given the overall pathological similarities between all of these diseases and the absence of tau and/or ubiquitin immunohistochemistry in some reports. The diseases described as "dementia lacking distinctive histopathology", "dementia lacking distinctive histological features", "familial dementia of adult onset with pathological features of a non-specific nature" and "dementia with microvacuolar pathology and laminar spongiosis" may all correspond to either FTD-Ub or FTD-NLS depending on the results of ubiquitin immunohistochemistry.[256-259] Genetic linkage to chromosome 3 has been reported in a Danish kindred originating in Jutland, apparently without distinctive pathological features (OMIM #600795).[260] A recent study using ubiquitin immuno-histochemistry did not show intraneuronal ubiquitinated inclusions in the chromosome 3 linked kindred, and conversely analysis of a large family with ubiquitin inclusion dementia excluded linkage to chromosome 3.[252, 261] This suggests that chromosome 3 linked dementia may correspond to FTD-NLS described in this series. Assuming continuing pathological-genetic correlation across FTD this series suggests that there are at least two further genes to be identified which may be responsible for familial FTD.

In conclusion, we have demonstrated three pathological subtypes of familial FTD and a close correlation between the presence of a tau mutation and the presence of tau pathology. Despite the suggestion that Pick's disease is a common familial dementia, this has not been supported by this study. There is genetic and pathological heterogeneity in familial FTD and, undoubtedly, further genes will be identified which are responsible for these disorders.

### *5.6* Sporadic FTD and focal cortical degenerations

A series of sporadic FTD cases were recruited for *tau* analysis, to determine if mutations in *tau* could be responsible for FTD cases without identified family histories. Included within this series were cases with other focal neurodegenerative syndromes including posterior cortical atrophy and

progressive apraxia, which had been included in the Dementia Research Group FTD and FTD-like syndromes group. There were 12 cases without pathological confirmation of diagnosis, and 11 cases of pathologically diagnosed PiD cases identified from the Neuropathology departments, Institute of Neurology and Institute of Psychiatry, some of which had incomplete clinical details.

## Results

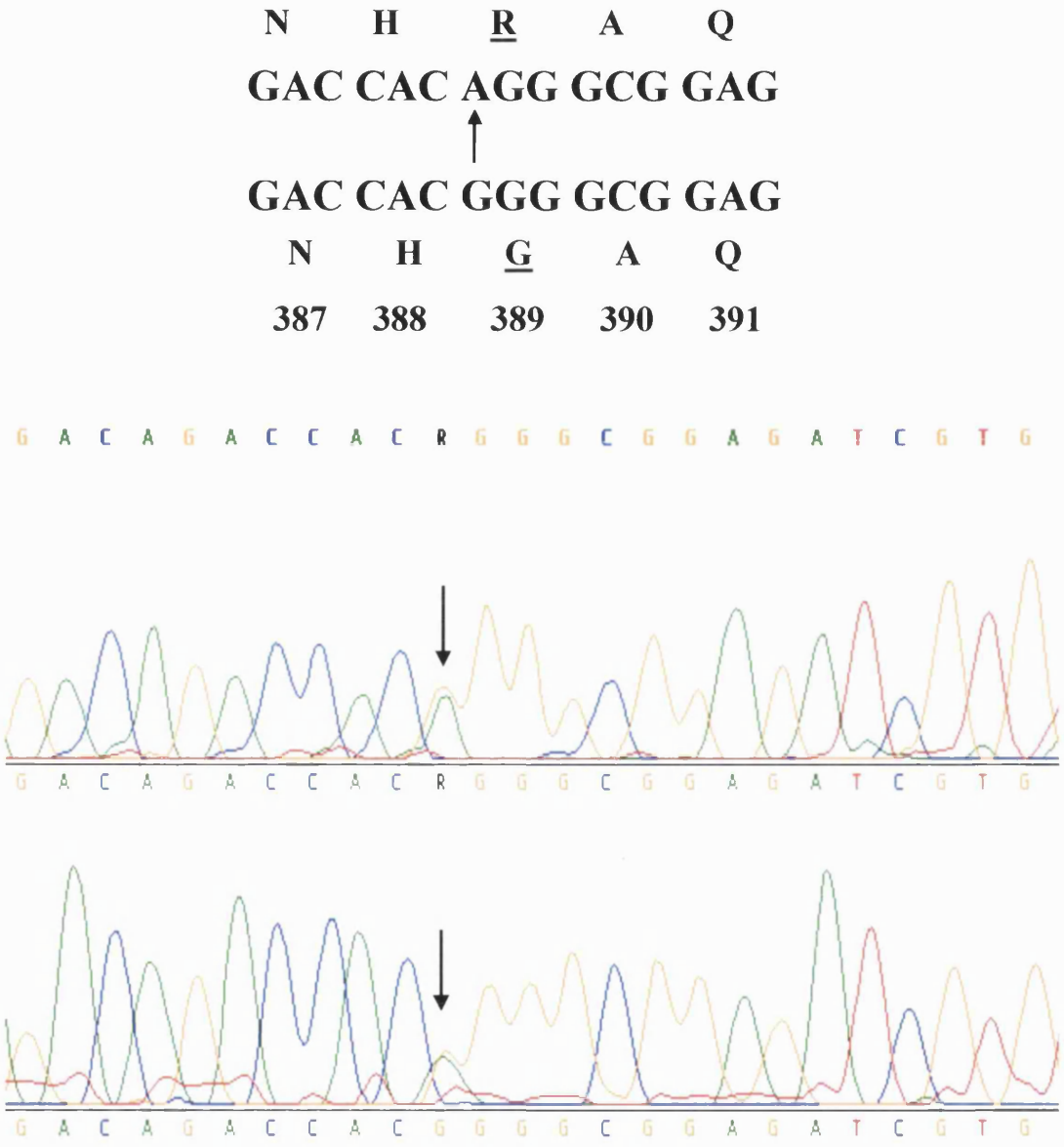
Among the non-pathologically confirmed FTD cases no pathogenic *tau* mutations were identified. The majority of pathologically diagnosed PiD cases did not have mutations in *tau*. Two cases without apparent family histories but with pathological diagnoses of PiD were identified to have the G389R *tau* mutation (Table 5-2). This mutation was identified by direct sequencing of the forward and reverse strand, but no restriction enzyme could be identified to enable confirmation of the genetic diagnosis. Further investigation of the pathological phenotype showed that both G389R mutation cases had 12-E8 positive Pick bodies, unlike each of the other PiD cases examined, which were 12-E8 negative.

**Table 5-2 Sequence analysis of *tau* in sporadic FTD and focal cortical degeneration**

| Case # | Age at onset | Clinical syndrome | Pathology | Tau |
|---|---|---|---|---|
| 1 | 52 | Memory impairment, dysphasia | NA | N |
| 2 | 67 | Apraxia, myoclonus | NA | N |
| 3 | 67 | Frontal syndrome | NA | N |
| 4 | 55 | Frontal syndrome, expressive dysphasia | NA | N |
| 5 | 66 | Dysphasia, orofacial dyspraxia | NA | N |
| 6 | 48 | Dysphasia, psychosis, myoclonus | NA | N |
| 7 | 67 | Frontal syndrome, semantic dysphasia | NA | N |
| 8 | 55 | Progressive dysphasia, posterior cortical syndrome | NA | N |
| 9 | 60 | Frontal syndrome, expressive dysphasia | NA | N |
| 10 | 67 | Frontal syndrome, semantic dysphasia | NA | N |
| 11 | 64 | Frontal syndrome, dyspraxia nominal dysphasia | NA | N |
| 12 | 66 | Posterior cortical syndrome | NA | N |

| Case # | Age at onset | Clinical syndrome | Pathology | Tau |
|---|---|---|---|---|
| 13 | 55 | Frontal syndrome with apathy, parkinsonism | PiD | N |
| 14 | 68 | NA | PiD | N |
| 15 | 64 | NA | PiD | N |
| 16 | NA | NA | PiD | N |
| 17 | NA | NA | PiD | N |
| 18 | NA | NA | PiD | N |
| 19 | 34 | Frontal syndrome | PiD; 12E8 +ve | G389R |
| 20 | 45 | Frontal syndrome, nominal dysphasia | PiD | N |
| 21 | 58 | Frontal syndrome, myoclonus | PiD | N |
| 22 | 40s | NA | PiD; 12E8 +ve | G389R |
| 23 | NA | NA | PiD | N |

NA – Not available, PiD – Pick's disease, G389R – Glutamate to Arginine mutation *tau* position 389




**ABI electropherogram showing A/G heterozygote in *tau* exon 13 codon 389, forward and reverse strands**

**Figure 5-6 *Tau* exon 13 G389R mutation**

## Discussion

The rarity of *tau* mutations in sporadic FTD is in accordance with other studies. The study performed by the Dutch group identified one *tau* mutation, ΔK280 in 53 apparently sporadic FTD cases.[248] The study performed by Houlden and colleagues identified no *tau* mutations in a sporadic series of 71 cases.[247] A detailed pathological breakdown of the non-PiD sporadic FTD cases is not available in the current series and the nosology of the sporadic FTD cases remains uncertain. Although PiD, CBD and FTDP-17 are well delineated entities it remains unclear whether there is a sporadic FTD-tau variant which is distinct from CBD, PSP and PiD.

## PiD

The genetic basis of PiD is of particular interest as PiD is the pathologically best established FTD and has been frequently described to occur in familial forms.[122] In addition, as described (Section 2.5) tau in PiD is deposited in predominantly 3 repeat tau protein isoforms, in contrast to the 4 repeat tau isoform deposition pattern, described in *tau* exon 10 splice site mutations.[103] Since no PiD families were identified, sporadic PiD cases identified on pathological grounds were studied. There are no formally agreed pathological criteria for PiD but recognized features include the presence of lobar atrophy, with relative sparing of the posterior superior temporal gyrus, frequent neocortical and mesial temporal Pick bodies, particularly in the dentate gyrus of the hippocampus.[122] In PiD tau inclusions are immunoreactive to a range of phosphorylation dependant and independent tau antibodies, but not to the phosphorylation dependant antibody 12-E8.[122] Antibody 12-E8 recognizes a phosphorylated serine 262 in tau exon 9.[103] Supportive biochemical evidence for the pathological diagnosis of PiD include the presence of abnormal hyperphosphorylated tau bands at 60 and 64kDa, which can be demonstrated to be made up of only three repeat tau isoforms following treatment with alkaline phosphatase.[122]

Two out of 11 pathologically diagnosed Pick's disease cases in this series were identified to have exon 13 mutation G389R mutations. Although both of these cases involved lobar atrophy with the formation of numerous Pick cells and Pick bodies, including involvement of the dentate fascia, these cases were atypical in the presence of Pick-like bodies which could be stained with tau antibody 12-E8 and the young age of onset. This amino acid mutation has also been identified by Murrell and co-workers, although due to a different base pair substitution.[262] They confirm the presence of atypical Pick body staining with antibody 12E8.[262] The family described in this paper like those described in the current work did not have a clear cut family history of fully penetrant autosomal dominant dementia. This suggests that this amino acid substitution may be a low penetrance pathogenic mutation. Tau protein analysis performed by Murrell and co-workers indicates that the tau protein deposited in the G389R cases, like PiD is in the form of 60 and 64 kDa tau bands, but this tau is probably composed of more 4R tau than the tau deposited in sporadic PiD.[262] It is particularly interesting that in these families Pick-like bodies can be associated with a PiD like hyperphosphorylated tau Western blot pattern, without selective 3 repeat tau protein deposition. Although this is a question of nosology, these data seem to be sufficient to classify the G389R cases as atypical PiD. Two further *tau* mutations described may produce a pathological phenotype that is similar to PiD, the exon 9 K257T and exon 10 ΔK280 mutations.[248, 263] The pathology described in the exon 9 K257T mutation case is very similar to PiD in that the Pick body like inclusions are not immunoreactive with antibody 12-E8.[263] However, although the phosphorylated tau deposited in K257T brain forms major bands at 55 and 64 kDa identical to those seen in PiD, analysis of the dephosphorylated tau indicated that this involves the deposition of both three and four repeat tau isoforms, although three repeat tau may predominate.[263] Another mutation, ΔK280 has been identified in a Dutch individual, and is predicted *in vitro* to lead to PiD like three repeat tau deposition.[248] The possible neuropathological similarity between ΔK280 and sporadic PiD has not been confirmed. Neither ΔK280 nor K257T mutations have been identified in the

pathologically classified cases in this study. If typical PiD is defined as involving the lack of 12E8 immunoreactive Pick bodies then typical PiD appears to be usually a sporadic condition, and not due to mutations in *tau*.

## 5.7 Progressive supranuclear palsy

There are a number of good reasons to investigate the sequence of *tau* in PSP: i.) PSP and FTDP-17 are pathologically and clinically similar conditions, ii.) the gene for familial PSP remains to be identified, iii.) the association of *tau* with PSP may relate to a pathogenic mutation in *tau* which has occurred on the H1 background, iv.) it has been suggested that familial PSP in fact encompasses a wider range of clinical phenotypes than is currently recognised.[264] A number of different PSP cases have therefore been sequenced in the search for *tau* mutations. Tau was sequenced in two families with clinically diagnosed PSP affecting more than one member, in five pathologically diagnosed cases of PSP in which there was a family history of parkinsonism or dementia and five sporadic PSP cases (Table 5-3).

**Table 5-3 Sequence analysis of *tau* in PSP**

| Case | Tau | FH | Clinical Features | Age at onset | Pathological Features |
|---|---|---|---|---|---|
| 1 | N | -ve | Atypical: IPD like | 53 | Typical |
| 2 | N | -ve | Typical: Tolosa | 72 | Typical |
| 3 | N | -ve | Atypical: IPD like | 76 | Typical + Vascular disease |
| 4 | N | -ve | Typical: NINDS | 62 | Typical |
| 5 | N | -ve | Typical: Tolosa | 66 | Typical |
| 6 | N | -ve | Atypical: gait disorder/ no eye movement disorder | 77 | Typical + Cortical involvement |
| 7 | N | -ve | Atypical: chorea | 60 | Typical |
| 8 | N | +ve: 1brother and father tremor | Typical: Tolosa | 63 | Typical + CBD features |
| 9 | N | +ve: 1 brother parkinsonism | Atypical: IPD like | 79 | Typical |
| 10 | N | +ve: father and nephew parkinsonism | Typical: NINDS | 66 | Typical |
| 11 | N | +ve: sister AD | Typical: NINDS | 69 | Typical |
| 12 | N | +ve: uncle PD | Typical: NINDS | 66 | Typical |
| 13 | N | +ve: brother | Typical: NINDS | 73 | Typical |

| Case | Tau | FH | Clinical Features | Age at onset | Pathological Features |
|---|---|---|---|---|---|
| | | parkinsonism | | | |
| 14 | N | +ve: mother and father tremor | Atypical: non-Ldopa responsive parkinsonism | 70 | Typical |
| 15 | N | +ve: brother PSP, brother AD | Typical: NINDS | 67 | Not available |
| 16 | N | +ve: cousin PSP | Typical: NINDS | 64 | Typical |
| 17 | +16 C-T | -ve | Typical: Tolosa | 40 | Typical (limited pathological study) |

**NINDS – National Institute for Neurological Disorders and Stroke, IPD – Idiopathic Parkinson's disease, CBD – Corticobasal degeneration, Tolosa – meets Tolosa criteria for PSP**

## Results

A further ***tau*** exon 10 +16 mutation was identified in PSP case 17, a sporadic young onset PSP case. However, no further mutations were identified in cases with a concordant family history of PSP, in cases with a family history of other neurodegenerative diseases, or in clinically atypical and pathologically typical cases.

## Discussion

This study has identified a ***tau*** mutation in an individual with a PSP-like presentation. This patient presented with lethargy and micrographia and then developed disturbances of gait, eye movement and bulbar dysfunction typical of PSP. The clinical diagnosis was of likely PSP, although he did not fulfil the NINDS PSP clinical research criteria in that falls were not a clinical feature in the first year of symptoms, but they were a prominent and disabling feature later in the disease course.[138] His age of onset did not preclude a diagnosis of NINDS probable PSP, since this stipulates an age of onset of 40 or over, but as the median age of onset of PSP is 63, and the youngest cases of pathologically proven PSP reported in the literature are aged 43 and 45 at onset, this would have been an exceptionally early presentation.[265] There was no autosomal dominant family history of neurodegenerative disease, prominent disinhibition, abulia, or dysphasia. Neuropsychological evaluation revealed a decline in verbal memory at an advanced disease stage, but also evidence of decreased word fluency and

cognitive slowing, consistent with PSP. Despite the clinical presentation, and the lack of a positive family history a genetic diagnosis of FTDP-17 was made. This case illustrates the very close clinical relationship between FTDP-17 and PSP. However, mutations in *tau* have not been identified in other PSP cases in this study, in common with other groups who have investigated PSP in clinically based series.[178, 193, 195]

FTDP-17 kindreds in which the pathogenic mutation is a *tau* exon 10 coding or splice mutation have particular clinical similarities to PSP.[233, 266] These conditions both involve degeneration of the basal ganglia and brainstem, with deposition of neurofibrillary tangles consisting of two major hyperphosphorylated tau bands at 64 and 68 kDa on Western blotting.[241] These bands consist predominantly of four repeat isoforms of *tau*, and in exon 10 splice mutations this occurs because of a change in the alternative splicing of *tau* RNA.[75] PSP may also involve a change in the alternative splicing of *tau*,[229] but this has not been demonstrated in all brain areas, or in all cases.[177, 229] In addition, FTDP-17 involves degeneration of frontal and temporal cortex and frequently involves marked personality change, obsessional symptoms and progressive dysphasia.[233] Although personality change and withdrawal may be early features of PSP, the most characteristic features are of early imbalance and a supranuclear gaze palsy and this probably reflects predominant damage to the brainstem. These features may be seen in FTDP-17, and some families and individuals affected appear to be indistinguishable from sporadic PSP, including the case described in this study.[231, 233, 237, 267] The FMSTD kindred is particularly close to PSP, with features including early gait disturbance, axial rigidity, bulbar symptoms and a vertical gaze disorder.[237] Both PSP and FTDP-17 involve the deposition of tau as NFTs in cortical and subcortical structures. However, many of the FTDP-17 kindreds described with supranuclear or oculomotor gaze abnormalities have atypical features for PSP such as prominent asymmetry, prominent cortical sensory signs, psychosis, L-DOPA induced dyskinesias, prominent neuropsychiatric symptoms or late gait disturbance.[268-270]

PSP distribution pathology has been described in a number of FTDP-17 families.[75, 200, 269] Exon 10 FTDP-17 involves extensive neuronal and glial tau deposition and this may include the tufted astrocyte type tau inclusion which has been considered to be relatively specific for PSP.[124] In addition, FTDP-17 exon 10 mutation cases may involve extensive oligodendroglial tau deposition and the formation of astrocytic plaques, considered to be more characteristic of CBD. The R406W and S305S mutation FTDP-17 families have close pathological similarities to PSP. In the family subsequently shown to have the R406W mutation, autosomal dominant dementia with widespread NFTs, NFTs were not prominent in the frontal or temporal neocortex, but neurofibrillary degeneration was prominent in subcortical structures including the subthalamic nucleus, globus pallidus and brainstem.[200] However significant mesial temporal tangle formation and cell loss was present which would be an unusual feature in PSP. In addition, the NFTs were paired helical filament type rather than the straight filaments usually seen in PSP.[200] Furthermore, the clinical picture was dominated by insidious memory impairment without clinical features suggestive of PSP. The S305S mutation family also involved relative sparing of the frontal and temporal neocortex with involvement of structures typically damaged in PSP. In addition, the S305S mutation is predicted to cause an increase in the 4R tau isoform expression ratio, suggesting that the tau western blot pattern in this family would be similar to PSP. One individual in the S305S kindred developed axial rigidity and a supranuclear gaze palsy, but this was accompanied by asymmetric limb clumsiness and then rigidity, suggestive of CBD. At the electron microscopic level a distinction can be made between exon 10 FTDP-17 tau filaments and PSP tau filaments, as FTDP-17 involves the deposition of the twisted ribbon filament.[271]

Although PSP has many similarities to FTDP-17 and there may be overlapping features, there are clinical and pathological differences, and the absence of *tau* mutations in the majority of families and individuals described here reinforces that distinction. Usually PSP or typical PSP like syndromes are not due to *tau* mutations. Taken together with the work of Hoenicka and colleagues in excluding *tau* in the largest PSP family described to date,[177] a separate gene may

determine neurofibrillary degeneration in familial PSP. Additionally this work confirms that the *tau* H1 predisposition effect is not due to a rare *tau* coding or immediate splice site mutation in exons 9-13 of *tau*.

## 5.8 Conclusions

The identification of *tau* mutations in FTDP-17 may help to elucidate the role of tau deposition in range of neurodegenerative disorders. Data presented in this study illustrates i.) the strong association between familial FTD involving tau deposition and the presence of a tau mutation, ii.) the genetic and pathological heterogeneity of familial FTD, iii.) the clinical overlap between PSP and FTDP-17, iv.) the pathological phenotype of typical and atypical PiD, including PiD due to the G389R mutation v.) the usual absence of mutations in PSP, sporadic and non-tau FTD.

