ELSEVIER

Neurobiology of Aging 30 (2009) 656–665

NEUROBIOLOGY OF AGING

www.elsevier.com/locate/neuaging

Rapid communication

Ubiquitin associated protein 1 is a risk factor for frontotemporal lobar degeneration

Sara Rollinson[a], Patrizia Rizzu[b], Stephen Sikkink[a], Matthew Baker[c], Nicola Halliwell[a], Julie Snowden[d], Bryan J. Traynor[e], Dina Ruano[b], Nigel Cairns[f], Jonathan D. Rohrer[g], Simon Mead[h], John Collinge[h], Martin Rossor[g], Ela Akay[a], Rita Guerreiro[e,i], Rosa Rademakers[c], Karen E. Morrison[j,k], Pau Pastor[l], Elena Alonso[l], Pablo Martinez-Lage[m], Neil Graff-Radford[c], David Neary[d], Peter Heutink[b], David M.A. Mann[d], John Van Swieten[n], Stuart M. Pickering-Brown[a,*]

[a] *Clinical Neurosciences, Faculty of Human and Medical Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, UK*
[b] *Section Medical Genomics, Department of Clinical Genetics, VU University Medical Center, Amsterdam, The Netherlands*
[c] *Department of Neuroscience, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224, USA*
[d] *Centre for Clinical Neurosciences, University of Manchester, Greater Manchester Neurosciences Centre, Hope Hospital, Salford M6 8HD, UK*
[e] *Laboratory of Neurogenetics, Neurogenetics Branch, National Institute of Neurological Disorders and Stroke National Institute on Aging, National Institutes of Health, Bethesda, USA*
[f] *Department of Pathology and Immunology and Alzheimer's Disease Center, Washington University School of Medicine, Saint Louis, MO 63110, USA*
[g] *Dementia Research Centre, Institute of Neurology, Queen Square, London WC1N 3BG, UK*
[h] *MRC Prion Unit, Department of Neurodegenerative Diseases, UCL Institute of Neurology, Queen Sqaure, London WC1N 3BG, UK*
[i] *Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal*
[j] *Department of Clinical Neurosciences, Division of Neuroscience, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK*
[k] *Queen Elizabeth Hospital, University Hospitals Birmingham NHS Foundation Trust, Birmingham B15 2TH, UK*
[l] *Neurogenetics Laboratory, Division of Neurosciences, Center for Applied Medical Research (CIMA), and Department of Neurology, Universitaria de Navarra, University of Navarra School of Medicine, Pamplona, Spain*
[m] *ACE, Barcelona, Spain*
[n] *Erasmus MC, Rotterdam, P.O. Box 2040, Rotterdam, The Netherlands*

Received 18 December 2008; accepted 3 January 2009
Available online 12 February 2009

Abstract

Frontotemporal lobar degeneration (FTLD) is now recognised as a common form of early onset dementia. Up to 40% of patients have a family history of disease demonstrating a large genetic component to its etiology. Linkage to chromosome 9p21 has recently been reported in families with this disorder. We undertook a large scale two-stage linkage disequilibrium mapping approach of this region in the Manchester FTLD cohort. We identified association of ubiquitin associated protein 1 (*UBAP1*; OR 1.42 95% CI 1.08–1.88, $P = 0.013$) with FTLD in this cohort and we replicated this finding in an additional two independent cohorts from the Netherlands (OR 1.33 95% CI 1.04–1.69, $P = 0.022$), the USA (OR 1.4 95% CI 1.02–1.92, $P = 0.032$) and a forth Spanish cohort approached significant association (OR 1.45 95% CI 0.97–2.17, $P = 0.064$). However, we failed to replicate in a fifth cohort from London (OR 0.99 95% CI 0.72–1.37, $P = 0.989$). Quantitative analysis of *UBAP1* mRNA extracted from tissue from the Manchester cases demonstrated a significant reduction of expression from the disease-associated haplotype. In addition, we identified a case of familial FTLD that demonstrated colocalisation of UBAP1 and TDP-43 in the neuronal cytoplasmic inclusions in the brain of this individual. Our data for the first time identifies UBAP1 as a genetic risk factor for FTLD and suggests a mechanistic relationship between this protein and TDP-43.

Keywords: Frontotemporal lobar degeneration; UBAP1; Ubiquitin associated protein 1; TDP-43; Risk factor

* Corresponding author. Tel.: +44 161 275 1341; fax: +44 161 275 5272.
E-mail address: SPB@Manchester.ac.uk (S.M. Pickering-Brown).

0197-4580/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.neurobiolaging.2009.01.009

1. Introduction

Frontotemporal lobar degeneration (FTLD) is the term used for a group of clinical syndromes characterized by changes in behaviour, personality and language with relative preservation of memory and spatial skills (Neary et al., 1998). FTLD is a common form of dementia in individuals under the age of 65 and around half of all patients present with a family history of a similar disease (Pickering-Brown et al., 2002). The genetic aetiology of FTLD is complex with 7 loci identified to date on chromosomes 3, 9p (two loci), 9q, 17q21 (two loci) and 17q24 (Pickering-Brown, 2007). Four of the genes that account for these linkages have been identified being *CHMP2B* on chromosome 3, *VCP* on chromosome 9p and *MAPT* and progranulin (*PGRN*) on chromosome 17q21 and collectively these account for 10–20% of all FTLD (Hutton et al., 1998; Watts et al., 2004; Skibinski et al., 2005; Baker et al., 2006; Gass et al., 2006). There are two common neuropathological subtypes observed in FTLD, one being tauopathy, the accumulation of abnormally phosphorylated and insoluble aggregates of tau protein in neurons and sometimes glia, the other being referred to as FTLD-U where ubiquitinated neuronal inclusions containing TAR DNA binding protein (TDP-43) are present in varying quantities (Cairns et al., 2007). FTLD-U has been further divided into three main subgroups determined by the distribution and morphological characteristics of the TDP-43 pathology (Mackenzie et al., 2006; Cairns et al., 2007). Familial tauopathy is often associated with mutations in *MAPT* whereas null-mutations of *PGRN* lead to FTLD-U (Hutton et al., 1998; Baker et al., 2006). Cases with mutations in *PGRN* have been designated as type 3 FTLD-U, whereas those cases from families linked to chromosome 9p have been designated as having type 2 FTLD-U pathology (Cairns et al., 2007). One characteristic that unites the two differing histological subtypes in FTLD is that both tau and TDP-43 inclusions are ubiquitinated to varying degrees. The observation that many proteins that accumulate within neurons and/or glia in neurodegenerative diseases are ubiquitinated has lead to the suggestion that dysfunction of the ubiquitin proteasome system could be an aetiological factor in this group of conditions (Petrucelli and Dawson, 2004).

