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ARTICLE

Refinement of the PARK3 locus on chromosome 2p13 and the analysis of 14 candidate genes

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Parkinson's disease (PD) is a common neurodegenerative disorder with clinical features of bradykinesia, rigidity, resting tremor and postural instability resulting from the deficiency of dopamine in the nigrostriatal system. Previously we mapped a susceptibility gene for an autosomal dominant form of PD to a 10.6 cM region of chromosome 2p (PARK3; OMIM 602404). A common haplotype shared by two North American kindreds (Families B and C) genealogically traced to Southern Denmark and Northern Germany suggested a founder effect. Here we report progress in the refinement of the PARK3 locus and sequence analysis of candidate genes within the region. Members of families B and C were genotyped using polymorphic markers, reducing the minimum common haplotype to eight markers spanning a physical distance of 2.5 Mb. Analysis of 14 genes within the region did not reveal any potentially pathogenic mutations segregating with the disease, implying that none of these genes are likely candidates for PARK3. *European Journal of Human Genetics* (2001) **9,** 659–666.

Keywords: PARK3; Parkinson's disease; chromosome 2p13; genetic and physical mapping

Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative disorders with a prevalence of greater than 2% among persons over the age of 65.[1,2] The pathological features of PD include the presence of intracytoplasmic inclusions, termed Lewy bodies and the loss of dopaminergic neurons in the substantia nigra pars compacta.[3] The mechanism(s) underlying the formation of Lewy bodies and selective death of nigral neurons remains unknown. Clinically, PD is characterised by bradykinesia, rigidity, resting tremor and postural instability, resulting from the deficiency of the neurotransmitter dopamine in the nigrostriatal system.

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The majority of PD patients present with sporadic PD, although there is increasing evidence that genetic factors have an important role in the susceptibility to PD.[4] Genetic components have been demonstrated in epidemiological surveys of PD,[5] twin studies[6] and by genetic linkage analyses of families in which the disease segregates as a Mendelian trait.[7] Six genetic loci on chromosomes 2p13, 4p15, 4p14, 4q21-23, 6q34 and 17q21 and four of the underlying mutant genes have been identified.[8] The study of multigenerational families with monogenically inherited forms of PD facilitates the cloning of mutant genes, allowing the creation of cellular and animal models of neurodegenerative disorders. Understanding the molecular aetiology of PD will lead to the identification of biomarkers for the condition, rationale drug design and the development of new treatment strategies.[9] Mutations in alpha-synuclein (PARK1, chromosome 4q21-23) and ubiquitin C-terminal hydrolase genes (UCH-L1, chromosome 4p14) are responsible for some forms of autosomal dominant PD.[10–12] Alpha-synuclein is a major

component of Lewy bodies[13] and is thought to play a role in neuronal plasticity and synaptic function.[14] Similarly, UCH-L1 is a major component of Lewy bodies[15] and is a key component of the proteosome proteolytic pathway.[16]

Previously, we mapped a third locus for autosomal dominant PD (PARK3) to chromosome 2p13.[17] The clinical features of affected individuals resemble those of sporadic PD, including a similar mean age of onset (61 years in these two families). Follow-up examinations over the years revealed that, in addition to parkinsonism, several members of 2p13 linked families showed signs of dementia.[18] Neuropathology revealed neuronal loss in the substantia nigra, typical brain-stem Lewy-bodies and the presence of neurofibrillary tangles and Alzheimer plaques.[18,19] Therefore, the underlying mutation may be associated with a range of phenotypes that includes varying degrees of dementia and Alzheimer's disease pathology.

The maximum multipoint lod score for all six families in the original study was 3.96, considering affected members only and allowing for genetic heterogeneity. Four of the six families analysed showed positive lod scores. Two of the families supporting linkage to this locus (Families B, Danish-North American; and C, German-American) were historically and genealogically traced to a relatively small area in Southern Denmark and Northern Germany.[20] Affected individuals shared a common haplotype over eight markers within the linked region, suggesting the possibility of a founder mutation. Based on the occurrence of the 'affected' haplotype in clinically asymptomatic members in these two linked families, the penetrance of the mutation was estimated to be 40%, suggesting that it might also play a role in apparently sporadic cases of PD. However, the founder haplotype has not been identified in other German patients with familial and sporadic PD residing in the area of Germany to which the origin of family C was traced,[21,22] arguing against a recent and prevalent founder-effect in Germany. Further population studies, including PD patients from Southern Denmark, will be required to identify a possible ancient founder-effect that may help to define the PARK3 locus.