### Mutations in tau their functional effects

Since the original description of *tau* mutations in 1998, a number of other mutations have been described together with molecular pathological data and *in vitro* data on their possible functional importance, summarized in Table 5-4. There are three main groups of tau mutations: exon 10 splicing mutations, exon 10 coding mutations and non-exon 10 coding mutations, and each has a separate molecular pathological profile(Table 5-5). Exon 10 splicing mutations may be exonic or intronic. As described, intronic mutations are believed to affect the alternative splicing of ***tau*** through disruption of a stem loop structure.[244, 272]

**Table 5-4 Tau mutations and their effects**

| Tau mutation | Microtubule interaction (either binding or stabilization) | Filament formation | 4R:3R tau RNA isoform ratio | Reference |
|---|---|---|---|---|
| G272V | ↓ | ↑ | NA | 75, 246, 273 |
| N279K | ↔ | - | ↑ | 274, 275 |
| ΔK280 | ↓ | - | ↓ | 248, 272 |
| L284L | ↔ | ↔ | ↑ | 272 |
| P301L | ↓ | ↑ | ↔ | 273 |
| P301S | ↓ | ↑ | NA | 75, 246 |

| Tau mutation | Microtubule interaction (either binding or stabilization) | Filament formation | 4R:3R tau RNA isoform ratio | Reference |
|---|---|---|---|---|
| S305N | ↔ | ↔ | ↑ | 246, 276, 275 |
| S305S | ↔ | ↔ | ↑ | 269 |
| Exon 10 +3 | - | - | ↑ | 271 |
| Exon 10 +12 | - | - | ↑ | 277 |
| Exon 10 +13 | - | - | ↑ | 75 |
| Exon 10 +14 | - | - | ↑ | 75 |
| Exon 10 +16 | - | - | ↑ | 75 |
| V337M | ↓ | ↑ | - | 246, 273 |
| G389R | ↓ | NA | NA | 262 |
| R406W | ↓ | ↔ | NA | 75, 246, 273 |

**Effect refers to the pure mutation containing protein or RNA. The alteration in the 4R:3R ratio may have secondary effects on microtubule interaction or filament formation**

In addition to the exon-intron boundary splicing mutations, exonic mutations have been described which affect the splicing of tau exon 10. These mutations, some of which do not lead to an amino acid substitution, are postulated to either increase the efficiency of an exon splice enhancer element (e.g. N279K) or decrease the efficiency of an exonic splice inhibitor (e.g. L284L).[272] Support for this hypothesis of the effect of exon 10 splicing mutations comes from both post mortem tau RNA analysis and from *in vitro* analysis of the exon 10 region containing the observed sequence changes.[272] The change in the alternative splicing of tau RNA leads to the deposition of 4R tau protein with a major doublet of protein bands seen on western blotting at 64 and 68 kDa. However, the mechanism of toxicity produced by the alteration in the tau 4R:3R ratio is unknown. Possibly this involves a change in the filament forming properties of pure cytoplasmic 4R tau as opposed to a mixture of 3R/4R tau. Alternatively, or additionally there may be separate 4R and 3R tau-microtubule binding sites, and a change in the 4R:3R ratio may lead to saturation of the 4R binding sites and an increase in free cytoplasmic *tau*.

Exon 10 coding and non-exon 10 coding mutations produce a single amino acid change within the tau sequence without an alteration in *tau* alternative splicing. Exon 10 coding mutations lead to a PSP-like 64/68 kDa western blot pattern

whereas non-exon 10 coding mutations lead to an AD-like 60/64/68 kDa tau triplet.[278] As described in Chapter 1 for AD, detailed molecular pathological examination has confirmed hypotheses generated from the study of *tau* mutations and their *in vitro* effects.

**Table 5-5 Pathological effect of tau mutations**

| | Exon 10 coding | Exon 10 splicing | Non-exon 10 |
|---|---|---|---|
| Cytoskeletal pathology | Neuronal/glial | Neuronal/glial | Neuronal |
| Electron microscopy | Twisted ribbon | Twisted ribbon | PHF |
| 4R tau: 3R tau RNA | Normal | Increased | Normal |
| Tau western blot | Doublet | Doublet | Triplet |

Experimental evidence has been gathered for two different effects of the tau coding mutations, firstly an acceleration of filament formation and secondly loss of binding to and alteration in the stabilization of microtubules. Loss of binding to microtubules might have the effect of destabilisation, although as FTDP-17 is a dominant disorder one normal sequence tau allele will be present and the extent of interaction between tau molecules in microtubule binding is not clear. This makes a "dominant negative" effect of tau mutations in destabilising microtubules and interfering with the action of the normal tau protein less likely. However, loss of binding to microtubules will lead to increased free cytoplasmic tau which could exert a deleterious effect. Enhancement of filament formation by *tau* mutations does seem to be an important effect, and this corresponds to the observed effect of pathogenic mutations in a range of other conditions. Both mechanisms may be important and contribute to neural toxicity, and the balance between these two mechanisms is difficult to determine. However, perhaps significantly, P301S has a significantly more marked effect on acceleration of filament formation than P301L, whereas for microtubule destabilization the situation is reversed. Given the earlier disease onset in families with P301S this suggests that filament formation may be a more important mechanism in the disease pathogenesis.

Data from the study of FTDP-17 can now be applied to the biochemical classification of tauopathies.

**Table 5-6 Biochemical and genetic classification of tauopathies**

| Tau isoforms | Three and four repeat tau | | Four repeat tau | | Three repeat tau | |
|---|---|---|---|---|---|---|
| Western blot (kDa | Triplet (59,64,68) | | Doublet (64,68) | | Doublet (59,64) | |
| Inheritance | Familial | Sporadic | Familial | Sporadic | Familial | Sporadic |
| Filaments | Paired helical | Paired helical | | Straight | | |
| | AD | | Exon 10 FTDP-17 | PSP | Myotonic dystrophy | Pick's disease (PiD) |
| | Non-exon 10 FTDP-17 | PDC | PSP | CBD | G389R<br>ΔK280<br>K257T | |
| | PDC (?) | PEP | | | | |

**For each of the sporadic tauopathies a familial genetic form exists which provides a model for understanding the disease pathogenesis and for developing new treatments. However, as the data in this chapter and**

Table 5-6 illustrate the sporadic conditions are distinct from the familial versions of these diseases and further work will need to be done to elucidate their more complex aetiology. If PSP is regarded as a sporadic version of exon 10 mutation FTDP-17 then this is likely to be one of very few conditions in which a common variant (H1) and rare mutations (Exon 10) of the same gene can lead to related conditions. In other words, there are very low penetrance and high penetrance changes present in the same gene. The explanation for the H1 *tau* predisposition effect is not known and further work is needed on genetic and environmental determinants of tau related neurodegeneration. One condition which may provide a greater insight into the aetiology and pathogenesis of tau related neurodegeneration is PDC and studies of the neurodegenerative diseases of Guam are presented in the next two chapters.

# CHAPTER 6 Clinical analysis of Parkinsonism dementia complex (PDC, bodig) and amyotrophic lateral sclerosis (ALS, lytlco) of Guam

## 6.1 Summary

This section summarises the clinical and pathological features of series of cases of PDC and ALS, and an analysis of five Guamanian Chamorro families. PDC is an atypical parkinsonian syndrome which shares common features with other tauopathies. In addition to its extrapyramidal features it involves prominent amnesia, sometimes with cognitive slowing. ALS on Guam has similar clinical and pathological features to motor neuron disease in other parts of the world. There is family clustering of ALS and PDC. Usually they do not occur together in the same individual and appear to be separate disorders. Five families with PDC were collected and analysed and a simulation linkage analysis was performed, which suggests that conventional linkage analysis would not be powerful enough to detect a PDC locus.

## 6.2 Introduction

The neurodegenerative disorders of Guam present an important opportunity for an aetiological study of tauopathies. Both ALS and PDC occur at a high incidence on the Western Pacific island of Guam. The aetiological factors which account for this disease cluster remain unexplained, and part of this thesis involves a collaborative attempt to determine a genetic basis for PDC. A study has been carried out of the clinical, pathological and genetic features of PDC and ALS. This is based on clinical analysis performed in collaboration with Dr. John Steele, who has practised neurology on Guam for over 17 years.

## 6.3 PDC

PDC was first described as a parkinsonian syndrome by Mulder in the 1950s during surveys of ALS on Guam.[279] Guam is the southernmost island of the Mariana chain in the Pacific ocean. Although an earlier report described a Chamorro patient diagnosed as having paralysis agitans who subsequently developed the features of PDC, [280, 281] Mulder was the first to note that parkinsonism was unusually common among Guamanian Chamorros and commented that it resembled post-encephalitic parkinsonism.[279] Mulder described disturbed sleeping habits, hyper-reflexia, extensor plantar responses and memory impairment, together with parkinsonism. In 1961 Hirano and colleagues carefully studied this syndrome and published details of the clinical and pathological features which they named PDC.[123, 282] PDC was distinguished by marked neuronal loss and NFT formation in the mesial temporal cortex, substantia nigra and globus pallidus. They reported striking akinesia and a "reptilian stare", a stooped posture and shuffling gait resembling PD. However, tremor was not a major characteristic and tone was found to be normal or only minimally increased in many patients.[282] Hyper-reflexia was usual and occurred in nearly all cases. Because NFTs were also present in Chamorros with ALS, the two syndromes of ALS and PDC were thought to be different manifestations of a single disease.[123] Elizan reviewed the clinical features of PDC in 1966,[283] and again emphasized the clinical and pathological differences from PD. She described minimal tremor and rigidity in early stages, concurrent organic mental changes and features of motor neuron disease. In the case series of PDC described by Elizan, 38% developed classical ALS. The presence of sub-clinical denervation changes in twelve patients with pure parkinsonism without ALS re-affirmed the idea that ALS and PDC on Guam were different manifestations of a single disease. Since these early studies, supranuclear gaze palsy and an unusual pigmentary retinopathy have been described as additional features in PDC.[121, 284] The majority of subsequent studies have concentrated on the aetiology of ALS and PDC, which occur at a high incidence on Guam. Viral and infectious agents,[20, 283] calcium deficiency,[285] and a cycad neurotoxin have not been found to be responsible for these diseases, and the cause remains obscure.[286] This

section describes a series of clinico-pathological cases which emphasise the range of features seen in patients with PDC.

**Results**

*Case 1*

A retired Chamorro soldier had lived away from Guam for 30 years. At the age of 53 he developed general slowness of movement and forgetfulness and was noted by his friends to have developed a sad expression. Within the first year of his symptoms he developed a small stepped unsteady gait with falls backwards, bradykinesia of the limbs and rigidity without tremor. He also had a brisk jaw jerk and facial reflexes, and progressively worsening memory impairment. Over the second year of his illness he developed progressive and marked difficulty in swallowing with frequent choking and subsequent weight loss. He developed wasting of the small muscles of the hands, and evidence of pyramidal dysfunction with hyper-reflexia and bilateral extensor plantar responses. He also developed a vertical supranuclear gaze palsy, and his neurological disease was similar to PSP.

*Pathology*

There was marked neurofibrillary degeneration with cell loss in the anterior olfactory area, amygdala, hippocampus and parahippocampal gyrus. The subthalamic nucleus, striatum and globus pallidus showed cell loss but NFTs were infrequent. There was dramatic cell loss in the substantia nigra and locus coeruleus with the very few remaining neurons having NFTs. Scattered tangles were seen in the basis pontis and tegmentum of the medulla. There was marked gliosis and nerve cell loss in the peri-aqueductal grey matter. Examination of the spinal cord showed mild posterior column degeneration and no evidence of anterior horn cell loss. There were no senile plaques or Lewy bodies.

*Case 2*

At 52 years of age this Chamorro man developed a stutter and excessive salivation. He was seen one year after symptom onset when the most striking

abnormality was difficulty with turning with one foot sticking to the floor. His extra-ocular movements were normal. Over the next two years his walking gradually worsened and he would fall backwards as he tried to turn. He then developed a supranuclear gaze palsy, initially involving upgaze but progressing so that five years after symptom onset he had developed a complete supranuclear gaze palsy. He also developed mild progressive memory impairment and severe bilateral blepharospasm which responded to treatment with botulinum toxin. In the last years of his illness he had marked rigidity of the neck which was held in a forward flexed position, and his limb bradykinesia became increasingly severe. There was little rigidity and no rest tremor, although there was a low amplitude rapid postural tremor. Electromyography showed no evidence of denervation and there was no Guam retinal pigment epitheliopathy (GRPE).

*Pathology*

The brain showed an old infarction of the left cerebral hemisphere and right cerebellar hemisphere. In the brain stem there was considerable depletion of pigment in both substantia nigra and locus coeruleus. Histology showed widespread changes characteristic of PDC. There were tau-immunoreactive tangles and associated neuropil threads involving all neocortical and subcortical regions. Tangles were especially numerous in the hippocampus and parahippocampus where ghost tangles were abundant, while remaining neurons contained tangles. In the dentate fascia there were occasional tangles in the granule cell layer. The amygdala, uncus and hypothalamic nuclei were similarly severely affected with tangle formation. The globus pallidus and subthalamic nucleus showed nerve cell depletion, astrogliosis and several tangle bearing neurons. Occasional tangles were identified in the striatum. Tangles were numerous in the nucleus basalis of Meynert. Tangles were identified in the majority of brain stem nuclei and few pigmented neurons remained in either the substantia nigra or locus coeruleus. There were occasional tangles in the cerebellar dentate neurons. Additional changes of longstanding infarction with areas of partial cystic collapse involved the areas of brain were seen in the left cerebral hemisphere and right cerebellar hemisphere.

*Case 3*

This Chamorro woman's symptoms began at the age of 53 years when she developed parkinsonism and dementia. Her disease progressed very rapidly such that within three years of the onset of her symptoms she had developed an akinetic mute state with flexion contractures of all limbs. When she was examined 10 years after the onset of her symptoms she was verbally unresponsive and the most striking feature was of dystonic posturing. There was marked rigidity of the neck which was held rotated to the left, the upper limbs were fixed in flexion with tight flexion of the fingers and adduction of the thumbs and the right leg was drawn up in flexion at the hip and the knee. The left leg was held in flexion at the knee and with marked plantar flexion at the ankle such that the ankle joint was dislocated. Some fingers of the hand were held in a boutonnière deformity with extension of the distal inter-phalangeal joint and flexion of the proximal inter-phalangeal joint. Examination of extraocular movements showed that there were frequent square wave jerks in the primary position and nystagmus on lateral gaze. The extra-ocular movements were full.

*Pathology*

Macroscopically there was general thinning of the cortical ribbon, with depigmentation of the locus coeruleus and the substantia nigra. There were widespread NFTs in the frontal cortex, temporal neocortex and basal ganglia. There was severe neuronal loss with gliosis and NFT formation in the entorhinal cortex. The midbrain showed almost complete loss of neurons in the substantia nigra, together with extensive neuronal loss and gliosis of the peri-aqueductal grey matter. No Lewy bodies or senile plaques were seen.

*Case 4*

At 44 years of age this Chamorro man developed hoarseness, a heavy sensation in the legs and general slowness. At his initial examination he had a fast, low monotonous voice, impairment of repetitive fine movements, mild rigidity of the neck and of the right leg. As his illness progressed he developed a profound supranuclear gaze palsy in both the horizontal and vertical planes, and

increasingly marked impairment of postural reflexes with retropulsion, falls backwards and slowness and difficulty in arising from a chair. In the last phases of his illness his memory and comprehension appeared to be normal but there was a marked delay in answering responses and following commands. He had a family history of PDC in his paternal grandfather, whose illness was described in the first report of PDC in the Mariana islands.

*Pathology*

The brain weight was normal, however there was cortical atrophy involving the posterior frontal and adjacent superior parietal region, more marked on the left side. There was depletion of pigment in substantia nigra and locus coeruleus. Histological examination showed widespread tau-immunoreactive NFTs in the absence of senile plaques. In cerebral cortex NFTs were most numerous in frontal and temporal regions including hippocampus and parahippocampus; the pre-α layer was especially severely affected and there were associated neuropil threads. NFTs were identified in all deep grey nuclei however, nerve cell depletion and gliosis was restricted to the globus pallidus and subthalamus. In the brain stem tangles were widespread and involved the tectal and tegmental nuclei of midbrain. The substantia nigra showed severe neuronal depletion. In the pons pigmented neurons were relatively well-preserved but there was severe peri-aqueductal involvement and the tegmental and pontine neurons contained tangles. NFT involvement of hippocampus and parahippocampus was less severe than is usually described for PDC and the histopathological appearances resembled those described for PSP.

*Case 5*

At 50 years of age this Chamorro man developed slowing of movement and difficulty in arising from a lying or sitting position. He had postural instability and tended to reel backwards. As his illness progressed over the subsequent four years, he developed slurred speech, blepharospasm, a mild intermittent resting tremor and difficulty in remembering day to day events. Evaluation at the age of 54 demonstrated GRPE, an impaired sense of smell (UPSIT 25/40) and a

supranuclear gaze palsy. As his illness progressed he developed a profound general bradykinesia, daytime somnolence and marked facial immobility with a disappearance of his blepharospasm. He developed axial rigidity with the neck held in an extended position and dystonic posturing of the hands and feet with flexion at the wrists, adduction of the thumbs and extension of the fingers and extension posturing of the toes. There was marked cogwheel rigidity and tremor, that had previously been apparent, disappeared. He died at the age of 58, 8 years after symptom onset. A post-mortem examination was not done.

*Case 6*

At 56 years of age this Chamorro farmer became aware of general slowing of his movements. Examination in the first symptomatic year showed a mildly stooped posture, bradykinesia, impassive facies and rigidity without tremor. Early parkinsonism was diagnosed. Over the subsequent nine years he developed striking generalized bradykinesia with a lack of spontaneous and associative movements, postural instability with falls forward and bradykinesia for repetitive fine finger movements. His facial expression was characterized by a taut immobility around the mouth and cheeks. He died four years later and in this period first developed memory impairment, a low amplitude resting and postural tremor, particularly of the left hand and foot and thinning of the small muscles of the hands. A tendency for delay in verbal and motor responses became very pronounced during the final 18 months of his illness and was accompanied by the emergence of axial rigidity and a supranuclear gaze palsy. He had a family history of ALS in one brother and one uncle and indirect ophthalmoscopy revealed the linear depigmentation of the retinal pigment layer characteristic of GRPE.

*Pathology*

Macroscopic examination showed atrophy of Ammon's horn, and atrophy and depigmentation of the locus coeruleus and substantia nigra. On light microscopy NFT formation was most prominent in the hippocampus and parahippocampal gyrus and the amygdala. NFT formation also occurred in frontal and temporal neocortex and brainstem nuclei including the pedunculo-pontine nuclei and

midline reticular formation. A small number of tangles were found in the caudate and putamen. Widespread senile plaques were seen in cortical areas. There was severe neuronal loss in the substantia nigra and locus coeruleus and the remaining neurons contained both Lewy bodies and NFTs. There was no neuronal loss from the dorsal motor nucleus of the vagus. The diagnosis was of PDC combined with Alzheimer type changes and Lewy bodies.

**Discussion**

These case reports illustrate the clinical features that can be seen in the neurofibrillary degeneration of PDC. Initially Hirano and colleagues likened the disease to PD, [282] but it is clear from these cases that there are a number of important differences. The facial masking of PDC is often highlighted early in the course of the illness and this may be different to PD in having a muscle tightness and asymmetry, which is sometimes described as a facial dystonia, and is similar to that seen in PSP. An associated pseudobulbar palsy may also explain this appearance. Similarly the stooped posture of PDC may be distinguished from that seen in PD by a forward looking stance with a flexed rigid neck such that the typical posture of PDC may resemble a "boxer's pose" - this is illustrated in Hirano's 1961 paper.[282] The gait disorder of PDC is often prominent and early. It does not usually include festination, but involves sticking of the feet while turning, postural instability with falls backwards and uncontrolled descent when sitting in a chair. Tremor can occur in PDC but is not a prominent feature. When present it may involve the face and lips or be more marked on action than at rest. It may particularly be enhanced by walking or performing the finger to nose test. Both Hirano and Elizan commented on the relative infrequency of tremor in PDC.[282, 283]

PDC has many more clinical similarities to other tauopathies such as PEP and PSP. As Mulder and Sacks have described, some patients have a more striking resemblance to PEP with a far more marked motor retardation than is seen in PD, with pyramidal signs and unusual dystonic postures particularly in terminal disease.[287, 288] However, unlike PEP marked L-DOPA responsiveness with concomitant adverse effects, sleep-wake reversal, oculogyric crises and

hyperkinetic movement disorders are rarely seen. The similarities to PSP are even more apparent and include a frequent supranuclear gaze palsy, blepharospasm and axial rigidity.[119, 121] The supranuclear gaze palsy may be identical to that seen in PSP. Gait disturbance, regarded as one of the hallmark features of PSP, is also very similar in PDC, involving a lurching unsteady gait, with prominent and early postural instability.

The most characteristic feature of PDC is the combination of prominent motor symptoms with dementia. PDC often involves marked cognitive slowing, particularly in the later stages of the disease. However, cognitive impairment often involves day to day memory impairment, presumably related to mesial temporal damage, and similar to that seen in AD. This distinguishes it from FTDP-17 which also produces dementia with motor symptoms, but usually involves prominent personality change with disinhibition and/or apathy related to frontal damage, with or without dysphasia. Unlike FTDP-17, PDC is not due to mutations in *tau*.[201] Overall the motor and cognitive features of PDC fit into the spectrum of features seen in other tauopathies. In some cases of PDC, the degree of mesial temporal involvement and clinically apparent amnesia may be less marked and in these cases the disease becomes clinically and pathologically highly similar to PSP. Recently an increasing overlap has been described between neurodegenerative disorders. Proteins which appear to be primarily abnormal in some conditions (such as α-synuclein in familial or sporadic PD) may be deposited as a secondary phenomenon in other disorders. Tau neurofibrillary tangles may be deposited in primary β-amyloid, BRI or α-synuclein deposition disorders and similarly α-synuclein in the form of Lewy bodies may be deposited in diseases such as AD or Down's syndrome. The description of Lewy body formation in a case of PDC in this study confirms that this neurodegenerative disease protein overlap also applies to PDC on Guam.