The identification of the additional causal genes and genetic risk factors will help elucidate the important aberrant biological pathways underlying FTLD. Therefore, in order to investigate whether we could identify any evidence of genetic association between genes in the chromosome 9p linkage region in our FTLD cohort from the North West of Great Britain we undertook a large scale two-stage linkage disequilibrium mapping approach of the minimal region defined from published families (Morita et al., 2006; Vance et al., 2006) and attempted to replicate our finding in an additional 4 independent cohorts from the Netherlands, USA, Spain and London, UK. This work represents the largest case controls study performed on FTLD to date.

2. Materials and methods

2.1. Subjects

All samples fulfilled current diagnostic criteria for FTLD (Neary et al., 1998) and were recruited with Ethical Committee approval and provided informed consent. All patients known to harbour a mutation in *MAPT* or *PGRN* genes were excluded. Manchester cohort: all patients were recruited between 1987 and 2007 through longitudinal neuropsychological and clinical assessment within the Cerebral Function Unit of the University of Manchester. The final study group comprised 214 patients (mean age at onset 59.6 years, range 23–83). The series comprised 116 men (mean age at onset 59.1 years, range 35–79), and 98 women (mean age at onset 60.1 years, range 23–83). By the time of this study 44 patients had come to post-mortem. Control data were drawn from a cohort of 286 mentally normal people (at the time of sample acquisition). All samples were of UK origin, Dutch cohort: 214 patients with FTLD comprising 118 females and 96 males with a mean age at onset 57.9 ± 9.0 (30–76) and a disease duration of illness 8.3 ± 3.9 with age at death being 65.0 ± 9.6 where known. Dutch controls consisted of 149 males and 149 females with an average age of 61.06 ± 2.89 years at the time of collection. Mayo Cohort: 176 patients with FTLD comprising of 88 males and 88 females with a mean age at onset of 63.55 (range 44–81 years). Mayo controls consisted of 108 females and 74 males with an average age of onset of 69.76 (range 30–87 years) at the time of collection. London cohort: 158 cases comprising 60 males and 98 females with an average age at onset of years (range 26–77 years). 192 controls comprising 70 males and 122 females with an average age at sampling of 38.4 SD 7.7. Spanish cohort: 75 patients with FTLD comprising of 39 males and 36 females with a mean age at onset of 66.12 (range 38–78 years). Spanish controls consisted of 75 females and 77 males with an average age of 69.76 (range 48–97 years) at the time of collection.

2.2. SNP selection and first round screening

Using the linkage data of Morita et al. (2006) and Vance et al. (2006) a 10.26 Mb region on chromosome 9p between markers D9S2154 and D9S1874 was identified as being a possible locus containing a genetic risk factor for FTLD and MND. To map the region using case-control linkage disequilibrium analysis a total of 190 frequency validated SNPs were selected from public databases including the NCBI SNP databases (www.ncbi.nlm.nih.gov/SNP), Ensembl (www.ensembl.org) and HapMap (phase 2.0 data) (www.hapmap.org – CEPH population), using a gene centric approach. A total of 133 genes (as of genome build 35) were found in the region. Linkage disequilibrium (LD) maps were obtained from HapMap, and where multiple genes fell within a single haplotype block, tag SNP's were selected to reduce

the total number of SNPs required for genotyping using the tagger application (de Bakker et al., 2005). A minimum of one SNP per gene or two SNPs per haplotype block were prioritized to give as complete coverage as possible of the region. Any SNPs also in coding regions or with a potential biological function were prioritized for genotyping selection, as were SNPs with a minor allele frequency of 0.2 or greater.

2.3. Second round screening

By analysis of individual markers and sliding window haplotype analysis three regions were chosen for further linkage study. As before SNPs were chosen using the tagger application to identify tag SNPs that capture the majority of variation across the candidate region. Where a gene fell across multiple haplotype blocks SNPs were chosen that spanned both blocks, additionally 5 kb up- and downstream of the gene was also included in the screening area so as to increase the likelihood of genotyping any putative regulatory regions.

2.4. Genotyping analysis

DNA samples were randomly assigned across genotyping plates with 5 cell line samples duplicated across the plates, and an additional 7 duplicate samples on each plate to test for genotyping consistency. 15 ng of each DNA was genotyped using the Sequenom MassArray genotyping technology according to manufacturer's instructions.

Genotyping quality control for each SNP assay included concordance of genotypes between replicate samples both internally per genotyping plate and externally between plates. An equal call of genotypes had to be observed in both the case and control groups of samples (the average ration of genotyping calls between the cases and controls being 1.002), with a minimum of 75% samples with an assigned genotype. All SNPs were required to be in Hardy–Weinberg in the control population with a minimum P value of 0.05, while cases could be out of equilibrium for inclusion into the study, no SNP was out of equilibrium in the cases and not in the controls.

3. Statistical analysis

Simple χ^2 and Hardy–Weinberg Equilibrium P values for each SNP variant were calculated using Haploview (Barrett et al., 2005) and Stata with Yates correction. Haplotype block structure was examined using haploview, a block defined using the confidence intervals option, where 95% confidence bounds on D' are generated, a block defined if 95% of informative comparisons are in strong linkage disequilibrium (Gabriel et al., 2002). For comparison with the generated data, data corresponding to genomic positions 26856230–37152282 on chromosome 9 were downloaded from the international haplotype mapping project (http://www.hapmap.org/). In order to correspond with the samples genotyped in the present study only CEPH samples were analysed. The CEPH samples comprise of Utah (USA) residents with ancestry from northern and western Europe. For multiple testing correction the false discovery rate as implemented using the Q-value algorithm run through R was implemented (Storey and Tibshirani, 2003). To reconstruct haplotypes from genotype data Phase version 2.1 was used (Stephens et al., 2001; Stephens and Donnelly, 2003), using 10,000 iterations and a burn in of 10,000. For single variant and haplotype odds ratios (OR) and 95% confidence intervals (95% CI) were estimated using un-conditional logistic regression adjusting for age and sex using Stata (version 9), using the most common variant/haplotype as the baseline. CLUMP (Sham and Curtis, 1995) was used to assess the χ^2 significance of the haplotypes between groups. Haplo.Stats (version 1.2.2) was used to carry out sliding-window haplotype analysis, used to estimate haplotype effects under the generalized linear model (http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm).

3.1. Sequencing

Sequencing was performed using standard PCR methods (primer sequences are found in supplementary Table 1) using BigDye 3.1 and a ABI3730 sequencer.