Here we describe progress in the refinement of the PARK3 locus and sequence analysis of candidate genes within the region. We report the refinement of PARK3 to a minimum common haplotype consisting of eight markers that span a physical distance of approximately 2.5 Mb and the sequence analysis of 14 candidate genes in the 2p13 region.

Methods

Patients

This study was approved by the local ethics committees of the institutions involved. Family B (Danish-North American) and Family C (German-American) have been longitudinally studied by ZKW since 1991.[20] Family members currently reside in the US and Canada. Informed consent was obtained, venous whole blood samples were taken and DNA was extracted following standard protocols. Clinical details of families B and C have been previously reported.[18,20] Briefly, all affected persons (Figure 1) had clinically definite parkinsonism as defined by Calne.[23] Positron emission tomography and autopsy findings of affected individuals have been published elsewhere and will not be discussed in this report due to space considerations.[24]

Genotype analysis

To refine the haplotype shared by families B and C, 17 microsatellite markers on chromosome 2p13 were genotyped in all available family members. Genotyping was performed from genomic DNA. Dideoxy cycle sequencing of PCR products amplified from genomic DNA or from cDNA was performed with the AmpliSequence sequencing kit (Perkin-Elmer) after purification with QIAquick[9] PCR Purification Kit (Qiagen) on ABI 310 and ABI 377 (ABI Inc., U.S.A.) automated sequencers with a fluorescence detection system. Markers are listed in Figure 1; primer sequences are available upon request from the authors. Haplotypes were constructed by hand and using the SIMWALK2 program[25] (Figure 1B,C).

Construction of an integrated genetic and physical map

Markers D2S292, D2S291, D2S2110, D2S1394, D2S2111, D2S145 and D2S2109 were found and positioned by searching the Genome Database GDB (http://gdbwww.gdb.org). Markers PAC3, CY172, PAC35 and CY7-PH3 were found from a previously described PAC contig. [26] Markers NT53, 7002, 6461, 6544, 5034, 7099 and 5041 were found by searching electronically for CA repeats within the homonymic BAC clones known to be located in that region using the National Center for Biotechnology Information's Electronic PCR resource (http://www.ncbi.nlm.nih.gov/).

Candidate gene analysis

Candidate genes were selected using GeneMap'99 (http://www.ncbi.nlm.nih.gov/genemap/). Fourteen genes were found to be mapped in or near the candidate region (Figure 2). The candidate gene's cDNA sequence was derived from Entrez (http://www.ncbi.nlm.nih.gov/Entrez/) and each sequence was BLASTed against the human genome database (http://www.ncbi.nlm.nih.gov/BLAST/).

cDNA from affected members of family B and C was obtained through reverse transcription PCR of total RNA (5 μg) from lymphoblastoid cell lines using the Superscript II reverse transcriptase (Gibco) according to manufacturers instructions. Total RNA was isolated from cells using Trizol reagent (Gibco) according to the manufacturer's instructions. Primers (LifeTech) were designed for cDNA (NP220, EGR4, DOK1, TIA1, SEMAW, GCS1, PIR-1, SLC1A4, Ccth, KIAA0919, AMSH and NN84AG) and/or genomic amplification (SEMAW, DCTN1 and SPR) in 300 to 400 base pair intervals. Primer sequences are available from the authors upon request. Candidate gene primers designed from cDNA