Some cases in this series have clinical features that might be taken to indicate incipient ALS such as brisk reflexes, extensor plantar responses and thinning of the small muscles of the hand. However, pathological examination of the spinal cord does not reveal anterior horn cell loss in these cases. These signs may well be part of the syndrome of PDC, and usually do not progress to the full blown

syndrome of clinical ALS with progressive and severe disability due to cortico-bulbar, cortico-spinal and amyotrophic features. From this analysis it appears that PDC and ALS are usually separate clinical syndromes. They may have existed together more frequently when ALS was very common on Guam in the 1950s and 1960s but as the prevalence of ALS has declined so it is now an uncommon accompaniment of PDC.

The pathological features of PDC have been described elsewhere in detail but this study confirms the pattern of damage involving the mesial temporal cortex, basal ganglia and brainstem. It is of interest that patients with different clinical syndromes such as pure dementia, PSP-like syndromes and PD-like syndromes cannot be distinguished pathologically.[289] This suggests that functional neuronal changes prior to tangle formation and cell death may be important in determining the neurological phenotype. A diagnosis of predominant PDC may be made with a characteristic pattern of NFT formation and cell loss in the mesial temporal cortex, basal ganglia and brainstem in conjunction with a typical clinical picture. These cases illustrate the range of clinical features in PDC, characteristic and overlapping with other tauopathies, but do not suggest that PDC and ALS are the same condition.

## 6.4 ALS

The description of a high prevalence focus of ALS on the Western Pacific island of Guam by Kurland and Mulder, [279, 290] was followed by over forty years of research in an attempt to define this disease. However, despite this work, the three central questions raised by the high prevalence of ALS, or more broadly motor neuron disease (MND), on Guam remain unanswered: its aetiology, its relationship to PDC and its relationship to classical ALS. The earliest reported diagnosis of progressive muscular atrophy on Guam was made in 1904 and a high incidence of ALS was reported by Zimmerman and colleagues.[291] The initial surveys of Kurland and Mulder found that the clinical features of ALS on Guam were identical to classical ALS, aside from the strong family history of ALS in the Guamanian patients.[279, 290] Zimmerman's pathological findings and

Mulder's clinical opinion that ALS on Guam was the same disease as that seen in Europe and America seemed less likely when Hirano and colleagues published their clinical and pathological description of PDC.[123, 282] They described the characteristic pattern of neurofibrillary degeneration and designated this syndrome PDC. They described an identical pattern of damage in Guamanian patients with ALS and concluded that the two clinical syndromes were different manifestations of a single disease, and represented two ends of a unitary clinical spectrum.[123, 282] In 1994 Oyanagi and colleagues re-evaluated the pathology of ALS on Guam using ubiquitin and tau immuno-histochemistry.[292] They described the pathology of classical ALS, including ubiquitin deposits, in the anterior horn cells of patients with Guamanian ALS. This pathology seemed to occur independently of the deposition of tau containing NFTs. In contrast, Oyanagi concluded that PDC and ALS were separate diseases and that ALS on Guam is classical ALS, and identical to the disease seen in America and Europe. This recent suggestion that ALS and PDC are separate diseases is further supported by epidemiological trends which show that the incidence of ALS and PDC are changing independently, with the incidence of ALS declining very rapidly in recent years.[293] The clinical profile of ALS/MND on Guam has not been considered since a review of the thirty year experience of both PDC and ALS, from 1950 to 1979, published in 1986.[294]

This section presents a retrospective review of 45 cases of motor neuron disease seen on Guam between 1983 and 1998, and considers their clinical and pathological similarity to classical MND elsewhere in the world. The clinical case records of every case of motor neuron disease encountered by Dr. John Steele during fifteen years of neurological practice (1983-1998) on Guam were reviewed and summarised, together with autopsy reports where available. These patients were recruited via referral from primary and hospital physicians and house to house surveys in the southern villages of Guam. Follow up was made by clinical review, telephone contact and interviews with relatives for patients who had left Guam; only one patient was completely lost to follow-up. Following informed consent, pathological confirmation of diagnosis was established for 16 cases and brief details of these pathological examinations are

presented, taken from autopsy records(Table 6-2). In eleven cases archival material was available for review. These cases were examined by Dr. Safa al-Sarraj and colleagues at the Department of Neuropathology, Institute of Psychiatry, London. They were processed, sectioned and stained using routine histological stains and examined immuno-histochemically using antibodies to tau (AT8, monoclonal mouse 1:200, Innogenetics) and ubiquitin (ubiquitin, polyclonal rabbit, 1:500, Dako). Where available hippocampus and spinal cord were studied along with motor cortex, medulla and cerebellum. In cases or individual case areas in which archival material was not available for review a pathological summary was produced based on the autopsy report using routine histological stains, usually including silver staining but not immunohistochemistry.

## Results

### *Clinical features*

Forty five patients (41 Chamorros and 4 non-Chamorros) were diagnosed as having motor neuron diseases (Table 6-1). Of these 37 met the El Escorial criteria [295] for clinically definite or probable ALS, 3 had a progressive muscular atrophy type syndrome and 3 had a diagnosis of possible ALS with a non-compressive monomelic mixed upper and lower motor neuron syndrome, or mixed upper and lower motor neuron signs without rostral cortico-bulbar/cortico-spinal involvement. These cases of MND are described together. For calculation of mean and median survival a 10 year cohort of 21 patients with disease onset between 1980-1989 was used; one patient has been lost to follow up. Only Chamorro patients had the following features: a mixed syndrome with extra-pyramidal signs and dementia; a positive family history of both ALS and PDC; and GRPE (although only one non-Chamorro was assessed for that condition). Patients with ALS are more likely to have a family history of ALS in at least one first degree relative than PDC (37.2 vs. 26.1%). In six patients with concurrent extrapyramidal signs, three presented with limb or bulbar weakness, one presented with memory impairment and two presented with general slowness. Three of these patients had tremor, cogwheel rigidity and bradykinesia

and in one of these patients the extrapyramidal signs were asymmetrical. A history of significant residence away from Guam was found in five patients who left Guam on average at 20 years of age (range 18-24), returned at age 41(range 37-42) and developed the disease at aged 52 (range 37-66). One case developed symptoms while resident overseas. The four non-Chamorro cases were two Caucasians and two Filipinos who came to Guam at ages 32, 39, 43 and 29 years (in 1955, 1971, 1969, 1970) and developed symptoms at age 63, 60, 52, and 57 years respectively (mean duration from immigration to disease onset is 20.25 years with a range of 21-28 years).

**Table 6-1 Clinical features of ALS on Guam**

| | n | Mean onset age (years) | Mean survival (years) | Median survival (years) | GRPE | Hyposmia | Extrapyramidal | Dementia | FH ALS | FH ALS+PDC | FH PDC |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Total | 45 | 49.4 | 9.4 | 5.0 | 47.0% | 53.0% | 13.3% | 8.8% | 22.2% | 15.0% | 6.7% |
| ALS | 39 | 49.1 | 9.6 | 4.5 | | | | | | | |
| PMA | 3 | 53.3 | 3.3 | 2.0 | | | | | | | |
| Suspected ALS | 3 | 49.3 | 13.7 | 6.0 | | | | | | | |
| Subgroups | | | | | | | | | | | |
| Chamorro | 41 | 48.6 | 9.8 | 5.5 | 52.6% | | 14.6% | 9.7% | 24.0% | 17.0% | 7.3% |
| Non-Chamorro | 4 | 58.0 | 6.0 | 1.5 | 0.0% | | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% |
| Bulbar onset | 7 | 55.6 | 3.3 | 2.0 | | | | | | | |
| Limb onset | 38 | 38.3 | 10.6 | 6.0 | | | | | | | |
| 1980-1989 cohort | 21 | 53.6 | 4.5 | 3.0 | | | | | | | |

**Abbreviations: n number, GRPE Guam retinal pigment epitheliopathy, FH family history, PMA progressive muscular atrophy**

**Pathology**

The classical features of ALS were present in the 16 cases examined (Table 6-2). These features included anterior horn cell loss and cortico-spinal tract degeneration. The hippocampi of nine cases were available for immunohistochemical examination. None had ubiquitin positive inclusions of the type that may be seen in MND or MND inclusion dementia. Six (67%) had moderately or markedly severe tau positive neurofibrillary tangle formation, more marked than that seen in Caucasian individuals of a similar age. The average age of death of individuals with immuno-histochemically defined moderately or markedly severe hippocampal neurofibrillary tangle formation was 54 years. However this neurofibrillary tangle formation was not associated with significant nerve cell loss, and this included case 9 which had clinically diagnosed mixed ALS/PDC. Two of the cases with markedly severe hippocampal NFT formation had concurrent senile plaque formation.

Spinal cord was available for examination in ten cases. Six of these cases had ubiquitin-positive tau-negative inclusions, with a filamentous skein like morphology typical of ALS/MND. Only one case out of ten was identified which had tau-positive ubiquitin-negative inclusions in anterior horn cells.

**Table 6-2 Pathological features of ALS on Guam**

| Case | Diagnosis | Ethnicity | Age at death | Survival (years) | Hippocampus | | | Frontal cortex | Substantia nigra | Senile plaques | Cortico-spinal degeneration | Anterior horn cells | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Tau/NFT | Ub | Cell loss | Tau/NFT | Cell loss | | | Tau | Ub | Cell loss |
| 1 | ALS | Cham | 48 | 11 | ++ | - | - | - | na | - | + | - | - | + |
| 2 | ALS | Cham | 69 | 2 | +* | na | - | + | na | - | + | - | + | + |
| 3 | ALS | Cham | 63 | 2 | +* | na | - | - | - | - | + | - | + | + |
| 4* | ALS | Cauc | 65 | 2 | +/- | na | - | +/- | - | - | + | - | na | + |
| 5 | ALS | Cham | 63 | 1 | +/- | - | - | - | - | + | + | - | + | + |
| 6 | ALS | Cham | 68 | 22 | ++ | - | - | +/- | +/- | + | + | - | + | + |
| 7* | ALS | Cham | 40 | 2 | +/- | na | +/- | +/- | +/- | - | + | - | na | + |
| 8 | ALS | Cham | 41 | 6 | +++ | - | - | + | +/- | - | + | - | + | + |
| 9 | ALS/PDC | Cham | 55 | 3 | +++ | - | - | + | + | - | + | na | na | na |
| 10 | ALS | Cham | 45 | 2 | +++ | - | - | - | - | - | - | - | - | + |
| 11 | ALS | Cham | 60 | 2 | +++ | - | - | +/- | +/- | + | + | - | - | + |
| 12 | ALS | Cham | 57 | 2 | +++ | - | - | + | +/- | + | + | + | - | + |
| 13* | ALS | Cham | 47 | 7 | +/- | na | +++ | + | +/- | + | na | na | na | na |
| 14* | ALS | Cham | 68 | 2 | +++ | na | na | + | + | + | + | - | na | + |
| 15* | PMA | Cauc | 61 | 1 | +/- | na | - | - | - | - | - | - | na | + |
| 16 | PMA | Cham | 64 | 2 | - | - | - | - | - | - | - | - | + | + |

**Abbreviations: - absent, +/- mild, + present, ++ moderately severe, +++ markedly severe ; na-not available; Cham - Chamorro; Cauc – Caucasian. * Archival material not available for immunohistochemical examination, NFT only evaluated by silver or haemotoxylin and eosin staining**

**Discussion**

This series describes patients with motor neuron diseases who developed symptoms on Guam from 1951 onwards, and who were followed up to 1998. Whether there are one or two neurodegenerative diseases on Guam has important implications for interpreting the pattern of the familial occurrence of these diseases and understanding the epidemiology. The observation of neurofibrillary tangles in ALS has led to the view that there is one disease on Guam with varied clinical manifestations and that it represents a unique form of ALS, known as "Western Pacific ALS".[123] These clinical and pathological observations, together with those of Oyanagi and colleagues suggest that this may not be the case, although this depends on the background level of neurofibrillary tangle formation among asymptomatic Chamorro subjects.

The clinical features of the majority of patients in this series meet the El Escorial criteria for the diagnosis of ALS,[295] with progressive bulbar and limb upper and lower motor neuron involvement without sensory signs or evidence of alternative pathologies. The only exception is the presence of extrapyramidal features, which would be an exclusion criterion under El Escorial, in 13% of cases. In addition, cases have been identified in this study with a progressive muscular atrophy phenotype. The average survival of the whole group is longer than is normally seen in ALS, but this is biased by the inclusion of long lived patients whose disease onset occurred before 1981 and the exclusion of deceased patients. Analysis of a ten year cohort, with disease onset between 1980 and 1989, shows a mean and median survival of survival of 4.5 and 3 years, which is comparable to that seen in classical ALS.[296] Similarly, the average age of onset of 49.4 years is similar to classical ALS, as is the reduced survival in patients with bulbar symptoms at onset. Although there is survival time of 41 years documented in one Guamanian individual with typical ALS, long survivors with ALS have been described in non-Guamanian series, with 20% surviving for over 5 years in one study.[297] Overall, these findings do not support a more benign course for ALS on Guam as compared with the rest of the world.

Four main features distinguish ALS on Guam: a high prevalence; a positive family history, an association with GRPE and the concurrence of parkinsonism and dementia. A frequent positive family history was noted in the early investigation of this disease, and it was assumed that the disease was most likely to be inherited.[279] Our series has confirmed that this familial predisposition continues, with a first degree family history of ALS in 37.2% of patients in this series. Although autosomal dominant ALS is well recognised, it accounts for only around 5% of all cases found outside of Guam.[298] The most common gene mutation identified in familial ALS is Cu/Zn superoxide dismutase (SOD-1), but analysis of this gene in 8 patients with ALS on Guam has not revealed a mutation in affected subjects.[299 300] A genetic effect on the aetiology of these diseases remains uncertain although a recent analysis has suggested that ALS and PDC may be due to the additive effect of two alleles.[301] It is of interest that these data show that in MND cases there is an excess occurrence of a family history of ALS as opposed to PDC in a ratio of 1.7 to 1. This is seen despite the fact that during 1986-1987 the prevalence of PDC was estimated to be double that of ALS.[302] This can be compared with the segregation ratios in a previously reported series of autopsy cases of PDC,[303] in which 8 (11%) of 73 true siblings of the PDC cases were diagnosed with ALS, while 33 (45%) were already diagnosed with PDC, which is close to the 50 % expected for autosomal dominant inheritance. Thus, PDC and ALS may cluster separately within either ALS or PDC families, as well as in families in which both diseases occur. These data are consistent with ALS and PDC being separate diseases. The common finding of a positive family history in patients with ALS, confirmed in a case control study, [304] suggests that a familial habit, vertically transmitted infection or genetic factor may play an important role in the development of the disease. Conversely, the residential history in four cases in this series suggests that ALS also occurs in Guamanians who have not had a lifelong exposure to the Guamanian environment.

GRPE was first described by Cox who noted this condition in the course of investigating the supranuclear gaze palsy of PDC.[284] This retinopathy occurs at an increased incidence among patients with ALS and/or PDC (~53%), and at a lower incidence in asymptomatic Guamanian Chamorros and is infrequent in

Chamorros residing on other islands of the Mariana group. It thus mirrors the geographical distribution of both PDC and ALS.[284] The increased incidence of GRPE in patients with ALS is confirmed in this study. The presence of GRPE is clearly not a prerequisite for the disease, but it may be a marker, perhaps of infection with a parasitic agent linked to the disease. Of note, GRPE is also seen in a large percentage of those Chamorros with parkinsonism and/or dementia.[284] Parkinsonism and dementia are present in 13% of our series of patients with ALS and it could be argued that this indicates that these syndromes are part of a single disease process. However, this family data together with that of other groups,[304] indicate that these diseases can cluster together in different individuals in the same families. It is possible that PDC and ALS are separate diseases with a common genetic or environmental predisposition, which occur together within the same families and occasionally occur together in the same individual. Dementia and parkinsonism are not invariable or even common accompaniments of ALS and these features do not necessarily appear in long term survivors with ALS. The notion that the presenting clinical syndrome is determined by different rates of degeneration in different parts of the nervous system with all parts finally becoming affected, seems unlikely. However, it remains possible that these two clinico-pathological phenotypes may reflect a regional variability in disease expression, with anterior horn cells producing ubiquitinated inclusions and hippocampal neurons producing tau neurofibrillary tangles in response to a single disease-provoking agent.

These pathological data confirm the long standing finding of tau neurofibrillary degeneration in Guamanian ALS cases, and this occurs to a level which is not seen in asymptomatic Caucasian individuals of a similar age. However neurofibrillary degeneration was not associated with significant nerve cell loss, clinical dementia or extrapyramidal syndromes. This is in contrast to the features of typical PDC, in which neurofibrillary degeneration is associated with substantial depletion of neurons in the temporal lobe, the CA1 region of the hippocampus, substantia nigra and brainstem nuclei, and significant dementia and movement disorders.[123] It has been suggested that asymptomatic control Chamorro individuals have a higher level of neurofibrillary tangle formation

without cell loss, and this phenomenon is positively associated with a family history of PDC.[6, 305] It may be that a small amount of neurofibrillary tangle formation without senile plaque formation is an accompaniment of ageing,[5] and it has been suggested that this process may be accelerated on Guam.[6] The presence of hippocampal neurofibrillary tangles may therefore be a background accompaniment to other neuropathological processes.[292] As this series does not include control cases it is difficult to be certain whether the neurofibrillary tangle formation seen in these ALS cases is similar to asymptomatic Chamorro control levels. This can only be properly evaluated with a prospective case control series.

One case out of ten in this series has been identified to have tau containing inclusions in anterior horn cells, as compared with six cases out of ten with ubiquitin inclusions. This is of particular interest since some families with frontotemporal dementia linked to chromosome 17 such DDPAC, resulting from a primary genetic abnormality of tau, may have a clinical anterior horn cell syndrome.[234] Furthermore, recently described transgenic mice which contain the tau P301L mutation have prominent clinical and pathological involvement of anterior horn cells with tau containing anterior horn cell inclusions.[306] This suggests that primary tau disorders may produce both anterior horn cell and extrapyramidal/dementia syndromes. We have identified anterior horn cell tau inclusions in only one Guamanian case with MND, as compared with six cases with ubiquitin immunoreactive anterior horn cell inclusions. These data are compatible with normal ALS type pathology in some, but not all cases. The ability to define the typical ubiquitinated anterior horn cell inclusions may depend on the number of spinal cord levels studied. Overall, the ubiquitinated anterior horn cell inclusions, typically seen in classical MND, [307] support a classical MND process on Guam rather than a tau NFT related anterior horn cell disease. Previous studies of tau deposition in the spinal cord of patients with Guamanian MND have shown neurofibrillary tangle formation to be most marked in the posterior horn,[308] with the anterior horn cells relatively spared. Conversely, dementia is well recognised in patients with MND, and despite the El Escorial criteria there are some reports of extrapyramidal features in patients with clinically and pathologically diagnosed MND.[309, 310] Some patients with FTD

have MND type inclusions in the hippocampus ("MND-inclusion dementia"), [253, 254] and the absence of ubiquitinated hippocampal inclusion in the Guamanian MND patients in this study suggests that the Guam disease does not overlap with MND inclusion FTD. As previously reported, MND predominantly affects the Chamorro as opposed to the Filipino or Caucasian populations on Guam (approximately 70,000 out of total island population of 130,000). Only four MND cases were encountered in the non-Chamorro population over this fifteen year series, and this does not support a widespread recent environmental exposure.

The clinical and pathological experience documented in this study leads to the conclusion that ALS and PDC are separate diseases that are predisposed to by a single, or related factors on Guam. Despite the presence of neurofibrillary tangles suggestive of early PDC in some patients with ALS, we believe that the frequent lack of overlap in clinical syndromes and pathology, even in long survivors with ALS indicates that these may be separate diseases.

### 6.5 Family analysis

Familial clustering of ALS was noted in the first major investigation in Guam,[290] but a primarily genetic cause for PDC has not been considered likely. However a number of lines of evidence support a genetic aetiology: i.) the disease clusters within some families in the South of the island;[303] ii.) adjacent villages which historically have not intermarried with similar lifestyles and environment have a dramatically different PDC prevalence (Umatac and Merizo, rates for PDC 274 vs. 42 /100,000 Chamorro 1956-1965) [311]; iii.) the disease has occurred in Guamanian migrants to California [312]; iv.) the disease has occurred on Guam over many years, occurring in patients born from ~1850 to 1940; and v.) case-control studies have shown that having an affected first degree relative is a risk factor for developing the disease.[304] Clinico-pathologically substantiated disease has remained confined to the minority Guamanian Chamorro population, but there have been occasional reports of a PDC like disease in Filipino and Caucasian residents of Guam.[313, 314] Pathological similarities between PDC, post-

encephalitic parkinsonism and progressive supranuclear palsy makes the importance of these few non-Chamorro cases uncertain. It seems clear that there has not been an epidemic of PDC among long-term Filipino and Caucasian residents of Guam. Some investigators have reported that the prevalence of PDC is in decline,[293] but from a genetic perspective this might be explained by increased social mobility and marrying out, allowing a decline in the co-occurrence of recessive genetic factors or a decreased concurrence between a genetic factor and an environmental co-factor.