3.2. Antibodies

An affinity purified polyclonal rabbit anti-UBAP1 peptide antibody (UBAP1-359) was generated by Eurogentec raised against peptides matching the C-terminal coding sequence of UBAP1 (CQDNALEDLMARAGAS). Also, a rabbit polyclonal UBAP1 antibody, 10782-1-AP (Protein Tech Inc, Chicago, IL) was used. For TDP-43 immunohistochemistry mouse anti-TARDBP monoclonal antibody (Clone M01; Abnova) was employed. Alexa-488 conjugated goat anti-mouse and Alexa-546 conjugated goat anti-rabbit secondary antibodies were obtained from Invitrogen Molecular Probes, UK.

3.3. Immunohistochemistry

5 μm sections were dewaxed with xylene then rehydrated through graded ethanol and rinsed in distilled water. Antigen retrieval was performed by microwaving sections for 20 min in 10 mM Sodium citrate buffer pH6.0 and allowing to cool. Sections were rinsed twice with PBS then permeabilized by incubating for 20 min in 0.2% Triton-X100. After 3 washes with PBS, non-specific epitopes were blocked by incubating in normal serum for 20 min. Sections were then incubated in primary antibodies (rabbit anti-UBAP1-359 (diluted 1:200 in PBS); mouse anti-TDP-43 (diluted 1:2000 in PBS)) overnight at 4 °C then rinsed 3 times with PBS containing 0.01% Tween-20 (PBS-TT). Sections were incu-

bated in secondary antibodies (Alexa-488 conjugated goat anti-mouse antibody; Alexa-546 conjugated goat anti-rabbit antibody (diluted 1:200 in PBS)) for 1 h at RT then rinsed 3 times with PBS-TT and mounted in Vectashield™ fluorescent mounting medium (Vectorlabs, UK) and coverslips sealed. Fluorescence was imaged and processed using excitation with argon (488 nm) and green helium neon (543 nm) lasers and sequential line-scanned to prevent fluorophore bleedthrough with a Leica DM IRE2 inverted laser scanning confocal microscope and LCS software (Leica Microsystems, UK).

3.4. Expression analysis

100–200 mg quantities of grey matter tissue were dissected and extracted using Trizol™ (Invitrogen, Darmstadt, Germany). 8 ml of the RNA were reverse-transcribed in a final volume of 20 μl using 200 U of Superscript III (Invitrogen), 300 ng of random hexamers (Roche), 40 U of RNaseOUT (Invitrogen), 10 nmols of dNTPs, 100 nmols DTT, in a final concentration of 1× first-strand Buffer (Invitrogen).

Real-time PCR analyses for UBAP1, β-actin and GAPDH (sequences available on request) were performed using the ABI 7500 Sequence Detection System instrument and software (Applied Biosystems, Foster City, CA, USA). Primers were designed using the Primer Express software (Applied Biosystems). To reduced issues of post-mortem related RNA degradation amplicons were designed to be short (75–100 bp), and towards the 3′ end of each gene and spanned introns. Samples were amplified in triplicate in 20 μl volumes using the Power Sybr green master mix (Applied Biosystems), and 10 pM of each forward and reverse primer, using Applied Biosystems standard cycling conditions for real time PCR (for primer sequences see supplementary information). Measurements of relative gene expression were calculated using the standard curve method. Hela cell cDNA was used to construct a standard curve over the logarithmic range of 100–0.001 ng, all curves calculated had a slope of between −3.1 and −3.6 with an minimum R^2 value of 0.997. For each sample the amplification cycle of threshold (CT) values were converted to ng amounts, with samples that amplified "off the curve" re-extracted and re-amplified, or discarded from analysis. To allow comparison across plates an aliquot of a reference sample (10 ng of Hela cDNA) was amplified on all plates. Both house keeping control genes, β-actin and GAPDH used to normalize the samples for UBAP1 expression. Since both β-actin and GAPDH gave similar results and displayed good correlation in expression between samples, only the results for UBAP1 expression normalized by GAPDH are presented here. For analysis of expression data each individual was included twice in the analysis (once for each haplotype), thus the N values indicate observations not individuals. Data were expressed as the average and standard error, while for intra-individual analysis, a two-sided *t* test was used.

4. Results

4.1. Stage one screen

A total of 190 SNP's were genotyped across the 10.296 Mbp region of 9p determined by linkage analysis to be associated with FTLD. After dropping those assays that did not meet the stringent quality control measures 151 SNPs were left for analysis, possessing an average genotype call of 92.4% (76.3–98.6% call). On examination of *P* values calculated using a case–control analysis, 10 *P* values were found to be significant after Yates correction ($P < 0.05$). None of these *P* values remained significant after correction for multiple testing (using the false discovery rate) (data not shown) and this is likely in part due to the relatively small number of cases available for analysis combined with the large number of tests carried out. To assess the effect of these SNPs, haplotype associations were calculated using haploview. 7 regions displayed strong linkage with a haplotype combination associated with FTLD ($P < 0.05$), associated with the genes *TEK*, *LRRN6C*, *DDX58*, *UBAP1*, *OR2S2*, *RECK* and *PAX5*. To assess the haplotype association further we performed a sliding window analysis of the genomic regions, including 2–7 SNPs in each block. We observed the strongest effect when we included markers rs4574933 (*UBAP1*) (global *P* value of = 0.0005), rs664513 (*TEK*) $P = 0.02$, and rs1359885 (*RECK*) $P = 0.0052$, whereas the other markers (and associated regions of linkage) failed to produce significance upon sliding window analysis.

4.2. Stage two screen

Hapmap and tagger programs were used to select an additional 50 SNPs to examine the haplotype structure of the genomic regions spanning *RECK* ($N = 5$, 36026906–36114448 bp), *TEK* ($N = 31$, 27099441–27220165 bp) and *UBAP1* ($N = 14$, 34169011–34242521 bp) for the second stage screen. Haplotype analysis both excluding and including the first round SNP assays was carried out using both haploview and haploscore. Upon analysis the haplotype block structure of both *TEK* and *RECK* showed concordance with that seen in the CEPH HAPMAP data, however neither gene displayed a haplotype that was associated with a significant effect for FTLD (data not shown). In contrast, in addition to the first round rs4574933 SNP, an additional 6 SNPs were found to have an allele association with FTLD (Table 1). Sliding window analysis confirmed this with a maximal global P value of 0.0002 using a sliding window size of 7 (Fig. 1). This 7 SNP haplotype includes 6 of the 7 significant SNP assays (rs10971977 to rs10814083), corresponding to the genomic region that contains and surrounds the *UBAP1* coding sequence. Haploview analysis confirmed that the haplotype structure around *UBAP1* agreed with that from by the CEPH hapmap data, with a 10 SNP haplotype block that spans all the *UBAP1* genomic sequence (Fig. 1).