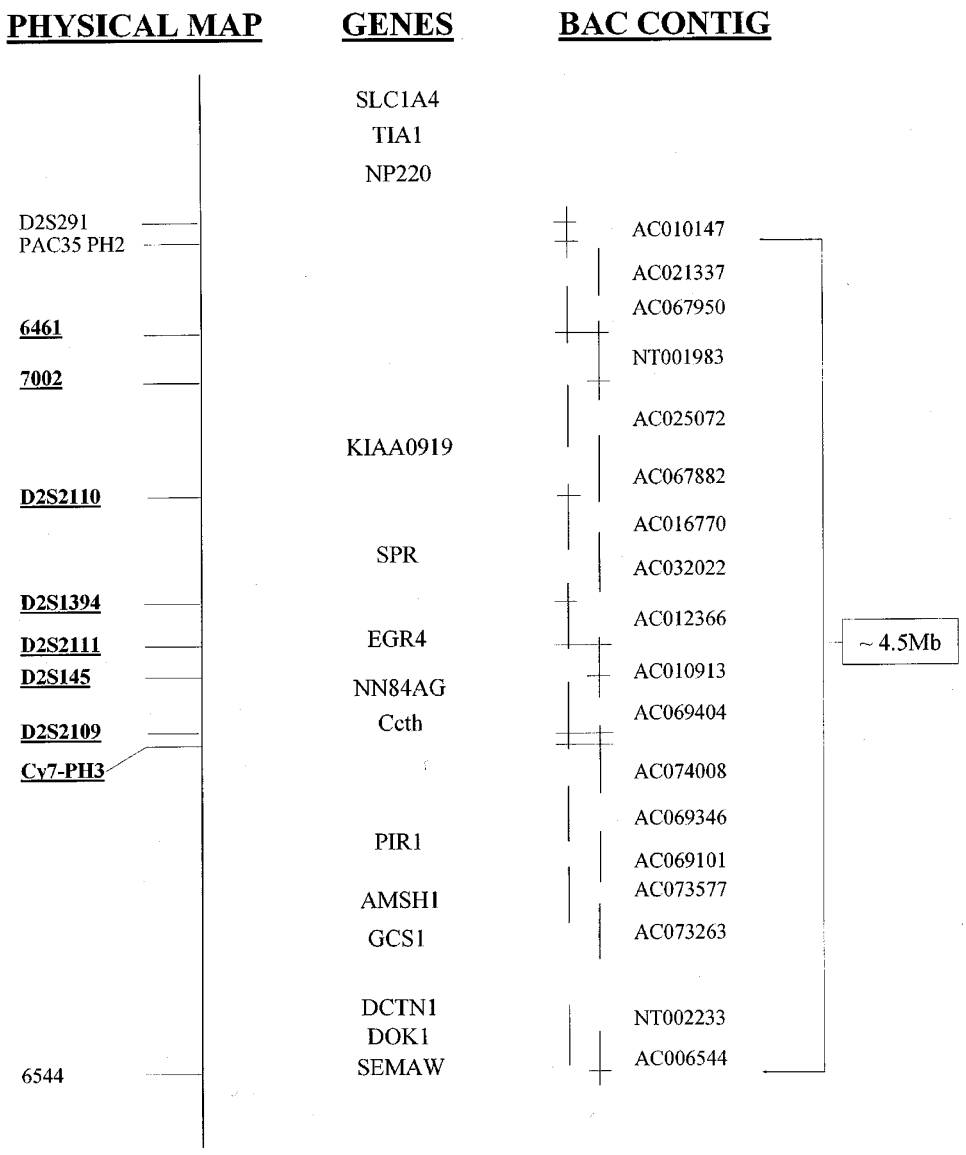

Figure 1 Integrated genetic and physical map of the PARK3 locus. Markers were assigned to individual BAC clones by PCR analysis or utilising NCBI Electronic PCR. The map positions of partially sequenced BAC clones and size of the critical region were derived from the NCBI database. The position and approximate size of the partially sequenced BAC clone contig were derived from the Washington University Genome Sequencing Center. Markers highlighted in bold represent the minimum common haplotype region.

sequence were initially screened with a whole brain total RNA library (Clontech) to check for neuronal expression, quality and correct predicted size of the PCR product. All primer pairs amplified the correct product using the whole brain control cDNA (data not shown).