A study of the genealogy of the families of Southern Guam was made in an attempt to recruit individuals and kindreds for genetic linkage analysis and in order to define the inheritance characteristics of the disease. Cases of ALS and PDC and unaffected individuals were identified from death certificates, interviews with family members and interviews and examination of patients referred to Dr. Steele, and the kindreds were constructed from this data.

**Results**

Five families were identified and DNA was collected from 19 individuals from these families. The family trees are presented illustrating affected individuals available for linkage analysis (Figure 6-1, Figure 6-2). The largest family was analysed in further detail. This is a four generation Chamorro family from the southern Guam village of Umatac, with 153 direct descendants of a founder born in around 1840 and 37 known affected individuals. Generation II contains eight members born between 1865 and 1884. One member may have had PDC and two may have had ALS with death certificate/family diagnoses of senility, muscle wasting and paralysis respectively. Subsequent generations have been followed, as completely as possible, from individual II-1 and individual II-8 and additional affected members have been traced from individuals II-3, II-5 and II-6. Generation III contains 30 individuals, born between 1879 and 1926. Five of these individuals had ALS and 5 had PDC. Generation IV contains 114 individuals, born between 1912 and 1955. Two individuals have developed ALS and 22 have developed PDC. Autopsy confirmation of diagnosis has been made in 5 individuals.

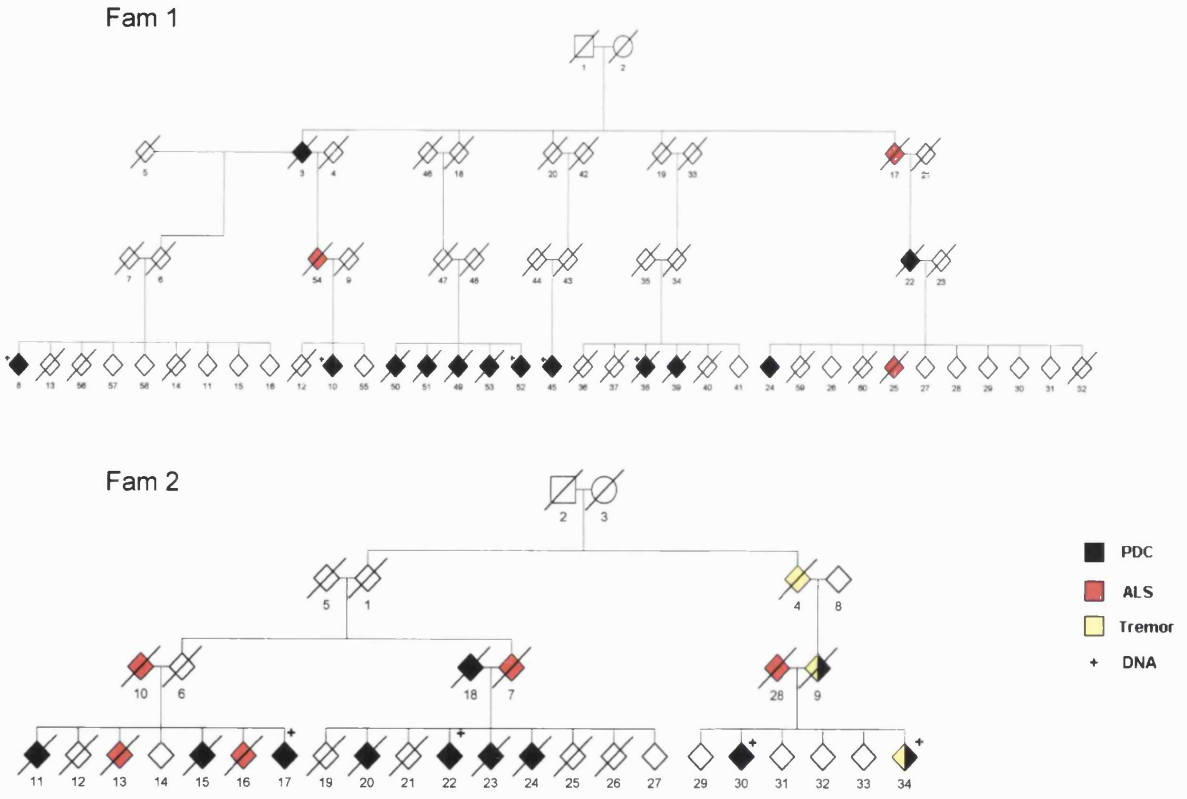



**Figure 6-1 Guam families 1 and 2**

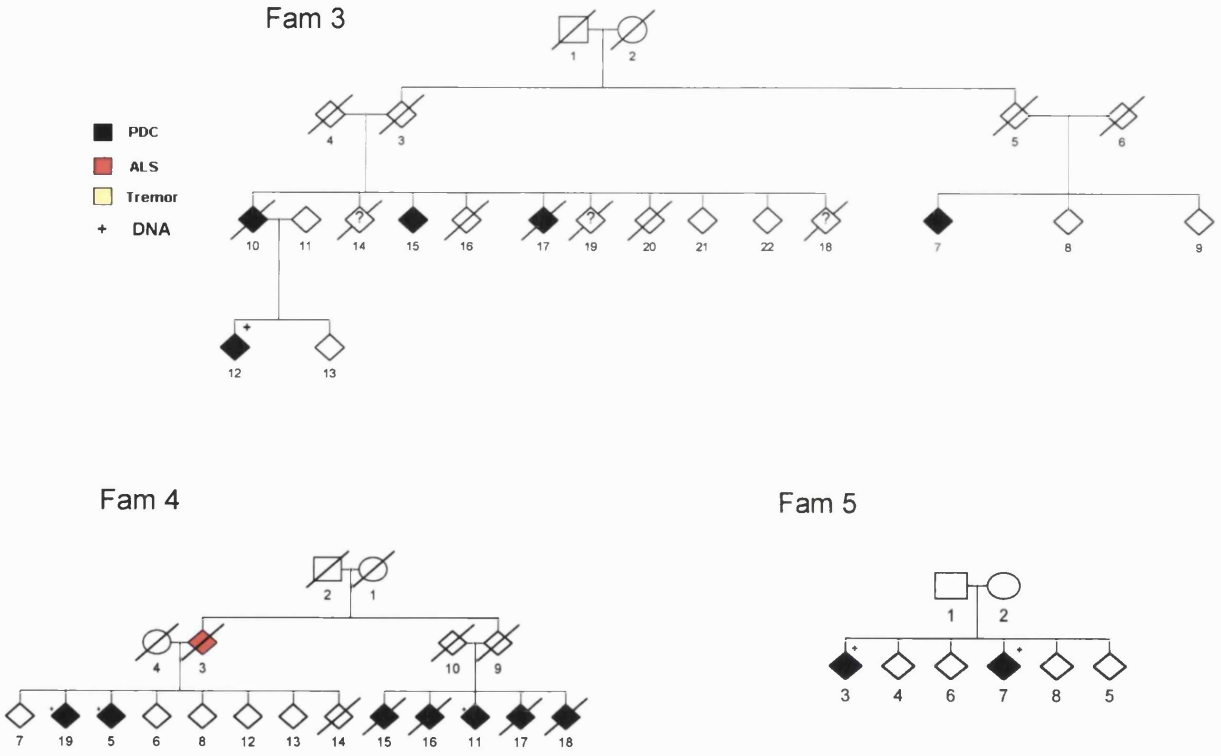



**Figure 6-2 Guam families 3, 4 and 5**

The male/female ratio was 1:1 overall, 1:1.2 for PDC alone and 1:0.5 for ALS. Age at onset information was available in 19 individuals. Considering ALS and PDC as one disease entity the average age of onset for generation III was 52.5 years and generation IV was 54 years. Considering ALS alone, mean onset age was 53.4 years in generation III and 30 years in generation IV (2 affected individuals). Considering PDC alone the mean onset age was 51 in generation III and 59.3 in generation IV. Of these 19 individuals, only 2 developed disease after the age of 65. Using the over 65 population in this family for calculation of segregation ratios, 42% of those at risk in generation 3 and 33% of those at risk in generation 4 developed ALS or PDC. Of 17 marriages in generation III, five were to apparently unrelated people who subsequently developed PDC. Simulation linkage analysis was performed on the available individuals from five families, indicating that the power of the families to detect linkage was low (Table 6-3).

**Table 6-3 Simlink: Analysis of Guam PDC families**

**Disease penetrance 0.4 by age 75, autosomal dominant linked marker lod score analysis**

| θ | Summed probability lod score >3.0 | Summed | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|---|
| | | Mean maximum lod score | | | | | |
| 0.00 | 0.0 | 1.9 | 1.2 | 0.21 | 0.29 | 0.23 | 0.13 |
| 0.025 | 0.0 | 1.6 | 1.0 | 0.23 | 0.29 | 0.22 | 0.11 |
| 0.050 | 0.0 | 1.2 | 0.83 | 0.19 | 0.25 | 0.22 | 0.10 |
| 0.50 | 0.0 | 0.1 | 0.08 | 0.10 | 0.15 | 0.1 | 0.08 |

**θ – recombination fraction**

## Discussion

The collection of these five families, analysis of family I and power analysis confirms the familial clustering of PDC, but illustrates some of the problems inherent in the proposed genetic analysis. Despite the clinico-pathological data suggesting that ALS and PDC may be separate diseases, the kindreds shows ALS to PDC transmissions, so that neither disease alone shows a conventional high penetrance autosomal dominant inheritance pattern. The kindreds contain married-in affecteds, which make it difficult to be certain of the phase of inheritance. Additionally, it is presumed that there is consanguinity within many

of the kindreds including family I kindred, but this is not established through current family records. The family I analysis confirms the disease prevalence changes within this family that have been seen within the Chamorro population as a whole.[293] The prevalence of ALS is decreasing, and the average age of onset of PDC is increasing. The change in the average age of onset of PDC is consistent with a cohort effect, which usually reflects an environmental exposure. There is no evidence for anticipation of age of onset in successive generations affected with PDC, as is seen in neurogenetic conditions which involve trinucleotide repeat expansions. There has been interest in the role of genetic factors in PDC since the original report by Plato and colleagues in the 1960s.[304, 315, 316] Most recently, ALS and PDC have been analysed in a sibship analysis based on historical NIH records. This included 2000 people through the island,[301] in a statistical model assuming that ALS and PDC are one disease entity. This analysis suggested that ALS and PDC could be due an effect of two additive alleles. Given the availability of affected individuals it was decided to concentrate on PDC, which bears many similarities to tauopathies in other parts of the world. DNA from 19 affected kindred members with PDC was collected. However, the simulation analysis illustrates the limited power of the current family collection to detect positive linkage to a PDC locus, but this is clearly dependant on the mode of inheritance, and age specific disease gene penetrance. Currently, these parameters are not known, and an alternative genetic approach may be more effective.

## 6.6 Conclusions

The family clustering of ALS and PDC seen in the families and cases collected in this study helps to support the theory that there is a significant genetic component in the aetiology of these conditions. The major problem with this theory, particularly if an autosomal dominant inheritance is hypothesised, is the declining prevalence of both PDC and more markedly of ALS. As discussed, this can be incorporated into a genetic hypothesis for these conditions only if significant outbreeding has led to a decline in necessary concurrent genetic (either two genes or two disease alleles for a single gene). The genealogical data currently available

is not extensive enough to track all the presumed consanguinity and interrelationships on the island. An environmental exposure is supported by the increasing age of onset for PDC which supports a cohort effect. However, this environmental exposure was presumably at its peak for the population born in the 1920s and 1930s and will now have disappeared, making its identification extremely difficult. Anecdotal reports suggest that the prevalence of pure dementia and pure movement disorders phenotypes is increasing among the elderly Chamorro population, and population based pathological studies will be needed to determine whether these are new phenotypic manifestations of PDC.

The clinical and pathological evidence in this chapter supports the concept that PDC and ALS may be separate diseases. The pathological data support ALS resembling ALS identified in other parts of the world. Despite the suggestive evidence from FTDP-17 and its animal models, tau NFT formation does not seem to frequently be associated with anterior horn cell damage. However, again this theory would be greatly assisted by population based neuropathological data which could confirm or refute whether the notion of "background" neurofibrillary tangle formation in Guamanian Chamorros is valid.

The pedigree simulation analysis suggests that it will be difficult to confirm or refute genetic linkage for PDC using the available family data set. For this reason it was decided to take an alternative genetic approach.

# CHAPTER 7 Genetic analysis of Parkinsonism dementia complex (PDC, bodig)

## 7.1 Summary

A genome wide association analysis was carried out in an attempt to determine a locus associated with PDC, using 22 cases and 19 controls, and a genome wide search with an approximate marker density of 5 cM. The two point analysis identified 14 loci with a p value of $< 0.015$. The multipoint analysis has highlighted two areas on chromosome 14q (14q11.1-14q22.2, 22.5Mb-60.1Mb, bounded by markers D14S1032 and D14S997) and chromosome 20 (20p12 – 20p 11.22, 15.6 Mb-19.5 Mb, bounded by markers D20S98 and D20S471), containing multipoint association lod scores of >2. These areas warrant further investigation in a second association sample and in conventional family based linkage studies.

## 7.2 Introduction

When PDC and ALS were first described it was thought that they would provide a paradigm which would help in the understanding of neurodegenerative disease throughout the world. However, while investigation into environmental factors on Guam has been unfruitful, the understanding of the aetiology of AD, PD and ALS has been greatly advanced by the description of genetic mutations responsible for some forms of these diseases.[317] As outlined above,(Section 6.5) there are a number of lines of evidence which support a genetic aetiology for PDC. The most promising candidate gene for PDC, *tau* has been excluded by direct sequencing and micro-satellite marker association.[201] Given the likely difficulty in a primary family based study of PDC, with the currently available DNA from family members and the genealogical data available,(Section 6.5) it was decided to use a linkage disequilibrium approach to mapping the PDC locus.

Population based disequilibrium studies involve mapping a disease locus by association at adjacent polymorphic genetic markers. They depend on a single

gene mutation arising on a founder chromosome, accounting for a given disease in the population under study.[185] The likelihood of successful linkage disequilibrium mapping is considerably higher in genetically isolated populations. Isolated populations are more likely to have a single disease/single gene and mutation relationship (genetic and allelic homogeneity). They are also more likely to have a sufficiently large shared chromosomal segment around any given marker (useful in the mapping of the contribution of many genes to complex traits) and around a single disease gene mutation, depending on the age of the population and the time of introduction of the mutation. An isolated population is defined as a population which is derived from a limited number of founder members, and which has expanded through growth rather than immigration.[185] The Chamorro population of Guam has historically been a relatively isolated population. Religious massacres and the chicken-pox epidemic of 1779 led to a reduction in the Chamorro population to 1,608,[318] from whom the current Chamorro islanders are descended - a so-called genetic "bottleneck". However, since the Second World War there has been considerable immigration to Guam. Under these circumstances, a common founder is assumed to have introduced a pathogenic mutation at an ancestrally distant point and the descendants will share both the pathogenic mutation and an extended adjacent chromosomal region. Genotyping of cases and controls, to determine shared alleles among cases, should identify the area in which the gene of interest lies.[185] This method has been successful in determining pathogenic mutations for diseases in a number of isolated populations.[319-321] This approach involves a case control association study, as described in Chapter 4, but extended to involve the whole genome. In this study affected and control individuals would be genotyped at polymorphic markers across the genome. Cases will share identical marker alleles around the disease locus which would be present to excess in comparison with control individuals. The strength of the association will depend on the background frequency of the disease associated allele in the control population, and will on average decay with increasing distance from the disease mutation, due to meiotic recombination. The background allele frequencies are unknown *a priori* and may depend on whether the mutation has arisen on an indigenous chromosome or has been introduced by an immigrant. Unlike family based linkage, even at points very close to the

disease mutation, a high background allele frequency may lead to no association between the disease and a disease chromosome marker allele. This is analogous to the situation discussed in 4.3, in which there is no association between the ***tau*** A0 allele and PSP in the Japanese population. For disease gene mutations of low penetrance, or genetic susceptibility factors, the mutation itself may not necessarily be associated with the disease in some populations. This phenomenon and allelic association mapping is illustrated in Collins and Morton's study of allelic association in mapping the common cystic fibrosis disease mutation ΔF508 in the outbred Caucasian population.[192] They estimate that ΔF508 was established in the population between 100-200 generations ago and illustrate that allelic association can be detected over a 1.7 Mb region spanning the disease mutation. Allelic association surrounding a disease mutation extends for much greater distances than linkage disequilibrium across anonymous genomic regions. Allelic association studies do not depend on the mode of inheritance of the disease or on the number of genes involved. If PDC depends on several genes these may all appear as associated areas in the genome wide search. Loci for autosomal recessive diseases are particularly easy to detect with association studies since there is a doubling of the amount of disease associated allele as compared with dominant diseases.

Twenty-two apparently unrelated cases and nineteen elderly, unrelated, neurologically normal controls were selected for the case control study. Cases and controls were examined by Dr. John Steele, the clinical features and family histories were recorded and autopsy confirmation was obtained where possible.

**Table 7-1 Clinical features of PDC cases for case control study**

| # | Sex | Age at onset | Age at sample | Age at death | Onset symptom | Subsequent symptoms and signs | Family History | Pathological confirmation |
|---|---|---|---|---|---|---|---|---|
| 1 | M | 74 | 75 | 77 | Forgetful, dementia | SNGP, anarthria and aphagia, axial rigidity, flexion contracture of the limbs and dystonic posturing of fingers and toes. Akinetic, mute and bedbound in late stages. | Mother (ALS), 3 Brothers (2 with PDC and 1 with ALS), 2 Sisters (PDC and ALS) | PDC |
| 2 | F | 74 | 75 | - | Forgetful, dementia | Slowly advancing cognitive impairment. | Father (PDC) | - |
| 3 | M | 57 | 63 | - | Writing tremor | Generalized tremor unresponsive to L-DOPA, poor immediate and day to day recall, blepharospasm, SNGP hypophonic dysarthria, axial rigidity and postural impairment. | Mother (ALS) Kindred A | - |
| 4 | F | 54 | 74 | 74 | Forgetful, dementia | Akinetic-rigid syndrome with anarthria/aphagia and vertical gaze palsy. | Father and Brother (PDC) Kindred A | PDC |
| 5 | F | 68 | 74 | 77 | Forgetful, dementia | Rigidity without tremor, limb contractures and akinesia Developed vertical gaze palsy and mutism in late stages | Father (PDC), Mother (ALS) and 3 Brothers (PDC) Kindred B | PDC: 2 brothers |
| 6 | F | 63 | 65 | 67 | Forgetful, dementia | Parkinsonism and ALS. | Mother (PDC)and Sister (ALS); Kindred A | PDC: mother |
| 7 | F | 55 | 58 | - | Tremor, Parkinsonism | Rigid-akinetic Parkinsonism with axial extension and limb flexion, vertical gaze palsy, and dysarthria. | Father (PDC), Mother (ALS), 2 Sisters (PDC) Kindred B | PDC: father |
| 8 | M | 69 | 70 | 70 | Falls backward, parkinsonism | SNGP, dysarthria, axial hyperextension, limb rigidity, bradykinesia and postural instability. | 2 Sisters (PDC and ALS) | - |
| 9 | F | 68 | 71 | 73 | Slow, parkinsonism | Prominent bradykinesia, postural instability and rigidity with minimal rest tremor, unresponsive to L-dopa. | Sister (PDC) | - |
| 10 | M | 55 | 63 | 63 | Slow, parkinsonism | Bradykinesia, vertical gaze palsy, anarthria and | Father, 1 Brother, 2 Sisters | PDC in sister |

| # | Sex | Age at onset | Age at sample | Age at death | Onset symptom | Subsequent symptoms and signs | Family History | Pathological confirmation |
|---|---|---|---|---|---|---|---|---|
| | | | | | | dysphagia, limb rigidity without tremor, flexion contractures, dystonic posturing and axial hyperextension. | and ½ Sister (PDC), one Brother (ALS) Kindred A | |
| 11 | F | 71 | 80 | 83 | Forgetful, dementia | Parkinsonism without tremor, akinetic mutism in late stages and was accompanied by myoclonus, axial rigidity, limb contractures and dystonic posturing of fingers and toes. | 2 Brothers (PDC) Kindred C | PDC in brother |
| 12 | F | 60 | 74 | 77 | Choking, pseudobulbar palsy | Pseudobulbar palsy, dementia and bradykinesia. Atypical parkinsonism with postural tremor, athetosis, akathisia and supranuclear vertical gaze palsy. Akinetic and rigid with limb contractures and dystonic deformity of fingers and toes. | 4 Paternal 2nd Cousins (PDC) Kindred C | PDC in maternal second cousin |
| 13 | F | 56 | 61 | 64 | Forgetful, dementia | Bradykinesia, rigidity without tremor and postural instability, not responsive to L-dopa. | Father (ALS), Sister (PDC) | - |
| 14 | F | 62 | 71 | 73 | Forgetful, dementia | Blepharospasm, anarthria, rest tremor of lips, face and arms, axial rigidity, dystonic flexion deformities of the fingers. | Father and 3 Brothers (PDC), Sister (ALS) | PDC |
| 15 | M | 65 | 75 | 77 | Depressed, forgetful, dementia | 6 years after onset, bradykinesia, cogwheel rigidity without tremor, akathisia, postural instability. | Mother (ALS) and Materna relatives (PDC and ALS) | PDC: maternal uncle |
| 16 | M | 63 | 66 | 69 | Forgetful, dementia | Impaired postural stability, mild symmetrical akinetic rigid syndrome, frontal release signs. In late stages, SNGP, anarthria, aphagia, nuchal extension, hyperreflexia, amyotrophy. | Father (ALS) 3 Sisters (PDC) Kindred B | - |
| 17 | M | 68 | 69 | 71 | Impaired judgement, dementia | Bradykinesia and lurching, ataxic gait. | 3 Sisters and 1 Brother (PDC) Kindred A | - |
| 18 | M | 55 | 59 | 61 | Tremor, parkinsonism | Parkinsonism with rest tremor, rigidity, bradykinesia, | Both Parents (PDC) | - |

| # | Sex | Age at onset | Age at sample | Age at death | Onset symptom | Subsequent symptoms and signs | Family History | Pathological confirmation |
|---|---|---|---|---|---|---|---|---|
| | | | | | | and postural instability. | | |
| 19 | F | 70 | 75 | - | Slowness, tremor, parkinsonism | Bradykinesia and hemiplegic dystonia right limbs, rest tremor, and cogwheel rigidity. | Many Maternal Relatives (PDC and ALS) | |
| 20 | M | 71 | 76 | - | Paranoia and tremor of hands | Dementia and Parkinsonism with rest tremor, akinetic-rigid syndrome. Blepharospasm, impaired upgaze and oro-lingual-mandibular tremor. | 2 Brothers (PDC) | PDC (2 brothers) |
| 21 | M | 66 | 72 | - | Backward falls, parkinsonism | Prominent dementia Parkinsonism, tongue tremor, impaired up gaze and spastic dysarthria. | Father (PDC) and many Paternal Relatives (PDC and ALS) | Paternal uncle |
| 22 | M | 65 | 70 | - | Nocturnal rigidity, parkinsonism | Mild dementia, and progress to a mute, rigid akinetic bed bound state without tremor. | 3 Maternal Cousins (PDC) Kindred C | Maternal uncle |