Table 1
Location, rs identifier, allele frequencies statistical analysis of SNPs genotyped in and around UBAP1 as part of the first round screening of the Manchester FTLD cohort.

Name	Gene	Location	Case, control ratio	Case, control freq.	Chi square	*P* value	Perm *P* value
rs10453201	UBAP2	34040345	314:94, 372:126	0.770, 0.747	0.624	0.4295	0.995
rs2275003	WDR40A	34114860	238:218,285:277	0.522, 0.507	0.221	0.6382	1
rs4879753		34133307	277:133, 325:179	0.676, 0.645	0.952	0.3292	0.984
rs13283064		34142464	141:301,133:411	0.319, 0.244	6.748	0.0094	0.108
rs10971969		34154375	74:326,77:415	0.185,0.157	1.274	0.259	0.954
rs7018487		34157733	303:115, 335:175	0.725, 0.657	4.947	0.0261	0.258
rs10971977	UBAP1	34170023	232:214,221:327	0.520, 0.403	13.546	2.00E−04	0.006
rs12375731	UBAP1	34194266	200:198,206:298	0.503, 0.409	7.902	0.0049	0.065
rs10814079	UBAP1	34216087	325:119,354:188	0.732, 0.653	7.076	0.0078	0.096
rs2380925	UBAP1	34239204	213:205,212:318	0.510, 0.400	11.344	8.00E−04	0.014
rs17258783	UBAP1	34243097	108:324, 107:429	0.250, 0.200	3.513	0.0609	0.474
rs4574933	UBAP1	34243488	130:324,112:444	0.286, 0.201	9.889	0.0017	0.022
rs10814083	UBAP1/C9ORF48	34246347	297:111, 333:161	0.728, 0.674	3.077	0.0794	0.566
rs10972030	C9orf48	34256323	284:156,312:224	0.645, 0.582	4.081	0.0434	0.385
rs17350373	C9orf48/	34274944	192:242,224:308	0.442, 0.421	0.444	0.5052	0.998
rs 10972040	KIF24	34282855	84:336, 85:445	0.200, 0.160	2.515	0.1127	0.693
rs11788425	NUDT2	34323855	191:219,227:289	0.466, 0.440	0.62	0.4309	0.995
rs3892388	NP_065753.1	34358984	356:54, 408:92	0.868, 0.816	2.573	0.325	0.304
rs12377	NP_065753.1	34362347	441:13,542:20	0.971,0.964	0.386	0.5342	1
rs885048	CI025HUMAN	34389338	85:343, 87:423	0.199, 0.171	1.219	0.2695	0.958
rs4879787	CI025_HUMAN	34416658	312:106, 375:133	0.746, 0.738	0.081	0.776	1

4.3. Replication in four additional cohorts

In order to evaluate whether the effect we observed was a type 1 error we genotyped the second round SNPs in two additional independent FTLD cohorts from The Netherlands and from the Mayo Clinic Jacksonville, USA. Significant association was observed for multiple SNPs after Yates correction in both of these additional cohorts. Importantly, the same 2 SNPs (rs7018487 and rs10814079) were consistently significantly associated with FTLD across the Manchester, Dutch and US populations (Table 2), and an additional SNP (rs10814083) was associated with FTLD in the Dutch and Jacksonville cohorts. rs7018487 was also associated ($P = 0.043$) in the Spanish cohort and rs10814079 approached significance ($P = 0.071$) in this population (Table 2). The use of the tagger application(Stephens et al., 2001) showed that these three SNPs are in strong linkage with each other ($R^2 > 0.98$) and define 2 haplotypes each containing all of *UBAP1*. Using these three SNPs as surrogate markers for the gene, haplotype analysis identified a significant association of the T_G_C haplotype in the Manchester and Dutch cohorts (OR 1.42 95% CI 1.08–1.88, $P = 0.013$, and OR 1.33 95% CI 1.04–1.69, $P = 0.022$, respectively), and significant association for the G_C_T haplotype of OR 1.4 95% CI 1.02–1.92, $P = 0.032$ for the Jacksonville cohort and this haplotype also approached significance in the Spanish cohort (OR 1.45 95% CI 0.97–2.17, $P = 0.064$) (Table 3). None of the SNPs or haplotypes were associated with disease in the

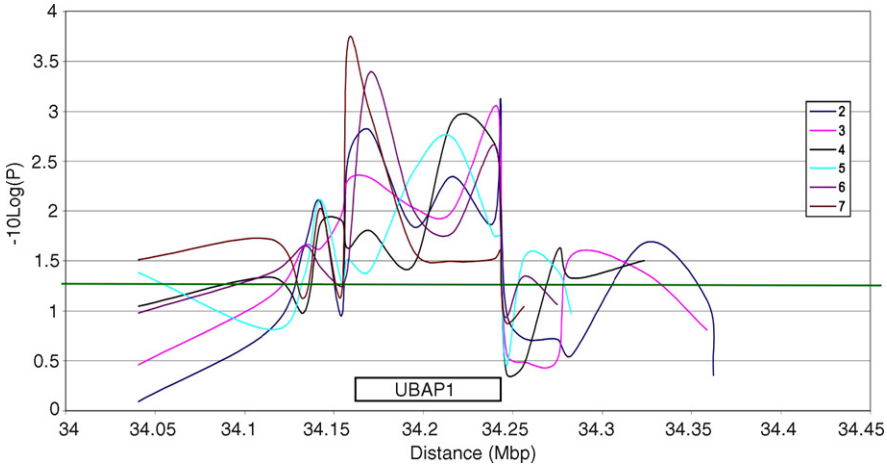

Fig. 1. Sliding window analysis (haplotype size $N = 2$ to N-7) generated with Haplo.score of the SNPs compromising the genomic region containing UBAP1 in the Manchester FTLD cohort. Green line equivalent to $P = 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
Replication of second round UBAP1 SNPs in the Manchester, Dutch, US, Spanish and London FTLD cohorts.