PCR was performed using 40 ng genomic DNA or cDNA as template, 0.8 μM of each forward and reverse primer, 1 unit of *Taq* polymerase, 5 μl of Q solution (Qiagen) and 5 mM dinucleotide triphosphates (dATP, dCTP, dGTP and dTTP) in a 25 μl reaction. Amplification was performed using a touchdown protocol over 35 cycles with a final extension at 72°C for 10 min. PCR products were then purified from excess primer using Qiagen PCR purification columns and the product concentration subsequently estimated on an ethidium bromide stained agarose gel with a low molecular weight mass marker (LifeTech). Sequencing was performed on an ABI377 automated sequencer. Heterozygote base calls and sequence alignment was performed with PolyPhred-Phrap.[27] Base calls were made with Phred (version 0.961028) assembled with Phrap (version 0.960731), scanned by PolyPhred (version 0.970312) and the results viewed with Consed 4.0. Opposite strand sequencing was used in all cases

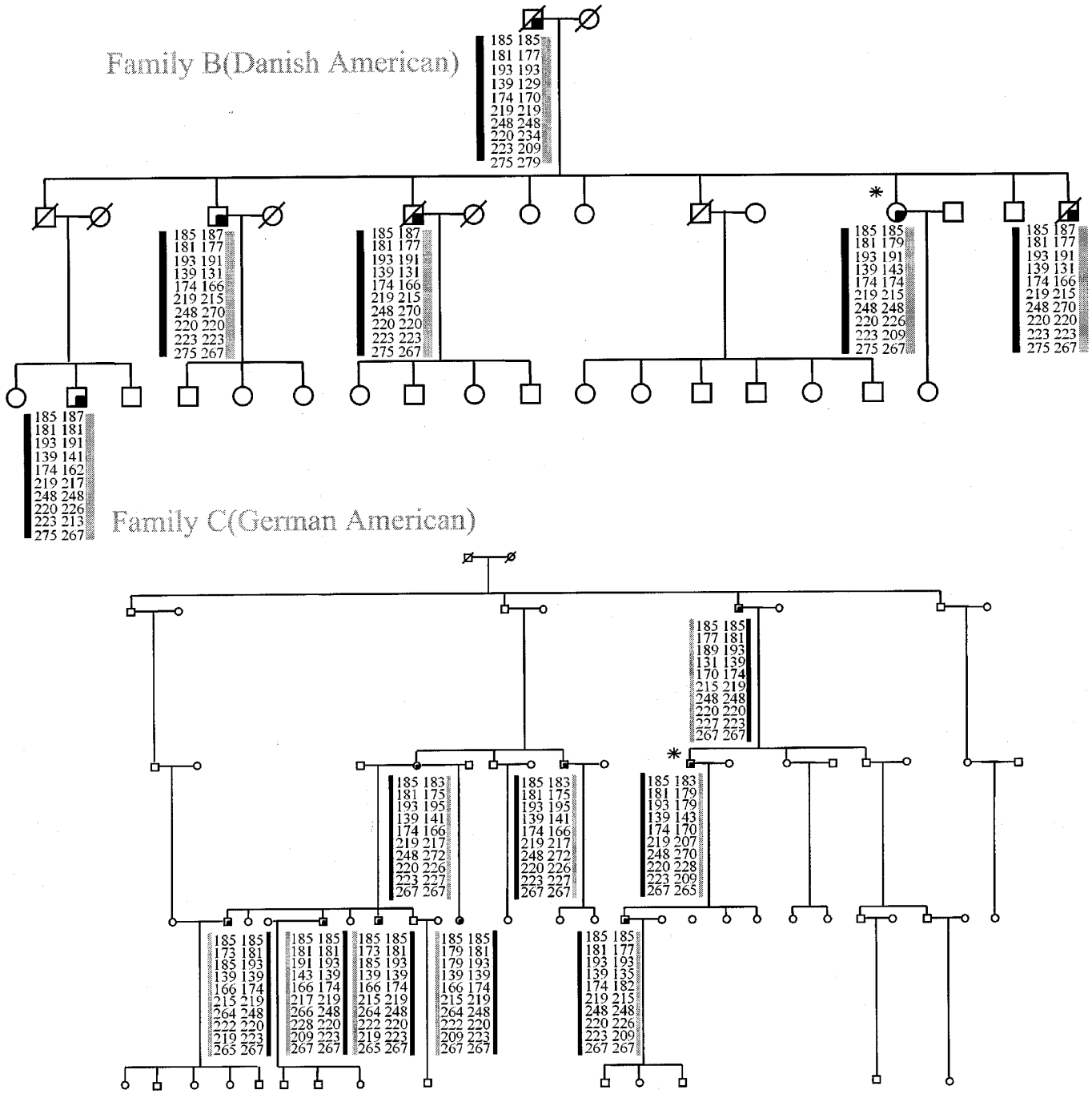

Figure 2 Segregation of a common haplotype. Data from 10 markers are shown in the following order in the above chart: D2-PAC35, 6461, 7002, D2S2110, D2S1394, D2S2111, D2S145, D2S2109, CY7-PH3, 6544. Individuals diagnosed with dementia are denoted with an asterisk. The haplotypes of individuals not diagnosed with Parkinson's disease are not shown to protect the family member's confidentiality.

and poor quality parts of the read were checked directly on chromatograms and repeated as required. All candidate genes were sequenced from at least two affected persons from family B and two affected persons from family C, in addition to one unaffected family member and one unrelated control. Genetic variations were confirmed using standard restriction fragment length polymorphism (RFLP) techniques, involving digestion of a PCR product using a restriction enzyme

predicted to be informative (data not shown). Variants segregating in both families B and C were first screened against NCBI's SNP database (http://www.ncbi.nlm.nih.gov/SNP/). If unreported, polymorphic variants were evaluated in a control series consisting of 36 German unaffected individuals.

Results

Haplotype analysis