SNGP – Supranuclear gaze palsy, Kindred A: Individuals 3, 4, 6, 10, 17 and 18 share a common great-grandfather; Kindred B: Individuals 7 and 16 share a common grandparent, Individuals 5,7 and 16 share a common great-grandparent; Kindred C: Individuals 11 and 22 share a common grandfather

All 22 cases were Chamorros with neurodegenerative symptoms beginning between ages 54 and 74. Eleven presented with dementia and, of these 10 subsequently developed Parkinsonism. Ten presented with Parkinsonism and of these 6 subsequently developed dementia, but the other 4 cases did not develop cognitive impairment. In one case the onset was with simultaneous dementia (with paranoia) and parkinsonism. The parkinsonism was usually atypical (i.e. unresponsive to L-dopa and involving predominant rigidity without tremor), and in 10 cases there were additional features suggesting PSP. By the late stages of the disease the majority suffered both dementia and severe disabling Parkinsonism. In only two patients was there a definite ALS accompaniment. Eleven cases were from the southern Guamanian village of Umatac, and 11 were from other parts of Guam. All had a family history of other members affected by PDC or ALS, and in 20 of the 22 this was in first degree family member. Thirteen of the 22 had pathological confirmation of PDC in themselves (3) or in a family member (10). Fifteen cases died between initial recruitment and in July 1999. Although all apparently unrelated individuals in this study are presumed to be related to a common founder, genealogical research following patient recruitment revealed that most of the subjects are definitely related and they are formed into three kindreds: A, B and C.

The research was planned in the following sequence: i.) exclude the most likely candidate gene, *tau* by direct sequencing and flanking marker association,[201] ii.) investigate the possible role of other loci involved in neurodegeneration by association analysis, iii.) identify new genomic areas of interest in a genome wide association study, iv.) provide confirmatory evidence for possible association in a replication data set and in family based studies, v.) identify sequence variants in associated areas which might impact on the disease pathogenesis.

For the case control study 834 microsatellite markers were analysed and compared in a case control design. The average marker spacing was 4.4 cM. The genotypes in cases and controls were compared using a two-point (*diseq*) and multipoint (*dismult)* association analysis package.

## 7.3 Results

The markers corresponding to established neurodegenerative disease loci showed no evidence of association.

**Table 7-2 Guam association data at known neurodegenerative disease loci**

| Disease locus | Gene | Ch | STS map position on chromosome | Marshfield map position locus | Marker analysed | Marshfield map position marker | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| PARK-1 | SNCA | 4 | 87 M | 99 cM | D4S414 | 101 cM | 0.0 | 0.500 | 0.0 |
| PARK-2 | Parkin | 6 | 170 M | 179 cM | D6S624 | 179 cM | 0.0 | 0.500 | 0.0 |
| PARK-3 | - | 2 | 67 M | 88 cM | D2S285 | 86 cM | 0.0 | 0.500 | 0.0 |
| PARK-4 | - | 4 | 29 M | 41 cM | D4S2397 | 43 cM | 0.0 | 0.500 | 0.0 |
| PARK-5 | UCH-L1 | 4 | 42 M | 50 cM | D4S2632 | 52 cM | 0.0 | 0.500 | 0.0 |
| PARK-6 | - | 1 | 15.5 M | 48.5 cM | D1S552 | 45 cM | 0.0 | 0.500 | 0.0 |
| PARK-7 | - | 1 | 5.4 M | 14 cM | D1S214 | 14.0 | 3.7 | 0.028 | 0.4 |
| APP | APP | 21 | 24 M | 21 cM | D21S1253 | 20.5 | 0.0 | 0.500 | 0.0 |
| PS-1 | PS-1 | 14 | 67 M | 84.9 | D13S158 | 0.0 | 0.5 | 0.0 | |
| PS-2 | PS-2 | 1 | 234 Mb | 245 cM | D1S2800 | 252.1 | 0.0 | 0.500 | 0.0 |
| FTD-3 | - | 3 | 74 M | 102.5 cM | D3S2406 | 102.6 | 6.4 | 0.006 | 0.3 |
| FTD-9 | - | 9 | 71.4 M | 80.3 cM | D9S922 | 80.3 | 0.0 | 0.500 | 0.0 |
| FTDP-17 | tau | 17 | 45.5 M | 65 cM | D17S791 | 64.2 | 0.0 | 0.500 | 0.0 |

Abbreviations: Ch - Chromosome, STS – sequence tagged site, LRT – likelihood ratio test

Seventeen markers were identified in the whole genome two point analysis which showed possible evidence of association with $p<0.015$, including a marker adjacent to the FTD-3 locus, D3S2406 (Appendix, Table 7-3).

**Table 7-3 Markers with p<0.015, Guam two point association**

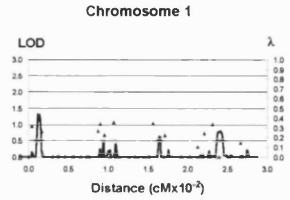
| Locus | LRT | p |
|---|---|---|
| D14S592 | 11.4090 | 0.0004 |
| ATA59H06 | 8.5143 | 0.0018 |
| D2S326 | 8.2000 | 0.0020 |
| D18S976 | 7.9300 | 0.0024 |
| D3S2406 | 6.4410 | 0.0056 |

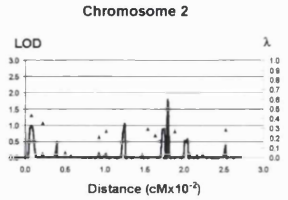
| Locus | LRT | p |
|---|---|---|
| D1S468 | 6.3840 | 0.0058 |
| D16S2624 | 6.1620 | 0.0065 |
| D12S375 | 6.1530 | 0.0066 |
| D3S1271 | 6.0980 | 0.0068 |
| D20S103 | 5.9200 | 0.0075 |
| D2S319 | 5.6490 | 0.0087 |
| D11S925 | 4.9000 | 0.0100 |
| D5S2505 | 5.2750 | 0.0108 |
| D2S160 | 5.1040 | 0.0119 |
| D4S403 | 5.0580 | 0.0123 |
| D4S412 | 4.8670 | 0.0137 |
| D13S171 | 4.8170 | 0.0141 |

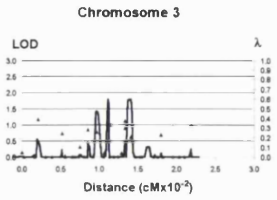
The association data was analysed in a genome wide multipoint like analysis (*dismult*) analysis which highlighted possibly associated areas, on chromosome 14q (14q11.1-14q22.2, 22.5Mb-60.1Mb, bounded by markers D14S1032 and D14S997) and chromosome 20 (20p12 – 20p 11.22, 15.6 Mb-19.5 Mb, bounded by markers D20S98 and D20S471), containing multipoint association lod scores of >2 (Figures 7-1, 7-2).

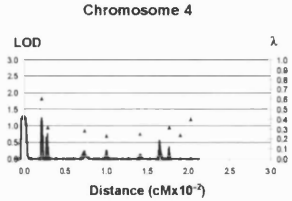
**Figures 7-1, 7-2 Guam genome wide multipoint analysis**

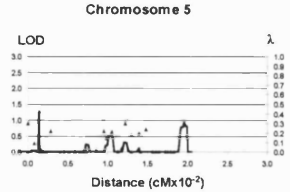
**Pages following**

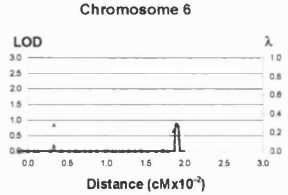

Chromosome 1
LOD
λ
Distance (cMx10-2)

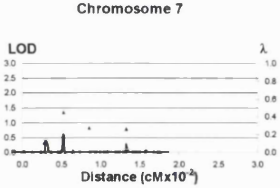

Chromosome 2
LOD
λ
Distance (cMx10-2)

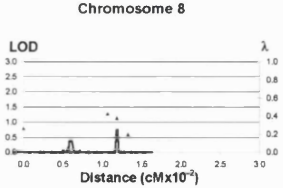

Chromosome 3
LOD
λ
Distance (cMx10-2)

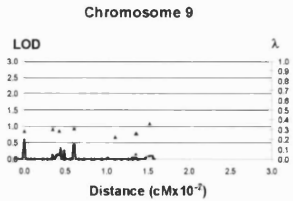

Chromosome 4
LOD
λ
Distance (cMx10-2)

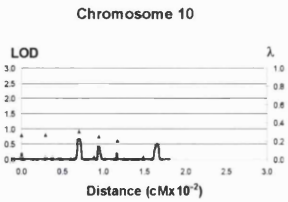

Chromosome 5
LOD
λ
Distance (cMx10-2)

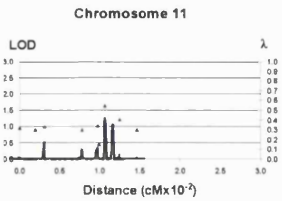

Chromosome 6
LOD
λ
Distance (cMx10-2)

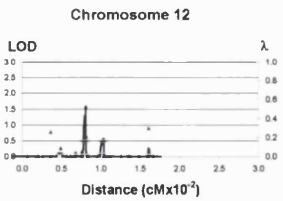

Chromosome 7
LOD
λ
Distance (cMx10-2)

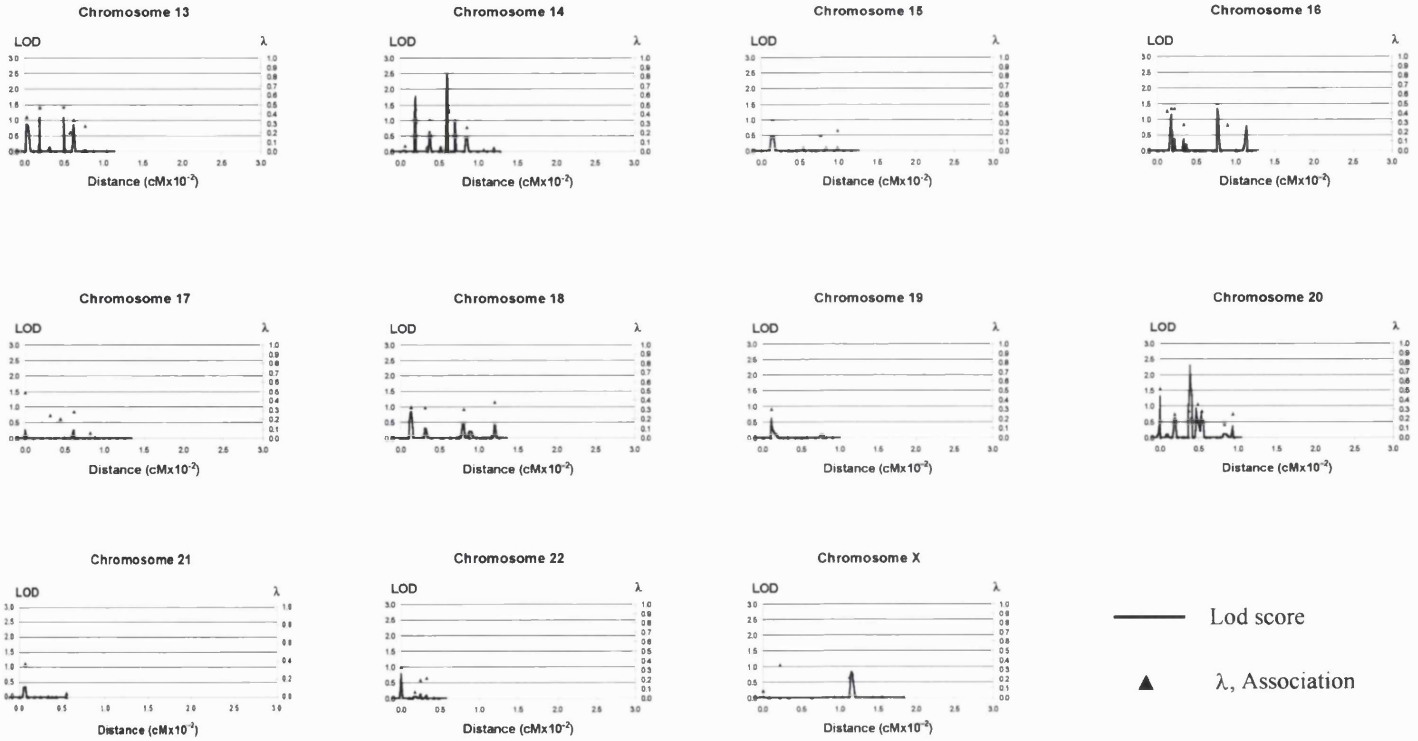

Chromosome 8
LOD
λ
Distance (cMx10-2)


Chromosome 9
LOD
λ
Distance (cMx10-2)


Chromosome 10
LOD
λ
Distance (cMx10-2)


Chromosome 11
LOD
λ
Distance (cMx10-2)


Chromosome 12
LOD
λ
Distance (cMx10-2)


Chromosome 13
Chromosome 14
Chromosome 15
Chromosome 16
Chromosome 17
Chromosome 18
Chromosome 19
Chromosome 20
Chromosome 21
Chromosome 22
Chromosome X
LOD
λ
Distance (cMx10-2)
Lod score
λ, Association

## 7.4 Discussion

This study provides evidence of genetic susceptibility to PDC on chromosomes 14 and chromosome 20, without evidence of association to major known neurodegenerative disease loci, although the FTD-3 locus is possibly associated. Differences in the pathology between FTD-3 and PDC suggest that it is unlikely that these will be allelic variants. Recent attempts to map complex diseases have used non-parametric techniques such as affected sib pair analysis. Different groups investigating diseases such as type II diabetes, multiple sclerosis and AD have produced different areas of interest in genome wide searches and replication studies and the meta-analysis of series of studies are now regarded as vital in assessing the importance of results of genome wide searches for non-Mendelian diseases. The areas identified in this study do not reach the absolute significance level assumed to be necessary to demonstrate positive association in the context of a genome wide search, and as discussed,(7.2) the association maps show fluctuation in the areas of interest. However, replication in a further association data set and in family based analysis may confirm the importance of these areas. This study crucially depends on the extent of the genetic effect on the aetiology of PDC. The epidemiological data described casts some doubt on a genetic predisposition effect, but no convincing environmental cause has been determined.

The study is also limited by the power of this allelic association study to detect a susceptibility locus to PDC. The power of genome wide association studies is difficult to predict *a priori*, since it depends on unknown factors such as the number of generations since the pathogenic mutant arose or was introduced into the population, and the background frequency of the associated alleles in the normal population. However, we assume that the mutation has propagated in the Guamanian population after 1780, approximately 11 generations before the current at risk cohort. Neglecting the effects of inbreeding and selection the average separation genetic separation between the most distant relations who share a single ancestor from 1780 will be approximately 22 meioses. By

definition this would imply an average shared chromosomal region of around 4 cM (at this distance recombination events occurring 4 times in every 100 meioses). The genealogical studies illustrate that the cases studied in the genome search are more closely related than 22 meioses, but introduces the possibility that false positive regions will be identified by virtue of kinship. By comparison with the work on the cystic fibrosis ΔF508 mutation in an "older" outbred population, a genome wide study with a 5 cM map density should be adequate to detect an association effect in this population. Even if the mutation in fact arose many generations before 1780 the propagation of the mutation within the Chamorro population following 1780 lends considerable power to this study.

## 7.5 Conclusions

This study illustrates the approach to a genome wide association study and highlights the advantages and potential pitfalls of this approach. It provides preliminary evidence for a susceptibility effect to PDC on chromosomes 14 and 20, which needs to be confirmed in a larger sample and replicated in Guamanian family based studies.

# CHAPTER 8 Conclusions

Molecular genetic analysis has been used to explore the aetiology of tau related neurodegeneration. Data is presented which explores an association between *tau* and PSP, describes ***tau*** mutations in FTD and the search for *tau* mutations in a number of other tauopathies. The features of PDC and ALS on Guam and a genome wide search for a genetic locus associated with PDC are also described. Correlation of pathological data with the results of mutational and genotype analysis has been carried out which has clarified the classification of different types of sporadic tauopathies and different forms of familial FTD. It is anticipated that molecular pathological investigation, involving protein and RNA analysis, will be central to understanding these neurodegenerative diseases. The genome wide association study of PDC provides some insight into linkage disequilibrium mapping and its potential importance as an investigative tool.

## 8.1 Impact of the human genome project and new technologies

The human genome project (HGP) will undoubtedly be of immense importance to the future investigation of neurodegenerative diseases. The most important single aspect of the recent advances in genetics is the publication of the genome sequence in a freely accessible form via the internet, dependant on the goodwill and cooperation of participating scientists. Allelic association studies are concerned with determining genetic predisposition to sporadic disease. The variation in genome sequence is of particular research relevance. Both the private sector Celera project and the public sector funded HGP have sequenced a number of different individuals. Comparisons between the sequences studied will confirm the shared consensus sequence but also genomic variability. This variation is estimated to involve 1 SNP every 1000 bp,[322] in addition to the variation inherent in micro and mini satellites and insertion/deletion polymorphisms. As discussed, SNPs which alter the protein coding sequence of genes or which determine the function of regulatory elements are likely to be particularly important. The data presented in chapter 4 illustrates the role of SNP variability analysis in

understanding the predisposition to neurodegenerative disease. Further detailed analysis of linkage disequilibrium and SNP variability around genes of interest will inform association studies. As an example of this, despite the negative data in this work on α-synuclein microsatellite polymorphisms and MSA, a recent analysis of SNP haplotypes in α-synuclein has reported a PD associated *α-syn* SNP haplotype.[323] There are ethical implications to the forthcoming work on genomic variation. SNP variability will undoubtedly include information on physical characteristics which do not impact on disease but may be subject to parental preference. It will provide information on differences between racial groups which may be used to reinforce prejudicial views. It will also include uneven disease related information which may be used to discriminate against individuals who carry identified disease susceptibility genes.

Much of the comparison of sequence and the identification of sequence variation can be carried out on published electronic data ("*in silico*") rather than with the need for fresh sequence experiments. Novel genes can be determined in unexplored areas of draft sequence using sequence prediction programmes, comparison with known human and other organism gene families. *In vivo* techniques will be needed to determine regional expression patterns and for adult neurodegenerative diseases to select non-developmental brain expressed proteins. The data presented in this thesis on splice variation mutations reinforces the fact that raw sequence data in itself is not sufficient – knowledge of changes in alternative splicing and regulation of gene expression is also important.

The physical techniques used in this project – PCR with restriction enzyme digestion, automated slab gel sequencing and automated slab gel fluorescent microsatellite marker quantification are already becoming outmoded. While these techniques are dramatically faster and more efficient than older techniques such as manual sequencing and radiolabelled DNA quantification, new and more powerful processes are already being used. 96 lane capillary systems allow high throughput sequencing, without the need for gel pouring or loading. The use of oligonucleotide etching onto assay plates ("gene chips") allows the quantification of multiple SNPs on a single slide.[324] These types of technologies are also being

applied to multiple cDNA arrays which allow the quantification of the relative amounts of transcription of multiple gene products – allowing the determination of the gene families which are involved in various physiological and pathological states.[325] Recent cDNA assay experiments of interest have included the determination of gene expression changes in AD hippocampus,[326] and in the ageing mouse brain.[327] It is anticipated that cDNA expression systems will be used to map changes in gene expression in sporadic conditions such as PSP and PiD. One of the problems with this type of gene expression data is that the expression of many different gene families may be altered, and many of the changes may be secondary to the underlying disease process, for example related to gliosis, rather than primarily important in the disease pathogenesis. The analysis of predisposing genomic variability will allow the selection of the gene/proteins from the mass of cDNA expression data which may be of aetiological importance. These rapidly developing technologies will undoubtedly be used to take forward the work presented in this thesis.