	Major allele controls	Major allele frequency (case, control)	Chi square	*P*
rs7018487				
Manchester cohort	T	0.726, 0.658	4.268	0.039
Dutch cohort	T	0.701, 0.637	5.110	0.023
Mayo cohort	T	0.624, 0.704	4.656	0.031
Spanish cohort	T	0.556, 0.659	4.062	0.043
London cohort	T	0.677, 0.685	0.025	0.875
rs10814079				
Manchester cohort	G	0.734, 0.654	6.291	0.012
Dutch cohort	G	0.697, 0.637	4.613	0.031
Mayo cohort	G	0.636, 0.733	5.304	0.021
Spanish cohort	G	0.573, 0.665	3.243	0.071
London cohort	G	0.678, 0.685	0.011	0.951
rs10814083				
Manchester cohort	C	0.728, 0.674	2.637	0.104
Dutch cohort	C	0.705, 0.639	5.479	0.019
Mayo cohort	C	0.626, 0.703	4.294	0.038
Spanish cohort	C	0.572, 0.654	2.599	0.106
London cohort	C	0.679, 0.674	0.004	0.951

London cohort. The fact that association was observed in 4 independent cohorts provides strong evidence that common genetic variation at the *UBAP1* locus confers risk for FTLD.

4.4. Molecular genetic and histological investigation

We sequenced the entire open reading frame of *UBAP1* in both the Manchester and Dutch cohorts and five variants were identified in separate familial cases (E87K, P96L, S391Afs21X and H149Q in the Manchester samples and a P256L in the Dutch cohort). Four of these were absent from 450 controls and are possibly mutations, however, the H149Q is found at an allele frequency of 0.0082% in cases and 0.0107% in controls and is therefore a polymorphism. Unfortunately, samples from other family members were unavailable to test for segregation. Nevertheless, these variants are at highly conserved residues (supplementary Fig. 1) suggesting functional significance.

To investigate any involvement of UBAP1 in the neuropathology of FTLD we immunostained 40 cases of FTLD with the 2 different UBAP1 antibodies and both produced identical results. UBAP1 immunoreactivity was present in pyramidal and non-pyramidal neurons throughout the cerebral cortex in the form of small granules or larger globules or a mixture of both. These were evenly scattered throughout the perikaryon and showed no focal accumulation at any particular region of their neurons. Occasional glial cells, probably astrocytes, also showed similar but smaller accumulations of UBAP1 immunoreactivity. In all but one case, UBAP1 antibodies failed to stain any pathological inclusions, i.e. TDP-43 cytoplasmic or intranuclear inclusions, tangles or Pick bodies. However, we did observe robust staining of

Table 3
Replication of haplotype analysis in Manchester, Dutch, US, Spanish and London FTLD cohorts. *N* is observed number of haplotypes not the number of cases.

Haplotype analysis: by cohort	Cases (%)	Controls (%)	OR	95% CI	*P*
Manchester cohort					
TGC	301 (73.06)	368 (62.25)	1.40	1.06–1.85	0.018
GCT	111 (26.94)	190 (33.69)	1.00		
Dutch cohort					
TGC	321 (70.09)	615 (63.80)	1.33	1.04–1.69	0.018
GCT	136 (29.69)	345 (35.79)	1.00		
Mayo cohort					
TGC	221 (62.78)	256 (70.33)	1.0		
GCT	130 (36.93)	107 (29.40)	1.4	1.02–1.92	0.032
Spanish cohort					
TGC	85 (56.67)	209 (65.72)	1.0		
GCT	64 (42.67)	108 (33.96)	1.45	0.97–2.17	*0.064*
London cohort					
TGC	221 (67.79)	252 (67.74)	1.0		
GCT	105 (32.21)	120 (32.26)	0.99	0.72–1.37	0.989

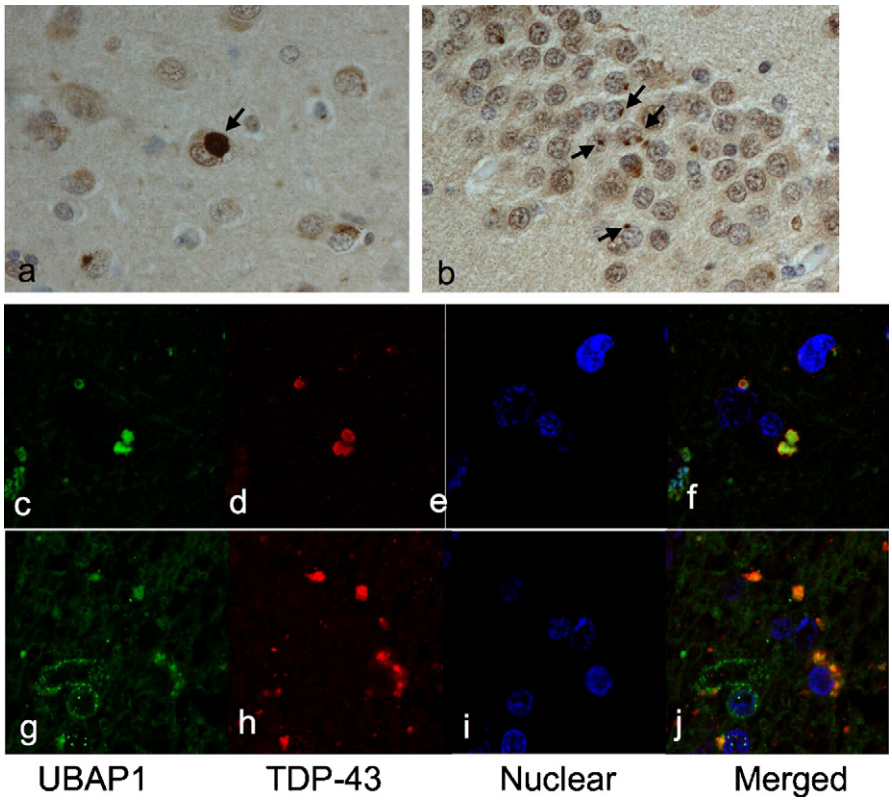

Fig. 2. Immunohistological analysis of UBAP1 in a case of familial FTLD (Momeni et al., 2006). UBAP1 positive neuronal cytoplasmic inclusions (arrows) in (a) frontal cortex and (b) hippocampus. Confocal analysis of UBAP1 (c and g) and TDP-43 (d and h) with merged images (f and j) demonstrating co-localisation.

TDP-43 positive cytoplasmic inclusions and dystrophic neurites with both UBAP1 antibodies in a single familial case (Fig. 2). We were able to demonstrate colocalisation of TDP-43 and UBAP1 in these pathological structures using double labelling immunoflorescence microscopy (Fig. 2).