Based on the original haplotype analyses, the disease gene should reside in a region defined by markers D2S2113, D2S291. D2S2111, D2S2110, D2S145 and D2S1394, which corresponds to a genetic distance of 3.2 cM.[17] Integrating all the available information, a physical and genetic map was constructed (Figure 2). Map positions of partially sequenced BAC clones suggest that the interval between the two closest markers not shared by both families (PAC35 PH2 telomeric and 6544 centromeric) is approximately 4 to 5 Mb, thus defining the critical PARK3 region. A gap of approximately 250 Kb exists between BACs AC073263 and NT002233. Based on the insert size of the BAC contig, we estimate that the minimum common haplotype of markers 6461 to Cy7-PH3 corresponds to a physical distance of approximately 2.5 Mb.

Sequencing of candidate genes

The following genes map to the 2p13 region (NCBI GeneMap'99) and were considered as potential candidates for PARK3. A summary of the genetic variants detected is presented in Table 1.

Table 1 Summary of candidate gene variants in families B and C with the associated BAC given for each gene, as determined by BLAST

Gene	*BAC Accession*	*Family B*	*Family C*	*Amplification*
SLC1A4	AC068347			cDNA
TIA1	AC016700			cDNA
NP220	AC055886	G/A at 5491 and T/C at 3447		cDNA
KIAA0919	AC067882			Genomic and cDNA
SPR	AC032022			Genomic and cDNA
EGR4	AC010913			cDNA
NN84AG	AC010913	C/T at 2254		cDNA
Ccth	AC069404	T/A at 1379		cDNA
PIR1	AC010169	G/A at 90		cDNA
AMSH	AC011137			cDNA
GCS1	AC005041	G/A at 847 C/T at 1009T		cDNA
DCTN1	AC005041			Genomic
DOK1	AC006544			cDNA
SEMAW	AC006544	G/T at 375		Genomic and cDNA

Single nucleotide polymorphism positions given correspond to the published mRNA sequence. With the exception of GCS1 polymorphisms, no SNPs result in amino acid substitutions. GCS1 polymorphisms do not segregate with disease in family B, suggesting the gene is likely to map out the common haplotype region.

SLC1A4 is a 524 amino acid protein involved in the transport of neutral amino acids.[28] No sequence variations in the corresponding gene were found among the individuals screened.

TIA1 (cytotoxic granule-associated RNA-binding protein) is a 386 amino acid protein that possesses three RNA binding domains. Both natural and recombinant TIA1 were found to induce DNA fragmentation in digitonin permeabilised thymocytes, thus TIA1 may be part of the granule components responsible for inducing apoptosis in lymphocyte target cells.[29] No polymorphisms were found in both the normal and alternatively spliced form of the gene.