## 8.2 Sporadic and familial neurodegenerative disorders

PSP and CBD are associated with the *tau* H1 haplotype and both involve the deposition of four repeat tau protein. It remains to be seen whether this is due to a change in the alternative splicing of *tau* or whether this is due to selective deposition of four repeat tau protein isoforms. The analysis of markers around *tau* performed to date suggests that the predisposition effect of H1 to PSP involves a population wide increase in susceptibility to PSP. It is anticipated that high throughput SNP mapping around *tau* will confirm that the decay of association with PSP around *tau* matches the decay in linkage disequilibrium in control *tau* H1 chromosomes. There are three approaches which can be considered to determine further genetic risk factors for PSP: i.) selected candidate gene analysis in sporadic disease, ii.) genome wide search, iii.) identification of genes of importance from familial studies. A candidate gene analysis would presently be based on the pathological features of the disease and would involve the analysis of variability in several genes whose protein products are present in PSP post mortem brain or whose function could be important in determining the

pathology e.g. proteins that govern the phosphorylation state of tau or the ubiquitin degradation pathway. As outlined above further candidate genes may be identified from gene expression data, and genetic association analysis has an important role in determining the important genes/proteins from these expression experiments.

A more systematic, but labour intensive approach would be the use of genome wide association analysis of susceptibility to PSP. As has been discussed in relation to PDC the marker density used is proportional to the relatedness of the affected individuals studied. For an outbred population in which patients with PSP were no more related than the average in the general population this would involve genetic analysis of markers with a separation of no greater than 100 kb. This would involve analysing around 40,000 markers in an attempt to cover the human genome in an evenly spaced fashion. This type of approach can only be undertaken using high throughput genotyping methods, and it remains to be seen if these types of studies are feasible in outbred populations. The identification of genes in autosomal dominant families with neurodegenerative disease is historically the most successful approach but has limitations in relation to the diseases considered in this thesis. It is also apparent that not all mutations/genes identified in familial disease are relevant to sporadic disease. The identification of mutations in APP and the presenilins has undoubtedly brought into sharp focus the molecular pathways which may lead to amyloid deposition in AD, but variability in APP or the presenilins is not associated with sporadic AD. A further problem is the availability of families suitable for genetic research. Neither of the families with PSP identified in this study are large enough to undertake a genome wide search, and only one family in the world literature seems to be of sufficient size for a classical genome search approach.[328] An alternative approach to selecting single genes of relevance to sporadic neurodegenerative disease may lie within animal models for neurodegeneration. Random mouse mutagenesis programmes now have the capability of producing neurodegenerative phenotypes of interest, with similarities to human disease, which will allow the selection and examination of relevant genes.[329]

The identification of genes for Mendelian disorders has involved establishing linkage between the disease and a genomic areas using polymorphic genetic markers, moving on to narrowing the area using additional genetic markers possibly newly identified for the purposes for the study, building a physical map of the area using selected yeast or bacterial artificial chromosomes and then identifying and selecting genes from the region of interest. In recent years it has been estimated that 50% of the genes in any given region of interest will be entirely novel. Each likely candidate gene then has to be analysed for evidence of pathogenic mutations which segregate with the disease phenotype. The mapping and gene selection of the region of interest was a highly labour intensive process which usually took several years to complete. The completion of the draft sequence of the human genome means that linked areas are automatically available in full physical sequence and usually both microsatellite and SNP markers will already have been identified or can be identified in the region which will facilitate fine mapping and disease region narrowing. Furthermore, sequence prediction programmes which use intra- and inter-species homology are able to identify, with a high degree of accuracy, genes of interest within the appropriate area. The identification of novel genes will become an obsolete part of the disease gene identification process. It is anticipated that the acceleration of the linkage to disease gene process means that the genes for FTD-NLS and FTD-Ub will be identified within the coming 1-2 years.

FTDP-17 is very closely related to PSP, CBD and PiD. Differences between these conditions are also highlighted and some controversy has ensued in the literature as to whether certain phenotypes associated with ***tau*** mutations are or are not PSP, PiD etc.[330] An FTDP-17 case clinically indistinguishable from PSP is presented in this thesis(5.7). However, an important result arising from this thesis is the absence of mutations in the majority of sporadic cases of PSP and PiD. This indicates that these conditions are not equivalent to FTDP-17. Currently it should be considered that ***tau*** mutations or possibly linkage to chromosome 17 define FTDP-17, even in patients without a family history.

## 8.3 Protein overload theory of neurodegeneration

Each gene that has been identified to be involved in neurodegeneration has normal functions which have been explored and theories of the role of the gene in neurodegeneration has been put forward. The role of APP and presenilin has recently been discussed in relation to the regulation of the Notch signalling pathway,[331] SOD-1 has been linked to oxidative stress,[332] and tau mutations have been analysed for their impact on microtubule interaction. However, one common factor seems to link together all mutations described in neurodegenerative disease genes. All accelerate the formation of protein aggregates, as occurs in FTDP-17. P301S, the mutation shown to have the most marked effect on the age of onset of FTDP-17 is associated with very rapid abnormal filament formation. Coincident with the focus on aggregate formation, several lines of evidence link together proteins involved in neurodegeneration and the ubiquitin – 26S proteasome system. The normal function of this system is the degradation and recycling of misfolded and damage proteins.[333] α-synuclein has sequence homology with ubiquitin,[209] and parkin, the major cause of autosomal recessive juvenile parkinsonism, has recently been shown to act as a ubiquitin protein ligase.[334] These data point to excessive protein aggregate formation or abnormal protein handling as being a central theme in neurodegeneration. As discussed in chapter 6 there is some evidence for an overlap in the deposition of proteins involved in neurodegeneration – patients with AD, PDC or Down's syndrome may have, in addition to amyloid or tau deposition, α-synuclein deposition and/or Lewy body formation.[335-337] It is tempting to speculate that this may be due to some clogging up of the normal protein degradation pathway, which leads to an overspill deposition of a number of different abnormal protein products. However, while accelerated protein aggregation is firmly established to be an effect of *tau* protein coding mutations it is unclear whether the alteration in the 4R:3R ratio seen in *tau* splice site mutations leads by an indirect mechanism to an acceleration of tau aggregate formation. Finally, this type of explanation of neurodegeneration fits in very well with Kirkwood's disposable soma theory of ageing outlined in 1.7 – an amount of protein product clearance is maintained which can maintain the organism to the "usual" expected lifespan – 40 or 50 years

of age but it is energetically wasteful to maintain abnormal protein clearance beyond that time. In some circumstances, particularly related to mutations in the genes encoding the proteins which are deposited in these diseases this process is accelerated. A specific prediction of this theory is that further neurodegenerative disease genes will be identified which are involved in protein clearance and degradation, encoding proteins involved in the ubiqutin-26S proteasome system. Furthermore, some genes will be identified which can lead to more than one neurodegenerative disease pathology and perhaps this will be of relevance to ALS and PDC on Guam.

## 8.4 Closing remarks

The investigation of genetic factors in neurodegenerative disease, and the molecular pathology of these conditions will become increasingly well understood in the coming years. The tools are now becoming available which will make this process faster and more certain. It is to be hoped that this can be accompanied by new disease modifying treatments which can radically improve the quality of life of neurodegenerative disease sufferers, their carers and families.

## SUMMARY OF THESIS

1. PSP is positively associated with the *tau* A0 allele, in a series of 53 PSP patients as compared with 75 pathologically normal controls. The A0 allele was not associated with pathologically diagnosed PD, or clinically diagnosed FTD or CBD

2. The A0 allele occurs on a segment of genetic variability designated the H1 haplotype, spanning 100 kb of DNA, defined by 6 SNPs. PSP is not associated with polymorphic genetic markers as close as 1 Mb to *tau.*

3. PSP is not associated with variability in ApoE or α-synuclein.

4. Neither ApoE nor *tau* variability has any effect on the age at onset of PSP.

5. Cases of pathologically diagnosed PSP with atypical clinical presentations and atypical tau protein deposition patterns have a lower frequency of the *tau* PSP susceptibility haplotype, and there may be a number of different parkinsonian phenotypes related to PSP like pathology.

6. *Tau* was sequenced in 22 clinically diagnosed FTD families of whom 11 had mutations in *tau:* exon 10 +14, exon 10 +16 and the P301S mutation. The diagnostic yield of ***tau*** sequencing in FTD families is likely to be around 50%.

7. Pathologically the FTD cases were split into FTD with tau inclusions, FTD with ubiquitin inclusions and FTD lacking distinctive histopathology. In cases with histopathological verification the presence of *tau* mutations was uniquely correlated with the presence of tau pathology.

8. In general, pathologically defined PiD cases did not have *tau* mutations, however two individuals with the G398R mutation were identified. Both of these cases had atypical immunohistochemical characteristics, with antibody 12-E8 positive Pick like inclusions.

9. In general, PSP cases did not have *tau* mutations although one young onset individual, clinically diagnosed to have PSP, was identified to have a *tau* exon 10 +16 mutation.

10. The clinical and pathological features of ALS and PDC on Guam were analysed. PDC shares clinical similarities with PSP and other tauopathies. PDC and ALS usually appear as separate clinical and pathological entities, although is be an increase in NFT formation in Guam ALS cases, which may also occur in Guamanian controls.

11. A genome wide association study of PDC was carried out which provided evidence for the association of PDC with chromosome 14 and chromosome 20, but excluded association with the major known loci for neurodegenerative disease.

# APPENDIX

## GUAM GENOME WIDE SEARCH TWO POINT ASSOCIATION RESULTS

**Table 8-1 Chromosome 1 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 4.2 | D1S468 | 6.4 | 0.006 | 0.3 | 144.4 | D1S2726 | 0.0 | 0.500 | 0.0 |
| 14.0 | D1S214 | 3.7 | 0.028 | 0.4 | 150.3 | D1S252 | 0.0 | 0.500 | 0.0 |
| 16.2 | D1S1612 | 6.5 | 0.005 | 0.3 | 151.9 | D1S534 | 0.0 | 0.500 | 0.0 |
| 24.7 | D1S2667 | 0.0 | 0.500 | 0.0 | 155.9 | D1S498 | 0.0 | 0.500 | 0.0 |
| 29.9 | D1S228 | 0.0 | 0.500 | 0.0 | 164.1 | D1S1653 | 3.4 | 0.033 | 0.3 |
| 37.1 | GATA29A05 | 0.0 | 0.500 | 0.0 | 169.7 | D1S484 | 0.0 | 0.500 | 0.0 |
| 45.3 | D1S199 | 0.0 | 0.500 | 0.0 | 170.8 | D1S1679 | 0.0 | 0.500 | 0.0 |
| 45.3 | D1S552 | 0.0 | 0.500 | 0.0 | 175.6 | D1S1677 | 1.1 | 0.152 | 0.2 |
| 55.1 | D1S234 | 0.0 | 0.500 | 0.0 | 181.5 | D1S196 | 0.1 | 0.400 | 0.0 |
| 56.7 | D1S1622 | 0.0 | 0.500 | 0.0 | 191.5 | D1S218 | 0.0 | 0.500 | 0.0 |
| 65.5 | D1S255 | 0.0 | 0.500 | 0.0 | 192.1 | D1S1589 | 0.0 | 0.500 | 0.0 |
| 72.6 | GATA129H04 | 0.0 | 0.500 | 0.0 | 202.2 | D1S518 | 0.0 | 0.500 | 0.0 |
| 75.7 | D1S2134 | 0.0 | 0.500 | 0.0 | 202.7 | D1S238 | 0.0 | 0.500 | 0.0 |
| 75.7 | D1S2797 | 0.0 | 0.500 | 0.0 | 212.4 | D1S413 | 0.0 | 0.500 | 0.0 |
| 76.3 | D1S197 | 0.0 | 0.500 | 0.0 | 212.4 | D1S1660 | 0.0 | 0.500 | 0.0 |
| 87.3 | D1S220 | 0.0 | 0.500 | 0.0 | 218.5 | D1S1678 | 0.4 | 0.277 | 0.1 |
| 89.5 | GATA165C03 | 1.4 | 0.116 | 0.3 | 220.7 | D1S249 | 0.0 | 0.500 | 0.0 |
| 93.9 | D1S209 | 2.1 | 0.074 | 0.3 | 227.0 | GATA124F08 | 0.9 | 0.170 | 0.2 |
| 95.3 | D1S230 | 0.0 | 0.500 | 0.0 | 231.1 | D1S425 | 3.2 | 0.040 | 0.3 |
| 102.0 | D1S1665 | 0.9 | 0.169 | 0.2 | 233.4 | D1S2141 | 0.0 | 0.500 | 0.0 |
| 104.8 | D1S216 | 0.0 | 0.500 | 0.0 | 239.7 | D1S549 | 4.0 | 0.023 | 0.3 |
| 106.5 | D1S2841 | 0.0 | 0.500 | 0.0 | 247.2 | D1S3462 | 0.0 | 0.500 | 0.0 |
| 109.0 | D1S1728 | 1.8 | 0.087 | 0.4 | 252.1 | D1S2800 | 0.0 | 0.500 | 0.0 |
| 113.7 | D1S207 | 0.0 | 0.500 | 0.0 | 254.6 | D1S235 | 0.0 | 0.500 | 0.0 |
| 113.7 | D1S551 | 0.0 | 0.500 | 0.0 | 267.5 | D1S547 | 0.0 | 0.445 | 0.0 |
| 126.2 | D1S2868 | 0.0 | 0.500 | 0.0 | 274.5 | D1S1609 | 1.0 | 0.162 | 0.1 |
| 134.2 | D1S206 | 0.0 | 0.500 | 0.0 | 277.8 | D1S423 | 0.0 | 0.500 | 0.0 |
| 136.9 | D1S1631 | 0.0 | 0.500 | 0.0 | | | | | |
| 140.2 | GATA176G01 | 0.0 | 0.500 | 0.0 | | | | | |

**Table 8-2 Chromosome 2 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 0.0 | GATA165C07 | 0.0 | 0.500 | 0.0 | 114.4 | GATA176C01 | 0.0 | 0.500 | 0.0 |
| 7.6 | D2S319 | 5.6 | 0.009 | 0.4 | 123.0 | D2S160 | 5.1 | 0.012 | 0.3 |
| 16.0 | GATA116B01 | 0.0 | 0.500 | 0.0 | 125.2 | D2S410 | 0.0 | 0.500 | 0.0 |
| 20.0 | D2S162 | 1.8 | 0.089 | 0.4 | 131.5 | D2S347 | 0.0 | 0.500 | 0.0 |
| 27.1 | D2S168 | 0.0 | 0.500 | 0.0 | 132.6 | D2S1328 | 0.0 | 0.500 | 0.0 |
| 27.6 | D2S1400 | 0.0 | 0.500 | 0.0 | 144.5 | D2S368 | 0.0 | 0.500 | 0.0 |
| 38.3 | D2S1360 | 0.0 | 0.500 | 0.0 | 145.1 | D2S1334 | 0.1 | 0.402 | 0.0 |
| 38.9 | D2S305 | 0.0 | 0.500 | 0.0 | 147.4 | D2S442 | 0.0 | 0.500 | 0.0 |
| 47.4 | D2S165 | 0.0 | 0.500 | 0.0 | 152.0 | D2S151 | 1.6 | 0.102 | 0.3 |
| 48.0 | D2S405 | 0.0 | 0.467 | 0.1 | 152.0 | D2S1399 | 0.0 | 0.500 | 0.0 |
| 55.0 | D2S367 | 0.0 | 0.500 | 0.0 | 161.3 | D2S142 | 2.1 | 0.073 | 0.2 |
| 55.5 | D2S1788 | 0.0 | 0.470 | 0.0 | 164.5 | D2S1353 | 0.0 | 0.500 | 0.0 |
| 64.3 | D2S1356 | 0.0 | 0.500 | 0.0 | 173.0 | D2S1776 | 0.0 | 0.500 | 0.0 |
| 70.3 | D2S391 | 0.0 | 0.500 | 0.0 | 177.5 | D2S326 | 8.2 | 0.002 | 0.4 |
| 73.6 | D2S2739 | 0.0 | 0.500 | 0.0 | 186.2 | D2S1391 | 0.0 | 0.500 | 0.0 |
| 79.0 | D2S441 | 0.0 | 0.500 | 0.0 | 186.2 | D2S364 | 0.0 | 0.500 | 0.0 |
| 80.7 | D2S337 | 0.0 | 0.500 | 0.0 | 194.5 | D2S117 | 0.0 | 0.500 | 0.0 |
| 86.0 | D2S285 | 0.0 | 0.500 | 0.0 | 200.4 | D2S1384 | 2.4 | 0.618 | 0.2 |
| 90.8 | D2S1394 | 0.9 | 0.173 | 0.2 | 204.5 | D2S325 | 0.0 | 0.500 | 0.0 |
| 94.1 | D2S286 | 0.0 | 0.500 | 0.0 | 210.4 | GATA30E06 | 0.0 | 0.500 | 0.0 |
| 99.4 | D2S1777 | 0.5 | 0.230 | 0.3 | 214.7 | D2S164 | 0.0 | 0.500 | 0.0 |
| 101.6 | D2S139 | 0.0 | 0.500 | 0.0 | 215.8 | D2S434 | 0.0 | 0.500 | 0.0 |
| 104.0 | D2S1790 | 0.0 | 0.500 | 0.0 | 221.1 | D2S126 | 0.0 | 0.500 | 0.0 |
| 111.2 | D2S113 | 0.0 | 0.500 | 0.0 | 227.0 | D2S1363 | 0.0 | 0.500 | 0.0 |
| | | | | | 232.9 | D2S396 | 0.0 | 0.500 | 0.0 |
| | | | | | 236.7 | D2S427 | 0.0 | 0.500 | 0.0 |
| | | | | | 240.8 | D2S206 | 0.0 | 0.500 | 0.0 |
| | | | | | 250.5 | D2S338 | 3.7 | 0.027 | 0.3 |
| | | | | | 251.9 | GATA178G09 | 0.0 | 0.500 | 0.0 |
| | | | | | 260.6 | D2S125 | 0.0 | 0.500 | 0.0 |

**Table 8-3 Chromosome 3 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 5.5 | D3S2387 | 0.0 | 0.415 | 0.0 | 102.6 | D3S2406 | 6.4 | 0.006 | 0.3 |
| 8.3 | D3S1297 | 0.0 | 0.500 | 0.0 | 112.4 | GATA128C02 | 0.1 | 0.410 | 0.0 |
| 22.3 | D3S1304 | 0.0 | 0.500 | 0.0 | 117.8 | D3S1271 | 6.1 | 0.007 | 0.5 |

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 119.1 | D3S2459 | 0.0 | 0.500 | 0.0 |
| 26.3 | GATA164B08 | 1.7 | 0.393 | 0.4 | 129.7 | D3S1278 | 0.0 | 0.500 | 0.0 |
| 36.1 | D3S1263 | 0.0 | 0.500 | 0.0 | 134.6 | D3S2460 | 0.0 | 0.441 | 0.0 |
| 44.8 | D3S3038 | 0.0 | 0.500 | 0.0 | 139.1 | D3S1267 | 1.3 | 0.125 | 0.3 |
| 44.8 | D3S1293 | 0.0 | 0.500 | 0.0 | 146.6 | D3S1292 | 0.5 | 0.229 | 0.2 |
| 52.6 | D3S1266 | 0.0 | 0.500 | 0.0 | 152.6 | D3S1764 | 0.0 | 0.500 | 0.0 |
| 57.9 | D3S2432 | 1.0 | 0.157 | 0.2 | 158.4 | D3S1569 | 0.0 | 0.500 | 0.0 |
| 61.5 | D3S1768 | 0.0 | 0.484 | 0.0 | 161.0 | D3S1744 | 0.0 | 0.500 | 0.0 |
| 62.1 | D3S1298 | 0.0 | 0.500 | 0.0 | 176.5 | D3S1763 | 0.0 | 0.500 | 0.0 |
| 70.6 | D3S2409 | 0.0 | 0.500 | 0.0 | 177.8 | D3S1614 | 0.0 | 0.480 | 0.0 |
| 71.4 | D3S1289 | 0.0 | 0.500 | 0.0 | 181.9 | D3S3053 | 0.5 | 0.200 | 0.2 |
| 78.6 | D3S1766 | 0.0 | 0.500 | 0.0 | 186.0 | D3S1565 | 1.5 | 0.107 | 0.2 |
| 80.3 | D3S1300 | 0.1 | 0.362 | 0.1 | 188.3 | D3S2427 | 0.0 | 0.500 | 0.0 |
| 89.9 | GATA148E04 | 0.0 | 0.500 | 0.0 | 201.1 | D3S1262 | 0.0 | 0.500 | 0.0 |
| 91.2 | D3S1285 | 3.2 | 0.038 | 0.3 | 201.1 | D3S1262 | 0.0 | 0.500 | 0.0 |
| 97.8 | D3S1566 | 0.0 | 0.500 | 0.0 | 207.7 | D3S1580 | 0.0 | 0.500 | 0.0 |
| | | | | | 209.4 | D3S2398 | 0.0 | 0.500 | 0.0 |
| | | | | | 215.8 | D3S2418 | 0.0 | 0.500 | 0.0 |
| | | | | | 224.9 | D3S1311 | 0.9 | 0.175 | 0.3 |