Analysis of the levels of *UBAP1* mRNA in 20 frontal cortex and 17 cerebellum FTLD-U tissue samples (tissues obtained from the Manchester Brain Bank representing genotyped samples that had undergone post-mortem examination) revealed that the T_G_C disease associated haplotype in our cohort produced significantly less mRNA than the reference haplotype in both frontal cortex ($P = 0.015$) and cerebellum ($P = 0.033$) (Fig. 3). However, analysis of *UBAP1* mRNA in 13 frontal cortex and 10 cerebellum tauopathy tissue samples revealed no significant difference ($P = 0.6$) between the two haplotypes (data not shown).

5. Discussion

We have undertaken a large scale linkage disequilibrium mapping study of the published minimal linkage region for FTLD linked to chromosome 9p using our large FTLD cohort from the North West region of Great Britain. This analysis has

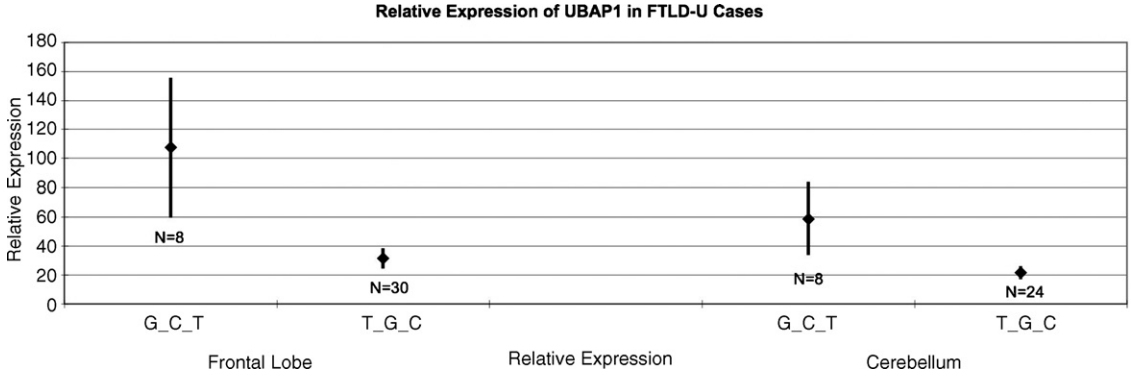

Fig. 3. Relative expression of UBAP1 mRNA in Manchester FTLD brain tissue analysed by UBAP1 haplotype. *N* values indicate observations not individuals.

identified haplotypes of *UBAP1* as a significant risk factor for FTLD with an OR of 1.42 95% CI 1.08–1.88, $P = 0.013$ in the Manchester cohort. Genetic association studies of human disease are often complicated by type 1 errors, i.e. false positive association, and are fraught with poor replication. However, we have found additional associations in another three independent unrelated cohorts from the Netherlands, Spain and the USA at the allele level. Furthermore, the Dutch and US samples were also significantly associated at the haplotype level with the Spanish sample approaching significance, bearing in mind the Spanish cohort was the smallest of the analysis. Collectively, these data argue against this association being a type 1 error. The T_G_C haplotype is associated with disease in both the Manchester and Dutch populations, however, it is the G_C_T haplotype that is associated with FTLD in the Jacksonville and Spanish cohorts. This difference argues that the SNPs used to define these haplotypes are not themselves the actual functional variants conferring risk, if they were one would predict the same allele/haplotypes to be associated in all three populations. The disparity in association presumably represents different founder effects with variants conferring disease risk arising independently on separate ancestral haplotypes between populations. The fact that the G_C_T haplotype was associated with disease in the US (Floridian) and Spanish cohorts maybe evidence of a Spanish founder effect in the Floridian population as Florida was first colonized and populated by the Spanish in the 16th century. Why we failed to detect any association in the London cohort is currently unclear, however, it maybe due to differences in population stratification or differences in ascertainment. Further genotyping of extra SNPs in and around UBAP1 are required to conclusively rule out association in the cohort.

Sequence analysis of the open reading frame of *UBAP1* has identified several putative mutations though samples from other family members were not available to demonstrate segregation. Nevertheless, these putative mutations are all at highly conserved residues with the P256L being fully conserved in all species examined (supplemental Fig. 1). As these patients are all currently living we are unable to determine the precise histological changes, i.e. TDP-43 or tauopathy, associated with these mutations.

Analysis of *UBAP1* mRNA extracted from cases obtained from the Manchester cohort revealed that the disease associated haplotype (T_G_C) correlated with significantly lower levels of expression compared to the non-disease associated haplotype. Our haplotype association data would predict a similar effect in tissue derived from the Dutch population but that the G_C_T haplotype would correlate with reduced expression in the US and Spanish cohorts. That this is observed in frontal lobe which is affected by pathology in FLTD and cerebellum, which is free from neuropathology, argues these findings are not just a product of the neurodegenerative process or due to post-mortem effects. These data strongly suggest that the key variant(s) in *UBAP1* that increases risk of FTLD operates by lowering *UBAP1* expression leading to loss/reduced function. This hypothesis is consistent with one of the putative mutations we have identified (S391Afs21X) which removes both of the ubiquitin associated domains of *UBAP1*, and this presumably has a negative effect on the function of this protein.

UBAP1 encodes a protein of 502 residues, predicted to have a molecular weight of 55KDa and was originally cloned from a tumour suppressor locus (Qian et al., 2001). While little is known of the actual function of the protein, the gene is likely to be a member of the ubiquitin-activated enzymes family whose members include proteins having connections to ubiquitin and the ubiquitination pathway. The protein itself has two ubiquitin-associated domains (UBA), between residues 389–430, and 451–498, and an Ubiquitin System Cue domain between residues 459–499, believed to be involved in the binding of ubiquitin-conjugating enzymes. The UBA domains are found in various proteins, including p62 which is found in certain TDP-43 positive inclusions in FTLD (Arai et al., 2003), and are involved numerous processes including the ubiquitin/proteasome pathway, growth control, receptor function, stress responses, DNA excision-repair and cell signalling via protein kinases (Schwartz and Ciechanover, 1999). It is believed that the ubiquitin proteamsome system (UPS) plays a vital role in protecting the CNS from the accumulation of toxic proteins (Petrucelli and Dawson, 2004). Furthermore, it has been demonstrated that mutations of certain genes involved in the UPS can lead to Parkinson's disease and clearly link a dysfunctional UPS to neurodegeneration (Thomas and Beal, 2007). Therefore *UBAP1* is an excellent candidate gene for FTLD.