**Table 8-4 Chromosome 4 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 4.7 | D4S412 | 4.9 | 0.014 | 0.4 | 104.9 | D4S1647 | 1.2 | 0.142 | 0.2 |
| 12.9 | D4S2366 | 0.0 | 0.500 | 0.0 | 108.0 | D4S1572 | 0.0 | 0.500 | 0.0 |
| 25.9 | D4S403 | 5.1 | 0.012 | 0.6 | 114.0 | D4S2623 | 0.0 | 0.500 | 0.0 |
| 33.0 | D4S2639 | 3.1 | 0.040 | 0.3 | 124.5 | D4S402 | 0.0 | 0.500 | 0.0 |
| 33.4 | D4S419 | 1.2 | 0.100 | 0.2 | 131.0 | D4S2394 | 0.0 | 0.500 | 0.0 |
| 42.7 | D4S2397 | 0.0 | 0.500 | 0.0 | 132.1 | D4S1575 | 0.0 | 0.500 | 0.0 |
| 43.6 | D4S391 | 0.0 | 0.500 | 0.0 | 143.3 | D4S1644 | 0.0 | 0.500 | 0.0 |
| 52.0 | D4S2632 | 0.0 | 0.500 | 0.0 | 144.6 | D4S424 | 0.0 | 0.500 | 0.0 |
| 57.0 | D4S405 | 0.0 | 0.500 | 0.0 | 146.0 | D4S1625 | 0.6 | 0.220 | 0.2 |
| 60.2 | D4S1627 | 0.0 | 0.500 | 0.0 | 158.0 | D4S413 | 0.0 | 0.500 | 0.0 |
| 64.2 | D4S428 | 0.0 | 0.500 | 0.0 | 158.0 | D4S1629 | 0.0 | 0.500 | 0.0 |
| 72.5 | D4S398 | 0.0 | 0.500 | 0.0 | 167.6 | D4S2368 | 0.0 | 0.500 | 0.0 |
| 72.5 | D4S3248 | 0.0 | 0.500 | 0.0 | 169.4 | D4S1597 | 2.5 | 0.060 | 0.5 |
| 78.4 | D4S2367 | 5.7 | 0.008 | 0.3 | 176.2 | D4S2431 | 0.0 | 0.500 | 0.0 |

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 79.0 | D4S392 | 0.0 | 0.464 | 0.0 | 181.4 | D4S415 | 1.3 | 0.128 | 0.3 |
| 90.0 | D4S3243 | 0.0 | 0.500 | 0.0 | 181.9 | D4S2417 | 0.0 | 0.500 | 0.0 |
| 93.5 | D4S2361 | 0.0 | 0.500 | 0.0 | 195.1 | D4S408 | 0.1 | 0.366 | 0.2 |
| 95.1 | D4S1534 | 0.0 | 0.500 | 0.0 | 195.1 | D4S1535 | 0.0 | 0.500 | 0.0 |
| 100.8 | D4S414 | 0.0 | 0.500 | 0.0 | 207.0 | D4S426 | 0.0 | 0.500 | 0.0 |
| | | | | | 208.1 | D4S1652 | 4.7 | 0.015 | 0.4 |

**Table 8-5 Chromosome 5 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 0.0 | D5S2488 | 1.5 | 0.113 | 0.3 | 95.4 | D5S428 | 0.7 | 0.194 | 0.2 |
| 7.8 | GATA145D10 | 0.2 | 0.315 | 0.1 | 97.8 | D5S1725 | 1.0 | 0.162 | 0.1 |
| 11.9 | D5S406 | 0.0 | 0.500 | 0.0 | 104.8 | D5S644 | 2.3 | 0.060 | 0.2 |
| 14.3 | D5S2505 | 5.3 | 0.011 | 0.4 | 112.0 | D5S433 | 0.0 | 0.500 | 0.0 |
| 19.0 | D5S807 | 0.0 | 0.500 | 0.0 | 113.0 | D5S1453 | 0.0 | 0.500 | 0.0 |
| 19.7 | D5S630 | 0.0 | 0.500 | 0.0 | 117.0 | D5S2501 | 0.0 | 0.435 | 0.0 |
| 22.9 | D5S817 | 0.0 | 0.497 | 0.0 | 122.0 | D5S421 | 1.8 | 0.092 | 0.3 |
| 28.8 | D5S416 | 0.5 | 0.248 | 0.2 | 129.8 | D5S1505 | 0.3 | 0.304 | 0.2 |
| 40.0 | D5S419 | 0.0 | 0.500 | 0.0 | 129.8 | D5S471 | 0.0 | 0.500 | 0.0 |
| 40.0 | GATA145D09 | 0.0 | 0.500 | 0.0 | 139.3 | D5S816 | 0.1 | 0.378 | 0.2 |
| 45.3 | D5S1470 | 0.0 | 0.500 | 0.0 | 140.7 | D5S393 | 0.0 | 0.500 | 0.0 |
| 52.0 | D5S426 | 0.0 | 0.500 | 0.0 | 147.5 | D5S436 | 0.0 | 0.500 | 0.0 |
| 58.6 | D5S418 | 0.0 | 0.500 | 0.0 | 147.5 | D5S1480 | 0.5 | 0.230 | 0.2 |
| 59.3 | D5S1457 | 0.0 | 0.500 | 0.0 | 155.9 | D5S673 | 0.0 | 0.500 | 0.0 |
| 64.7 | D5S407 | 0.0 | 0.500 | 0.0 | 159.8 | D5S820 | 0.0 | 0.500 | 0.0 |
| 69.2 | D5S2500 | 0.0 | 0.500 | 0.0 | 164.2 | D5S422 | 0.0 | 0.500 | 0.0 |
| 74.1 | D5S647 | 0.2 | 0.300 | 0.2 | 172.1 | D5S1471 | 0.0 | 0.500 | 0.0 |
| 82.0 | D5S424 | 0.0 | 0.500 | 0.0 | 174.8 | D5S1456 | 0.0 | 0.500 | 0.0 |
| 85.3 | D5S1501 | 0.0 | 0.477 | 0.0 | 174.8 | D5S400 | 0.0 | 0.500 | 0.0 |
| | | | | | 179.1 | D5S429 | 0.0 | 0.500 | 0.0 |
| | | | | | 182.9 | D5S211 | 0.0 | 0.500 | 0.0 |
| | | | | | 195.5 | D5S408 | 3.8 | 0.026 | 0.3 |

**Table 8-6 Chromosome 6 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 1.4 | D6S344 | 0.0 | 0.500 | 0.0 | 79.9 | D6S257 | 0.0 | 0.500 | 0.0 |
| 9.2 | F13A1 | 0.0 | 0.500 | 0.0 | 80.5 | D6S1053 | 0.0 | 0.500 | 0.0 |

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 14.1 | D6S309 | 0.0 | 0.500 | 0.0 | 99.0 | D6S462 | 0.0 | 0.500 | 0.0 |
| 18.2 | D6S470 | 0.3 | 0.300 | 0.2 | 102.8 | D6S1056 | 0.0 | 0.500 | 0.0 |
| 29.9 | D6S289 | 0.0 | 0.500 | 0.0 | 109.2 | D6S434 | 0.0 | 0.498 | 0.0 |
| 34.2 | D6S1959 | 0.9 | 0.177 | 0.3 | 112.2 | D6S1021 | 0.0 | 0.500 | 0.0 |
| 35.7 | D6S422 | 0.0 | 0.500 | 0.0 | 118.6 | D6S474 | 1.8 | 0.090 | 0.2 |
| 42.3 | GATA163B10 | 0.0 | 0.500 | 0.0 | 122.0 | D6S287 | 0.0 | 0.500 | 0.0 |
| 44.4 | D6S276 | 0.0 | 0.500 | 0.0 | 128.9 | D6S1040 | 0.0 | 0.500 | 0.0 |
| 53.8 | GGAA15B08 | 0.0 | 0.500 | 0.0 | 130.0 | D6S262 | 0.0 | 0.500 | 0.0 |
| 60.4 | D6S426 | 0.0 | 0.500 | 0.0 | 137.0 | D6S292 | 0.0 | 0.500 | 0.0 |
| 63.3 | D6S1017 | 0.0 | 0.500 | 0.0 | 137.7 | D6S1009 | 0.0 | 0.500 | 0.0 |
| 66.4 | D6S271 | 0.0 | 0.500 | 0.0 | 144.5 | D6S308 | 0.0 | 0.500 | 0.0 |
| 73.1 | GATA11E02 | 0.0 | 0.500 | 0.0 | 146.1 | GATA184A08 | 0.0 | 0.486 | 0.0 |
| | | | | | 164.8 | D6S1581 | 0.0 | 0.500 | 0.0 |
| | | | | | 173.3 | D6S1277 | 0.0 | 0.500 | 0.0 |
| | | | | | 179.1 | D6S264 | 0.0 | 0.500 | 0.0 |
| | | | | | 187.2 | D6S1027 | 0.0 | 0.500 | 0.0 |
| | | | | | 190.1 | D6S281 | 4.1 | 0.022 | 0.2 |

**Table 8-7 Chromosome 7 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 5.3 | D7S531 | 0.0 | 0.500 | 0.0 | 91.0 | D7S2204 | 0.0 | 0.500 | 0.0 |
| 7.4 | D7S1819 | 0.0 | 0.486 | 0.0 | 98.4 | D7S820 | 0.0 | 0.500 | 0.0 |
| 7.4 | D7S517 | 0.0 | 0.500 | 0.0 | 109.1 | D7S821 | 0.0 | 0.500 | 0.0 |
| 17.7 | D7S513 | 0.0 | 0.500 | 0.0 | 112.3 | D7S515 | 0.0 | 0.500 | 0.0 |
| 28.7 | D7S507 | 0.0 | 0.500 | 0.0 | 113.9 | D7S1799 | 0.0 | 0.500 | 0.0 |
| 29.3 | GATA137H02 | 0.0 | 0.500 | 0.0 | 124.1 | D7S486 | 0.0 | 0.500 | 0.0 |
| 33.0 | D7S1802 | 0.1 | 0.352 | 0.1 | 128.4 | D7S1842 | 0.0 | 0.500 | 0.0 |
| 34.7 | D7S493 | 1.3 | 0.100 | 0.3 | 134.6 | D7S530 | 0.0 | 0.500 | 0.0 |
| 41.7 | D7S1808 | 0.0 | 0.500 | 0.0 | 137.0 | D7S1804 | 0.0 | 0.500 | 0.0 |
| 50.3 | D7S817 | 0.0 | 0.500 | 0.0 | 137.8 | D7S640 | 1.8 | 0.090 | 0.3 |
| 53.5 | D7S484 | 0.0 | 0.500 | 0.0 | 147.2 | D7S684 | 0.0 | 0.500 | 0.0 |
| 57.8 | D7S2846 | 2.8 | 0.048 | 0.4 | 149.9 | D7S1824 | 0.0 | 0.500 | 0.0 |
| 59.9 | D7S510 | 0.0 | 0.500 | 0.0 | 154.0 | D7S2195 | 0.0 | 0.500 | 0.0 |
| 69.0 | D7S519 | 0.0 | 0.500 | 0.0 | 162.3 | D7S636 | 0.0 | 0.500 | 0.0 |
| 69.6 | D7S1818 | 0.0 | 0.495 | 0.0 | 163.0 | GATA189C06 | 0.0 | 0.468 | 0.0 |
| 78.7 | GATA118G10 | 0.0 | 0.500 | 0.0 | 173.7 | D7S1823 | 0.0 | 0.500 | 0.0 |
| 90.4 | D7S669 | 0.8 | 0.192 | 0.3 | 178.4 | D7S550 | 0.0 | 0.500 | 0.0 |

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 182.0 | D7S559 | 0.0 | 0.500 | 0.0 |

**Table 8-8 Chromosome 8 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 0.7 | D8S264 | 0.0 | 0.500 | 0.0 | 82.3 | D8S1136 | 0.0 | 0.463 | 0.0 |
| 8.3 | D8S277 | 0.0 | 0.500 | 0.0 | 91.5 | D8S279 | 0.0 | 0.500 | 0.0 |
| | | | | | 94.1 | GATA14E09 | 0.0 | 0.496 | 0.0 |
| 21.3 | D8S550 | 0.0 | 0.500 | 0.0 | 101.0 | D8S1119 | 0.0 | 0.500 | 0.0 |
| 22.4 | D8S1130 | 0.0 | 0.500 | 0.0 | 103.7 | D8S270 | 0.0 | 0.500 | 0.0 |
| 26.4 | D8S1106 | 0.0 | 0.500 | 0.0 | 110.2 | GAAT1A4 | 0.0 | 0.500 | 0.0 |
| 37.0 | D8S1145 | 0.0 | 0.500 | 0.0 | 118.2 | D8S556 | 0.0 | 0.500 | 0.1 |
| 41.6 | D8S258 | 0.0 | 0.500 | 0.0 | 120.0 | D8S1132 | 0.0 | 0.500 | 0.0 |
| 60.3 | D8S1477 | 0.0 | 0.500 | 0.0 | 125.3 | D8S592 | 0.0 | 0.500 | 0.1 |
| 60.9 | D8S283 | 0.0 | 0.466 | 0.0 | 130.0 | D8S514 | 0.6 | 0.200 | 0.3 |
| 67.3 | D8S1110 | 0.0 | 0.431 | 0.0 | 135.1 | D8S1179 | 0.0 | 0.500 | 0.0 |
| 71.0 | D8S285 | 0.0 | 0.500 | 0.0 | 139.5 | D8S1128 | 0.0 | 0.500 | 0.0 |
| 77.9 | D8S1113 | 0.0 | 0.500 | 0.0 | 143.8 | D8S284 | 0.2 | 0.346 | 0.2 |
| 79.4 | D8S260 | 0.0 | 0.500 | 0.0 | 148.1 | D8S256 | 0.0 | 0.500 | 0.0 |
| | | | | | 154.0 | D8S272 | 0.0 | 0.500 | 0.0 |
| | | | | | 164.5 | D8S373 | 0.0 | 0.500 | 0.0 |

**Table 8-9 Chromosome 9 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 9.8 | D9S288 | 2.1 | 0.072 | 0.3 | 80.3 | D9S922 | 0.0 | 0.500 | 0.0 |
| 14.2 | GATA62F03 | 0.0 | 0.500 | 0.0 | 83.4 | D9S167 | 0.0 | 0.500 | 0.0 |
| 18.1 | D9S286 | 0.0 | 0.500 | 0.0 | 91.9 | D9S257 | 0.0 | 0.500 | 0.0 |
| 21.9 | D9S921 | 0.0 | 0.500 | 0.0 | 94.9 | D9S283 | 0.0 | 0.500 | 0.0 |
| 32.2 | D9S157 | 0.0 | 0.500 | 0.0 | 103.4 | D9S287 | 0.0 | 0.500 | 0.0 |
| 38.0 | D9S925 | 0.0 | 0.500 | 0.0 | 104.5 | D9S910 | 0.0 | 0.500 | 0.0 |
| 42.7 | D9S171 | 0.0 | 0.500 | 0.0 | 120.0 | D9S930 | 3.0 | 0.041 | 0.2 |
| 44.3 | D9S1121 | 2.4 | 0.060 | 0.3 | 120.0 | D9S279 | 0.0 | 0.500 | 0.0 |
| 51.8 | D9S161 | 0.6 | 0.216 | 0.3 | 128.0 | D9S934 | 0.0 | 0.500 | 0.0 |
| 58.3 | D9S1118 | 0.0 | 0.500 | 0.0 | 136.5 | D9S282 | 0.0 | 0.500 | 0.0 |
| 65.8 | D9S273 | 0.0 | 0.500 | 0.0 | 140.9 | D9S290 | 0.0 | 0.500 | 0.0 |
| 66.3 | D9S301 | 0.0 | 0.500 | 0.0 | 146.0 | ATA59H06 | 8.5 | 0.002 | 0.3 |
| 70.3 | D9S175 | 0.4 | 0.268 | 0.3 | 147.9 | D9S164 | 0.0 | 0.500 | 0.0 |

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 75.9 | D9S1122 | 0.0 | 0.500 | 0.0 | 161.7 | D9S158 | 0.7 | 0.200 | 0.4 |
| | | | | | | D9S158 | 0.4 | 0.256 | 0.3 |

**Table 8-10 Chromosome 10 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 0.0 | D10S1435 | 0.8 | 0.186 | 0.3 | 93.9 | D10S1432 | 1.9 | 0.083 | 0.2 |
| 2.1 | D10S249 | 0.0 | 0.500 | 0.0 | 100.9 | D10S2327 | 0.0 | 0.500 | 0.0 |
| 13.5 | D10S591 | 0.0 | 0.500 | 0.0 | 101.8 | D10S201 | 0.0 | 0.500 | 0.0 |
| 19.0 | D10S189 | 0.0 | 0.500 | 0.0 | 112.6 | GATA115E01 | 0.0 | 0.500 | 0.0 |
| 28.3 | D10S1412 | 0.0 | 0.500 | 0.0 | 115.3 | D10S583 | 0.0 | 0.500 | 0.0 |
| 29.2 | D10S547 | 1.5 | 0.100 | 0.3 | 124.3 | D10S192 | 0.0 | 0.500 | 0.0 |
| 37.9 | D10S191 | 0.0 | 0.500 | 0.0 | 126.0 | D10S1239 | 0.0 | 0.500 | 0.0 |
| 45.7 | D10S548 | 0.0 | 0.500 | 0.0 | 128.7 | D10S597 | 0.0 | 0.500 | 0.0 |
| 49.0 | D10S1423 | 0.0 | 0.500 | 0.0 | 134.7 | D10S1237 | 0.0 | 0.500 | 0.0 |
| 59.0 | D10S1426 | 0.0 | 0.500 | 0.0 | 138.5 | D10S190 | 0.0 | 0.500 | 0.0 |
| 60.6 | D10S208 | 0.0 | 0.500 | 0.0 | 142.8 | D10S1230 | 0.0 | 0.500 | 0.0 |
| 63.3 | D10S1208 | 0.0 | 0.500 | 0.0 | 147.6 | D10S587 | 0.0 | 0.500 | 0.0 |
| 70.2 | D10S220 | 1.4 | 0.117 | 0.3 | 148.2 | D10S1213 | 0.0 | 0.470 | 0.0 |
| 77.0 | D10S1221 | 0.0 | 0.500 | 0.0 | 157.9 | D10S217 | 0.0 | 0.500 | 0.0 |
| 80.8 | D10S561 | 0.0 | 0.500 | 0.0 | 165.3 | D10S1248 | 2.2 | 0.068 | 0.2 |
| 88.4 | GATA121A08 | 0.0 | 0.480 | 0.0 | 170.9 | D10S212 | 0.0 | 0.500 | 0.0 |

**Table 8-11 Chromosome 11 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 2.1 | D11S1984 | 7.7 | 0.003 | 0.3 | 80.0 | D11S937 | 1.1 | 0.146 | 0.3 |
| 8.9 | D11S2362 | 0.0 | 0.500 | 0.0 | 85.5 | D11S901 | 0.0 | 0.500 | 0.0 |
| 12.9 | D11S1338 | 0.0 | 0.500 | 0.0 | 91.5 | D11S1358 | 0.0 | 0.500 | 0.0 |
| 17.2 | D11S1999 | 0.0 | 0.500 | 0.0 | 99.0 | D11S898 | 1.5 | 0.109 | 0.3 |
| 21.5 | D11S1981 | 2.1 | 0.074 | 0.3 | 100.6 | D11S2000 | 0.2 | 0.322 | 0.2 |
| 21.5 | D11S902 | 0.0 | 0.500 | 0.0 | 105.7 | D11S1986 | 0.0 | 0.500 | 0.0 |
| 33.0 | ATA34E08 | 2.4 | 0.062 | 0.3 | 108.6 | D11S908 | 4.7 | 0.015 | 0.5 |
| 33.6 | D11S904 | 0.0 | 0.500 | 0.0 | 113.1 | D11S1998 | 0.0 | 0.500 | 0.0 |
| 43.2 | D11S1392 | 0.0 | 0.500 | 0.0 | 117.2 | D11S925 | 4.9 | 0.010 | 0.3 |
| 45.9 | D11S935 | 0.0 | 0.500 | 0.0 | 120.9 | D11S1345 | 0.0 | 0.500 | 0.0 |
| 52.0 | D11S905 | 2.6 | 0.050 | 0.2 | 123.0 | D11S4464 | 0.0 | 0.497 | 0.0 |
| 58.4 | D11S1985 | 0.0 | 0.500 | 0.0 | 126.2 | D11S934 | 1.5 | 0.110 | 0.5 |
| 60.1 | D11S4191 | 0.0 | 0.500 | 0.0 | 127.3 | D11S4151 | 0.0 | 0.500 | 0.0 |

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 67.5 | D11S987 | 0.0 | 0.500 | 0.0 | 131.3 | D11S912 | 0.0 | 0.500 | 0.0 |
| 73.6 | D11S1314 | 0.0 | 0.500 | 0.0 | 141.9 | D11S1320 | 0.6 | 0.220 | 0.4 |
| 76.1 | D11S2371 | 0.0 | 0.486 | 0.0 | 147.8 | D11S968 | 0.0 | 0.500 | 0.0 |