The identification that UBAP1 and TDP-43 proteins co-localise together in neuronal cytoplasmic inclusions in a case of familial FTLD is important as it directly implicates UBAP1 in the metabolism of TDP-43. It also suggests that UBAP1 related FTLD will likely be of the FTLD-U neuropathological subtype. This hypothesis is also supported by our expression data where significant reduction of *UBAP1* mRNA is only seen in FTLD-U and not tauopathy. Furthermore, this is also consistent with the FTLD families known to be linked the chr9p which are of the FTLD-U subtype. It is a very common feature that the protein products of genetic risk factors for neurodegenerative disease are found in the cytoplasmic pathological inclusions of these diseases (Singleton et al., 2004) and our current data are supportive of this hypothesis with UBAP1 in at least some cases of FTLD. The family with UBAP1 positive neuropathology is the same family recently reported to have a mutation in the IFT74 gene (Momeni et al., 2006). Given the lack of replication of mutations in this gene it is now thought unlikely that IFT74 is the gene responsible for linkage to this region and that the original variant is not pathogenic (Momeni et al., 2006; Xiao et al., 2007). Furthermore, the presence of UBAP1 in the neuropathological lesions in the case argues for a functional role of UBAP1 in the pathogenesis of disease in this individual/family. Nevertheless, sequence analysis of the open reading frame of *UBAP1* in a disease haplotype carrying affected member from this family has failed to identify any variation so far. However, this

analysis does not exclude pathological copy number or regulatory sequence variants of *UBAP1* in this pedigree. Since our expression data suggests a reduction in expression being aetiologically relevant we are currently investigating *UBAP1* for such variation. Unfortunately, fresh frozen brain tissue is not available from this family for biochemical or expression analysis.

The identification of *UBAP1* as a risk factor for FTLD is an important discovery because for the first time it provides evidence for a link between the UPS and FTLD. This observation, therefore, suggests the UPS is a potential future therapeutic target for this group of diseases. It will be interesting to confirm whether *UBAP1* mutations lead to a TDP-43 or tauopathy based histology and to establish whether this protein has a wider role in neurodegenerative disease. Furthermore, it will be vital to investigate *UBAP1* in other populations and in families with FTLD definitively linked to chr9p. Finally, if pathogenic variations are absent in these latter linked families, this does not refute our finding of *UBAP1* being an independent genetic risk factor for FTLD located in this chromosomal region. If this scenario was true it would be analogous to the situation on chromosome 17 where the two genes, *MAPT* and *PGRN*, causing FTLD are only 1.7Mb apart (Hutton et al., 1998; Baker et al., 2006).

Disclosure statement

Dr. Pickering-Brown has filed a patent relating to UBAP1 in dementia but there are no other actual or potential conflicts of interest for him or any other of the authors. All patients included in this study were recruited with local Ethical Committee approval and provided informed consent.

Acknowledgments

SPB and DMAM received funding from the Medical Research Council and the Alzheimers Research Trust to support this work. This research was also supported (in part) by the Intramural Research Program of the NIH, National Institute on Aging (Z01-AG000949-02) and National Institute of Neurological Disorders and Stroke. RG received support from rant #SFRH/BD/27442/2006 from *Fundacao para a Ciencia e Tecnologia*, Portugal. This work was also supported by NIH grants P50 AG16574 (NG-R and RR) and the Pacific Alzheimer's Disease Research Foundation grant #C06-01 (RR). This work was supported by grants from the Government of Navarra ("*Ayudas para la Realización de Proyectos de Investigación"* 2006–2007) to PP.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at doi:10.1016/j.neurobiolaging.2009.01.009.

References

Arai, T., Nonaka, T., Hasegawa, M., Akiyama, H., Yoshida, M., Hashizume, Y., Tsuchiya, K., Oda, T., Ikeda, K., 2003. Neuronal and glial inclusions in frontotemporal dementia with or without motor neuron disease are immunopositive for p62. Neurosci. Lett. 342, 41–44.

Baker, M., Mackenzie, I.R., Pickering-Brown, S.M., Gass, J., Rademakers, R., Lindholm, C., Snowden, J., Adamson, J., Sadovnick, A.D., Rollinson, S., Cannon, A., Dwosh, E., Neary, D., Melquist, S., Richardson, A., Dickson, D., Berger, Z., Eriksen, J., Robinson, T., Zehr, C., Dickey, C.A., Crook, R., McGowan, E., Mann, D., Boeve, B., Feldman, H., Hutton, M., 2006. Mutations in progranulin cause tau-negative frontotemporal dementia linked to chromosome 17. Nature 442, 916–919.

Barrett, J.C., Fry, B., Maller, J., Daly, M.J., 2005. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21, 263–265.

Cairns, N.J., Bigio, E.H., Mackenzie, I.R., Neumann, M., Lee, V.M., Hatanpaa, K.J., White 3rd, C.L., Schneider, J.A., Grinberg, L.T., Halliday, G., Duyckaerts, C., Lowe, J.S., Holm, I.E., Tolnay, M., Okamoto, K., Yokoo, H., Murayama, S., Woulfe, J., Munoz, D.G., Dickson, D.W., Ince, P.G., Trojanowski, J.Q., Mann, D.M., 2007. Neuropathologic diagnostic and nosologic criteria for frontotemporal lobar degeneration: consensus of the Consortium for Frontotemporal Lobar Degeneration. Acta Neuropathol. (Berl.) 114, 5–22.

de Bakker, P.I., Yelensky, R., Pe'er, I., Gabriel, S.B., Daly, M.J., Altshuler, D., 2005. Efficiency and power in genetic association studies. Nat. Genet. 37, 1217–1223.

Gabriel, S.B., Schaffner, S.F., Nguyen, H., Moore, J.M., Roy, J., Blumenstiel, B., Higgins, J., DeFelice, M., Lochner, A., Faggart, M., Liu-Cordero, S.N., Rotimi, C., Adeyemo, A., Cooper, R., Ward, R., Lander, E.S., Daly, M.J., Altshuler, D., 2002. The structure of haplotype blocks in the human genome. Science 296, 2225–2229.

Gass, J., Cannon, A., Mackenzie, I.R., Boeve, B., Baker, M., Adamson, J., Crook, R., Melquist, S., Kuntz, K., Petersen, R., Josephs, K., SM, P.-B., Graff-Radford, N., Uitti, R., Dickson, D., Wzsolek, Z., Gonzalez, J., Beach, T.G., Bigio, E., Johnson, N., Weintraub, S., Mesulam, M., White III, C.L., Woodruff, B., Caselli, R., Hsiung, G.Y., Feldman, H., Knopman, D., Hutton, M., Rademakers, R., 2006. Mutations in progranulin are a major cause of ubiquitin-positive frontotemporal lobar degeneration. Hum. Mol. Genet. 15, 2988–3001.

Hutton, M., Lendon, C.L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., Hackett, J., Adamson, J., Lincoln, S., Dickson, D., Davies, P., Petersen, R.C., Stevens, M., de Graaff, E., Wauters, E., van Baren, J., Hillebrand, M., Joosse, M., Kwon, J.M., Nowotny, P., Che, L.K., Norton, J., Morris, J.C., Reed, L.A., Trojanowski, J., Basun, H., Lannfelt, L., Neystat, M., Fahn, S., Dark, F., Tannenberg, T., Dodd, P.R., Hayward, N., Kwok, J.B., Schofield, P.R., Andreadis, A., Snowden, J., Craufurd, D., Neary, D., Owen, F., Oostra, B.A., Hardy, J., Goate, A., van Swieten, J., Mann, D., Lynch, T., Heutink, P., 1998. Association of missense and 5′-splice-site mutations in tau with the inherited dementia FTDP-17. Nature 393, 702–705.

Mackenzie, I.R., Baborie, A., Pickering-Brown, S., Plessis, D.D., Jaros, E., Perry, R.H., Neary, D., Snowden, J.S., Mann, D.M., 2006. Heterogeneity of ubiquitin pathology in frontotemporal lobar degeneration: classification and relation to clinical phenotype. Acta Neuropathol. (Berl.) 112, 539–549.

Momeni, P., Schymick, J., Jain, S., Cookson, M.R., Cairns, N.J., Greggio, E., Greenway, M.J., Berger, S., Pickering-Brown, S., Chio, A., Fung, H.C., Holtzman, D.M., Huey, E.D., Wassermann, E.M., Adamson, J., Hutton, M.L., Rogaeva, E., St George-Hyslop, P., Rothstein, J.D., Hardiman, O., Grafman, J., Singleton, A., Hardy, J., Traynor, B.J., 2006. Analysis of IFT74 as a candidate gene for chromosome 9p-linked ALS-FTD. BMC Neurol. 6, 44.

Morita, M., Al-Chalabi, A., Andersen, P.M., Hosler, B., Sapp, P., Englund, E., Mitchell, J.E., Habgood, J.J., de Belleroche, J., Xi, J., Jongjaroenprasert, W., Horvitz, H.R., Gunnarsson, L.G., Brown Jr., R.H., 2006. A locus

on chromosome 9p confers susceptibility to ALS and frontotemporal dementia. Neurology 66, 839–844.

Neary, D., Snowden, J.S., Gustafson, L., Passant, U., Stuss, D., Black, S., Freedman, M., Kertesz, A., Robert, P.H., Albert, M., Boone, K., Miller, B.L., Cummings, J., Benson, D.F., 1998. Frontotemporal lobar degeneration: a consensus on clinical diagnostic criteria. Neurology 51, 1546–1554.

Petrucelli, L., Dawson, T.M., 2004. Mechanism of neurodegenerative disease: role of the ubiquitin proteasome system. Ann. Med. 36, 315–320.

Pickering-Brown, S.M., 2007. The complex aetiology of frontotemporal lobar degeneration. Exp. Neurol. 114, 39–47.

Pickering-Brown, S.M., Richardson, A.M., Snowden, J.S., McDonagh, A.M., Burns, A., Braude, W., Baker, M., Liu, W.K., Yen, S.H., Hardy, J., Hutton, M., Davies, Y., Allsop, D., Craufurd, D., Neary, D., Mann, D.M., 2002. Inherited frontotemporal dementia in nine British families associated with intronic mutations in the tau gene. Brain 125, 732–751.

Qian, J., Yang, J., Zhang, X., Zhang, B., Wang, J., Zhou, M., Tang, K., Li, W., Zeng, Z., Zhao, X., Shen, S., Li, G., 2001. Isolation and characterization of a novel cDNA, UBAP1, derived from the tumor suppressor locus in human chromosome 9p21–22. J. Cancer Res. Clin. Oncol. 127, 613–618.

Schwartz, A.L., Ciechanover, A., 1999. The ubiquitin-proteasome pathway and pathogenesis of human diseases. Annu. Rev. Med. 50, 57–74.

Sham, P.C., Curtis, D., 1995. Monte Carlo tests for associations between disease and alleles at highly polymorphic loci. Ann. Hum. Genet. 59, 97–105.

Singleton, A., Myers, A., Hardy, J., 2004. The law of mass action applied to neurodegenerative disease: a hypothesis concerning the etiology and pathogenesis of complex diseases. Hum. Mol. Genet. 13 (1), R123–126.

Skibinski, G., Parkinson, N.J., Brown, J.M., Chakrabarti, L., Lloyd, S.L., Hummerich, H., Nielsen, J.E., Hodges, J.R., Spillantini, M.G., Thusgaard, T., Brandner, S., Brun, A., Rossor, M.N., Gade, A., Johannsen, P., Sorensen, S.A., Gydesen, S., Fisher, E.M., Collinge, J., 2005. Mutations in the endosomal ESCRTIII-complex subunit CHMP2B in frontotemporal dementia. Nat. Genet. 37, 806–808.

Stephens, M., Donnelly, P., 2003. A comparison of bayesian methods for haplotype reconstruction from population genotype data. Am. J. Hum. Genet. 73, 1162–1169.

Stephens, M., Smith, N.J., Donnelly, P., 2001. A new statistical method for haplotype reconstruction from population data. Am. J. Hum. Genet. 68, 978–989.

Storey, J.D., Tibshirani, R., 2003. Statistical significance for genomewide studies. Proc. Natl. Acad. Sci. U.S.A. 100, 9440–9445.

Thomas, B., Beal, M.F., 2007. Parkinson's disease. Hum Mol Genet 16 (2), R183–194.

Vance, C., Al-Chalabi, A., Ruddy, D., Smith, B.N., Hu, X., Sreedharan, J., Siddique, T., Schelhaas, H.J., Kusters, B., Troost, D., Baas, F., de Jong, V., Shaw, C.E., 2006. Familial amyotrophic lateral sclerosis with frontotemporal dementia is linked to a locus on chromosome 9p13.2–21.3. Brain 129, 868–876.

Watts, G.D., Wymer, J., Kovach, M.J., Mehta, S.G., Mumm, S., Darvish, D., Pestronk, A., Whyte, M.P., Kimonis, V.E., 2004. Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. Nat. Genet. 36, 377–381.

Xiao, S., Sato, C., Kawarai, T., Goodall, E.F., Pall, H.S., Zinman, L.H., Robertson, J., Morrison, K., Rogaeva, E., 2007. Genetic studies of GRN and IFT74 in amyotrophic lateral sclerosis. Neurobiol. Aging 29, 1279–1282.