**Table 8-12 Chromosome 12 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 0.0 | D12S352 | 0.0 | 0.500 | 0.0 | 83.2 | D12S1052 | 0.0 | 0.500 | 0.0 |
| 6.4 | D12S372 | 0.0 | 0.500 | 0.0 | 86.4 | D12S326 | 0.0 | 0.500 | 0.0 |
| 12.6 | D12S99 | 0.0 | 0.500 | 0.0 | 95.0 | D12S1064 | 0.0 | 0.500 | 0.0 |
| 17.7 | GATA49D12 | 0.0 | 0.500 | 0.0 | 95.6 | D12S351 | 0.0 | 0.500 | 0.0 |
| 19.7 | D12S336 | 0.0 | 0.500 | 0.0 | 103.5 | D12S1300 | 0.0 | 0.500 | 0.0 |
| 26.2 | D12S391 | 0.0 | 0.500 | 0.0 | 104.7 | D12S346 | 0.0 | 0.500 | 0.0 |
| 30.6 | D12S364 | 0.0 | 0.500 | 0.0 | 109.5 | PAH | 0.0 | 0.500 | 0.0 |
| 36.1 | D12S310 | 0.0 | 0.500 | 0.0 | 111.9 | D12S78 | 0.0 | 0.500 | 0.0 |
| 36.1 | D12S373 | 2.3 | 0.068 | 0.3 | 125.3 | D12S79 | 0.0 | 0.500 | 0.0 |
| 48.7 | D12S1042 | 0.5 | 0.250 | 0.1 | 125.3 | D12S2070 | 0.0 | 0.500 | 0.0 |
| 53.1 | D12S345 | 0.0 | 0.500 | 0.0 | 134.5 | D12S86 | 0.0 | 0.500 | 0.0 |
| 56.3 | D12S1301 | 0.0 | 0.500 | 0.0 | 136.8 | D12S395 | 0.0 | 0.500 | 0.0 |
| 61.3 | D12S85 | 0.0 | 0.500 | 0.0 | 147.2 | D12S324 | 0.0 | 0.500 | 0.0 |
| 66.0 | D12S368 | 0.0 | 0.500 | 0.0 | 148.0 | D12S2078 | 0.0 | 0.500 | 0.0 |
| 75.0 | D12S1294 | 0.0 | 0.500 | 0.0 | 159.6 | D12S367 | 0.0 | 0.500 | 0.0 |
| 75.2 | D12S83 | 0.0 | 0.500 | 0.0 | 160.7 | D12S1045 | 1.1 | 0.143 | 0.3 |
| 80.5 | D12S375 | 6.2 | 0.007 | 0.5 | 165.7 | D12S392 | 0.0 | 0.500 | 0.0 |

**Table 8-13 Chromosome 13 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 6.0 | D13S175 | 0.0 | 0.500 | 0.0 | 55.9 | D13S156 | 4.4 | 0.018 | 0.5 |
| 8.9 | D13S787 | 4.5 | 0.017 | 0.4 | 63.9 | D13S170 | 1.4 | 0.100 | 0.2 |
| 17.2 | D13S217 | 0.0 | 0.500 | 0.0 | 63.9 | D13S317 | 0.0 | 0.500 | 0.0 |
| 25.1 | D13S171 | 4.8 | 0.014 | 0.5 | 68.7 | D13S265 | 3.9 | 0.030 | 0.3 |
| 25.8 | D13S1493 | 0.0 | 0.480 | 0.0 | 79.5 | D13S159 | 0.0 | 0.500 | 0.0 |
| 31.4 | D13S894 | 0.0 | 0.500 | 0.0 | 81.0 | D13S793 | 0.0 | 0.500 | 0.0 |
| 38.3 | D13S263 | 0.0 | 0.500 | 0.0 | 82.9 | D13S779 | 1.7 | 0.098 | 0.3 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 39.0 | D13S325 | 0.0 | 0.500 | 0.0 | | 84.9 | D13S158 | 0.0 | 0.500 | 0.0 |
| 45.6 | D13S153 | 0.0 | 0.500 | 0.0 | | 93.5 | D13S173 | 0.0 | 0.500 | 0.0 |
| 45.6 | D13S788 | 0.0 | 0.500 | 0.0 | | 93.5 | D13S796 | 0.0 | 0.500 | 0.0 |
| 55.3 | D13S800 | 0.0 | 0.500 | 0.0 | | 110.6 | D13S285 | 0.0 | 0.500 | 0.0 |

**Table 8-14 Chromosome 14 Guam association study results**

| cM | Locus | LRT | p | λ | | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|---|
| 6.46 | D14S261 | 0.000 | 0.5000 | 0.0000 | | 69.18 | D14S1026 | 0.000 | 0.5000 | 0.0000 |
| 12.46 | D14S742 | 0.142 | 0.3531 | 0.0607 | | 69.18 | D14S1024 | 0.000 | 0.5000 | 0.0000 |
| 13.89 | D14S283 | 0.000 | 0.5000 | 0.0000 | | 69.18 | D14S63 | 3.596 | 0.0290 | 0.4332 |
| 25.87 | D14S1280 | 8.036 | 0.0230 | 0.4619 | | 69.82 | D14S1046 | 0.000 | 0.5000 | 0.0000 |
| 26.59 | D14S80 | 1.724 | 0.0946 | 0.3213 | | 69.82 | D14S57 | 0.000 | 0.5000 | 0.0000 |
| 28.01 | D14S608 | 0.000 | 0.5000 | 0.0000 | | 73.03 | D14S1069 | 0.000 | 0.5000 | 0.0000 |
| 40.11 | D14S70 | 0.042 | 0.4186 | 0.0409 | | 75.61 | D14S588 | 0.000 | 0.5000 | 0.0000 |
| 40.68 | D14S599 | 0.000 | 0.5000 | 0.0000 | | 76.28 | D14S258 | 3.529 | 0.0302 | 0.3391 |
| 44.06 | D14S306 | 3.371 | 0.0332 | 0.3456 | | 86.29 | D14S53 | 0.000 | 0.5000 | 0.0000 |
| 47.51 | D14S288 | 0.000 | 0.5000 | 0.0000 | | 87.36 | D14S74 | 0.000 | 0.5000 | 0.0000 |
| 55.82 | D14S587 | 0.000 | 0.5000 | 0.0000 | | 91.62 | D14S606 | 2.004 | 0.0780 | 0.2570 |
| 56.36 | D14S276 | 0.000 | 0.5000 | 0.0000 | | 95.89 | D14S1279 | 0.000 | 0.5000 | 0.0000 |
| 57.98 | D14S1056 | 0.000 | 0.4875 | 0.0145 | | 95.89 | D14S68 | 0.000 | 0.5000 | 0.0000 |
| 60.43 | D14S980 | 0.000 | 0.5000 | 0.0000 | | 105.00 | D14S280 | 0.000 | 0.5000 | 0.0000 |
| 63.25 | D14S274 | 0.000 | 0.5000 | 0.0000 | | 105.53 | D14S617 | 0.000 | 0.5000 | 0.0000 |
| 66.81 | D14S592 | 11.409 | 0.0004 | 0.4966 | | 113.17 | GATA168F06 | 0.004 | 0.4733 | 0.0199 |
| 67.99 | D14S997 | 0.000 | 0.5000 | 0.0000 | | 117.30 | D14S65 | 0.000 | 0.5000 | 0.0000 |
| 68.59 | D14S1059 | 0.000 | 0.5000 | 0.0000 | | 125.88 | D14S78 | 0.000 | 0.5000 | 0.0000 |
| | | | | | | 125.88 | GATA136B01 | 0.044 | 0.4170 | 0.0360 |
| | | | | | | 134.30 | D14S292 | 0.000 | 0.5000 | 0.0000 |

**Table 8-15 Chromosome 15 Guam association study results**

| cM | Locus | LRT | p | λ | | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|---|
| 6.1 | D15S128 | 0.0 | 0.500 | 0.0 | | 75.9 | D15S211 | 0.0 | 0.500 | 0.0 |
| 20.2 | D15S165 | 2.4 | 0.062 | 0.5 | | 78.9 | D15S205 | 0.0 | 0.500 | 0.0 |
| 31.5 | ACTC | 0.0 | 0.500 | 0.0 | | 82.8 | D15S655 | 1.0 | 0.153 | 0.2 |
| 43.5 | D15S659 | 5.1 | 0.012 | 0.3 | | 86.8 | D15S127 | 0.0 | 0.500 | 0.0 |
| 45.6 | D15S126 | 0.0 | 0.500 | 0.0 | | 90.0 | D15S652 | 0.0 | 0.478 | 0.0 |
| 51.2 | D15S117 | 0.0 | 0.500 | 0.0 | | 100.6 | D15S816 | 0.0 | 0.500 | 0.0 |

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 52.3 | D15S643 | 0.0 | 0.500 | 0.0 | 100.6 | D15S130 | 0.0 | 0.500 | 0.0 |
| 60.2 | GATA151F03 | 0.0 | 0.476 | 0.0 | 104.9 | D15S657 | 0.5 | 0.232 | 0.2 |
| 62.4 | D15S153 | 0.0 | 0.500 | 0.0 | 112.6 | D15S120 | 0.0 | 0.500 | 0.0 |
| 71.3 | D15S131 | 0.0 | 0.500 | 0.0 | 122.1 | D15S642 | 0.0 | 0.500 | 0.0 |

**Table 8-16 Chromosome 16 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 10.4 | D16S423 | 0.0 | 0.500 | 0.0 | 67.6 | D16S415 | 0.0 | 0.500 | 0.0 |
| 11.5 | D16S2616 | 0.0 | 0.500 | 0.0 | 71.8 | D16S3253 | 0.0 | 0.500 | 0.0 |
| 18.1 | D16S407 | 0.0 | 0.500 | 0.0 | 81.2 | D16S2620 | 0.0 | 0.500 | 0.0 |
| 18.1 | D16S404 | 1.3 | 0.100 | 0.2 | 83.6 | D16S503 | 0.0 | 0.500 | 0.0 |
| 22.7 | D16S748 | 0.0 | 0.500 | 0.0 | 87.6 | D16S2624 | 6.2 | 0.007 | 0.5 |
| 28.3 | D16S405 | 4.3 | 0.019 | 0.4 | 92.1 | D16S515 | 0.0 | 0.500 | 0.0 |
| 30.0 | D16S764 | 0.0 | 0.500 | 0.0 | 100.4 | D16S516 | 0.3 | 0.307 | 0.3 |
| 40.7 | D16S3046 | 0.0 | 0.500 | 0.0 | 110.4 | D16S511 | 0.0 | 0.500 | 0.0 |
| 43.9 | D16S403 | 1.3 | 0.124 | 0.3 | 113.5 | D16S402 | 0.0 | 0.500 | 0.0 |
| 44.5 | D16S420 | 0.0 | 0.500 | 0.0 | 124.7 | D16S539 | 0.0 | 0.500 | 0.0 |
| 46.9 | D16S401 | 1.1 | 0.100 | 0.3 | 125.8 | D16S520 | 0.0 | 0.500 | 0.0 |
| 59.7 | D16S411 | 0.0 | 0.500 | 0.0 | 130.4 | D16S621 | 0.0 | 0.500 | 0.0 |

**Table 8-17 Chromosome 17 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 0.6 | D17S849 | 0.0 | 0.500 | 0.0 | 56.5 | D17S1293 | 0.0 | 0.500 | 0.0 |
| 0.6 | D17S1308 | 2.5 | 0.058 | 0.5 | 62.0 | D17S1299 | 1.2 | 0.139 | 0.3 |
| 10.7 | D17S1298 | 0.0 | 0.500 | 0.0 | 64.2 | D17S791 | 0.0 | 0.500 | 0.0 |
| 14.7 | D17S938 | 0.0 | 0.500 | 0.0 | 66.9 | ATC6A06 | 0.0 | 0.500 | 0.0 |
| 21.0 | D17S945 | 0.0 | 0.500 | 0.0 | 75.0 | D17S787 | 0.0 | 0.500 | 0.0 |
| 22.2 | D17S974 | 0.0 | 0.500 | 0.0 | 82.0 | D17S1290 | 0.0 | 0.500 | 0.0 |
| 22.2 | D17S1852 | 0.0 | 0.500 | 0.0 | 82.6 | D17S944 | 0.0 | 0.500 | 0.0 |
| 32.0 | D17S947 | 0.0 | 0.500 | 0.0 | 89.3 | ATA43A10 | 0.0 | 0.487 | 0.0 |
| 32.0 | D17S799 | 1.5 | 0.112 | 0.2 | 93.3 | D17S949 | 0.0 | 0.500 | 0.0 |
| 44.6 | GATA185H04 | 1.7 | 0.093 | 0.2 | 100.0 | D17S1301 | 0.0 | 0.500 | 0.0 |
| 49.7 | D17S925 | 0.0 | 0.494 | 0.0 | 106.8 | D17S802 | 0.0 | 0.500 | 0.0 |
| 50.7 | D17S1294 | 0.0 | 0.500 | 0.0 | 116.9 | D17S784 | 0.0 | 0.500 | 0.0 |
| 53.4 | D17S798 | 0.0 | 0.500 | 0.0 |  | D17S784 | 0.0 | 0.500 | 0.0 |

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 126.5 | D17S928 | 0.0 | 0.500 | 0.0 |

**Table 8-18 Chromosome 18 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 0.0 | D18S59 | 0.0 | 0.500 | 0.0 | 71.3 | D18S474 | 0.0 | 0.500 | 0.0 |
| 2.8 | GATA178F11 | 0.0 | 0.500 | 0.0 | 74.0 | D18S851 | 0.0 | 0.497 | 0.0 |
| 6.9 | D18S481 | 0.0 | 0.500 | 0.0 | 80.4 | D18S858 | 2.0 | 0.078 | 0.3 |
| 8.3 | D18S63 | 0.0 | 0.500 | 0.0 | 84.8 | D18S64 | 0.0 | 0.500 | 0.0 |
| 9.3 | D18S52 | 0.0 | 0.500 | 0.0 | 88.6 | D18S862 | 0.0 | 0.500 | 0.0 |
| 12.8 | D18S976 | 9.4 | 0.001 | 0.3 | 96.5 | D18S68 | 0.0 | 0.500 | 0.0 |
| 18.7 | D18S452 | 0.0 | 0.500 | 0.0 | 102.0 | D18S878 | 0.0 | 0.500 | 0.0 |
| 28.1 | D18S843 | 0.0 | 0.500 | 0.0 | 105.0 | D18S61 | 0.0 | 0.500 | 0.0 |
| 31.2 | D18S464 | 1.8 | 0.089 | 0.3 | 106.8 | ATA82B02 | 0.0 | 0.500 | 0.0 |
| 39.0 | D18S542 | 0.0 | 0.500 | 0.0 | 109.2 | D18S469 | 0.0 | 0.500 | 0.0 |
| 52.9 | D18S478 | 0.0 | 0.500 | 0.0 | 114.3 | D18S1161 | 0.0 | 0.500 | 0.0 |
| 54.4 | D18S877 | 0.0 | 0.500 | 0.0 | 115.9 | GATA177C03 | 0.0 | 0.500 | 0.0 |
| 62.8 | D18S57 | 0.0 | 0.500 | 0.0 | 116.4 | D18S844 | 0.0 | 0.471 | 0.0 |
| 64.5 | D18S535 | 0.0 | 0.500 | 0.0 | 120.1 | D18S462 | 1.5 | 0.114 | 0.4 |
| | | | | | 126.0 | D18S70 | 0.0 | 0.500 | 0.0 |

**Table 8-19 Chromosome 19 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 9.8 | D19S591 | 0.0 | 0.500 | 0.0 | 54.0 | D19S414 | 0.0 | 0.500 | 0.0 |
| 11.0 | D19S209 | 0.0 | 0.500 | 0.0 | 58.7 | D19S245 | 0.0 | 0.500 | 0.0 |
| 20.0 | D19S216 | 0.0 | 0.500 | 0.0 | 62.0 | D19S220 | 0.0 | 0.500 | 0.0 |
| 20.8 | D19S1034 | 2.5 | 0.057 | 0.3 | 66.3 | D19S420 | 0.0 | 0.500 | 0.0 |
| 32.9 | D19S586 | 0.0 | 0.500 | 0.0 | 78.1 | D19S246 | 0.0 | 0.500 | 0.0 |
| 32.9 | D19S714 | 0.0 | 0.500 | 0.0 | 87.7 | D19S589 | 0.5 | 0.224 | 0.2 |
| 36.2 | D19S221 | 0.0 | 0.500 | 0.0 | 92.6 | D19S418 | 0.0 | 0.500 | 0.0 |
| 42.3 | D19S226 | 0.0 | 0.495 | 0.0 | 100.0 | D19S210 | 0.0 | 0.500 | 0.0 |
| 51.9 | D19S433 | 0.0 | 0.500 | 0.0 | 100.6 | D19S254 | 0.0 | 0.500 | 0.0 |

**Table 8-20 Chromosome 20 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 2.1 | D20S103 | 7.6 | 0.003 | 0.6 | 39.3 | D20S470 | 6.9 | 0.004 | 0.3 |
| 2.8 | D20S117 | 0.0 | 0.500 | 0.0 | 45.0 | D20S477 | 0.0 | 0.500 | 0.0 |
| 9.0 | D20S482 | 0.0 | 0.472 | 0.0 | 50.8 | D20S195 | 1.8 | 0.089 | 0.4 |
| 11.0 | D20S889 | 0.4 | 0.300 | 0.5 | 54.1 | D20S478 | 1.0 | 0.162 | 0.1 |
| 16.7 | D20S95 | 0.0 | 0.500 | 0.0 | 55.7 | D20S107 | 1.0 | 0.161 | 0.3 |
| 21.2 | D20S115 | 3.1 | 0.040 | 1.0 | 61.8 | D20S119 | 0.0 | 0.500 | 0.0 |
| 24.7 | D20S851 | 0.0 | 0.500 | 0.0 | 66.2 | D20S178 | 0.0 | 0.500 | 0.0 |
| 30.6 | D20S189 | 0.0 | 0.500 | 0.0 | 75.0 | D20S196 | 0.0 | 0.500 | 0.0 |
| 32.3 | D20S186 | 0.0 | 0.500 | 0.0 | 79.9 | D20S480 | 0.0 | 0.500 | 0.0 |
| 32.9 | D20S604 | 0.0 | 0.500 | 0.0 | 84.8 | D20S100 | 0.5 | 0.235 | 0.1 |
| 39.3 | D20S118 | 0.3 | 0.307 | 0.3 | 95.7 | D20S171 | 0.0 | 0.500 | 0.0 |
| | | | | | 98.1 | D20S173 | 0.0 | 0.500 | 0.0 |

**Table 8-21 Chromosome 21 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 3.0 | D21S1432 | 0.0 | 0.500 | 0.0 | 35.5 | D21S1252 | 0.0 | 0.500 | 0.0 |
| 9.7 | D21S1256 | 2.2 | 0.070 | 0.4 | 36.8 | D21S1440 | 0.0 | 0.500 | 0.0 |
| 13.1 | D21S1437 | 0.0 | 0.500 | 0.0 | 40.5 | GATA188F04 | 0.0 | 0.500 | 0.0 |
| 19.4 | D21S1914 | 0.0 | 0.500 | 0.0 | 45.9 | D21S266 | 0.0 | 0.500 | 0.0 |
| 20.5 | D21S1253 | 0.0 | 0.500 | 0.0 | 57.8 | D21S1446 | 0.0 | 0.443 | 0.0 |
| 27.4 | D21S263 | 0.0 | 0.500 | 0.0 | 57.8 | GATA129D11 | 0.0 | 0.500 | 0.0 |

**Table 8-22 Chromosome 22 Guam association study results**

| cM | Locus | LRT | p | λ | cM | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 4.1 | D22S420 | 3.0 | 0.041 | 0.3 | 36.2 | D22S683 | 0.1 | 0.361 | 0.2 |
| 18.1 | GCT10C10 | 6.9 | 0.004 | 0.6 | 37.8 | D22S283 | 0.0 | 0.500 | 0.0 |
| 21.5 | D22S315 | 0.1 | 0.355 | 0.1 | 45.8 | D22S445 | 0.0 | 0.500 | 0.0 |
| 28.6 | D22S689 | 0.4 | 0.265 | 0.2 | 46.4 | D22S423 | 0.0 | 0.500 | 0.0 |
| 31.3 | D22S280 | 0.0 | 0.500 | 0.0 | 51.5 | D22S274 | 0.0 | 0.500 | 0.0 |

**Table 8-23 Chromosome X Guam association study results**

| cM fem | Locus | LRT | p | λ | cM fem | Locus | LRT | p | λ |
|---|---|---|---|---|---|---|---|---|---|
| 0.0 | DXS6814 | 0.0 | 0.417 | 0.1 | 93.2 | DXS6800 | 0.0 | 0.500 | 0.0 |
| 7.7 | DXS1060 | 0.0 | 0.500 | 0.0 | 103.6 | DXS6789 | 0.0 | 0.500 | 0.0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 8.8 | GATA124B04 | 0.0 | 0.500 | 0.0 | 111.8 | DXS1106 | 0.0 | 5.000 | 0.0 |
| 22.0 | DXS987 | 0.0 | 0.500 | 0.0 | 112.9 | DXS6797 | 0.2 | 0.337 | 0.2 |
| 22.0 | GATA175D03 | 1.3 | 0.128 | 0.4 | 116.2 | GATA172D05 | 3.7 | 0.028 | 0.3 |
| 33.0 | DXS1226 | 0.0 | 0.500 | 0.0 | 143.2 | DXS1047 | 0.4 | 0.270 | 0.2 |
| 39.5 | GATA124E07 | 0.1 | 0.374 | 0.2 | 154.3 | GATA31E08 | 0.0 | 0.500 | 0.0 |
| 63.6 | DXS6810 | 0.0 | 0.500 | 0.0 | 155.9 | DXS1227 | 0.0 | 0.500 | 0.0 |
| 71.3 | GATA144D04 | 0.0 | 0.500 | 0.0 | 165.1 | GATA182E04 | 0.0 | 0.500 | 0.0 |
| 83.3 | DXS7132 | 0.0 | 0.500 | 0.0 | 184.3 | DXYS154 | 0.0 | 0.500 | 0.0 |

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