NP220 (mRNA for nuclear protein) is a large DNA-binding protein of 1978 amino acids. NP220 may be important for organising chromosomes, localising genes, and regulating DNA transcription and replication.[30] Two polymorphisms were found in both families B and C: the first affects a *Nla*III site at position 3447 in the mRNA sequence, the second affects a *Ban*I site at position 5491. The 3447 polymorphism is either C or T, 57% T in a control population. The 5491 polymorphism is either G or A, 74% G in a control population. Neither of the polymorphisms results in a change to the amino acid sequence of the protein.

KIAA0919 was originally identified as part of a cDNA project in which the coding sequence of human brain expressed genes were identified.[31] The gene is composed of 16 exons and its encoded protein consists of 882 amino acids. A rat homologue of KIAA0919, rsec 15 was recently cloned and has been shown to be part of the mammalian exocyst complex.[32] Sequence variations were found in KIAA0919.

SPR (Sepiapterin reductase) consists of 261 amino acids and is composed of 3 exons spanning approximately 4 kilobases. The gene catalyses the final one or two reductions in tetrahydrobiopterin biosynthesis to form 6,7,8-tetrahydrobiopterin, an essential cofactor for aromatic amino acid hydroxylases such as phenylalanine hydroxylase, tyrosine hydroxylase and tryptophan hydroxylase.[33] No polymorphisms were found in the exons of the encoding gene. In addition, two kilobases of DNA upstream of SPR exon one were sequenced with no variations found.

EGR4 (early growth response 4) is a 486 amino acid zinc finger transcription factor, which is involved in cell proliferation and cell cycling.[34] No sequence variants were found in the mRNA sequence.

NN84AG (retinoic acid response protein) was originally found to be upregulated by retinoic acid in F9 embryonal carcinoma cells. It is thought to be involved in the regulation of other genes, however its specific biological function is unknown.[35] One polymorphism was identified in the 3′ untranslated region that segregates in both families B and C. This C or T polymorphism at position 2254, which affects an *Alu*I site with the addition of a mismatched primer, is present in a control population as 94% C.

Ccth is a 543 amino acid subunit of the chaperonin complex CCT or TCP-1 ring complex (chaperonin containing

t-complex polypeptide 1/TRiC), which is involved in chaperoning monomeric protein folding.[36] One polymorphism was found in families B and C at position 1379 of the mRNA sequence, but this change does not alter the translated sequence. This T or A variant has been previously described in the public SNP database, accession no.1530568, ID no.7851 and is a common polymorphism.

PIR1 (protein tyrosine phosphatase) is involved in the regulation of cellular processes such as signal transduction, cell cycle progression and tumor suppression. PIR1 can bind RNA *in vitro* and may participate in mRNA metabolism.[37] One polymorphism was found in the 5′ untranslated region in family B and C members, which disrupts a *Nae*I site. This G or A variant was present at 50% G in a control population.

AMSH (Associated Molecule with the SH3 domain of STAM) encodes a 424 amino acid protein that contains an SH3 domain. It has been suggested that AMSH plays a critical role in cytokine-mediated intracellular signal transduction.[38] No polymorphisms in this gene were found.

GCS1 (Glucosidase I) is an 834 amino acid protein involved in the N-linked oligosaccharide processing pathway.[39] Two polymorphisms were found in the coding region of GCS1 in members of family B but not in family C: the first, a G or A variant at position 847, affects a *Hph*I site and changes amino acid number 238 from asparagine to aspartic acid. The second polymorphism, a C or T variant at position 1009, affects an *Apa*I site and converts a proline 292 to serine.

DCTN1 (Dynactin) is encoded by 32 exons and several functionally distinct isoforms are produced by alternate splicing of the transcript in human neurons.[40] Dynactin is a required activator for vesicular transport along microtubules catalysed by the molecular motor cytoplasmic dynein.[41] No polymorphisms in the encoding gene were found.

DOK1 (docking protein 1) a 481 amino acid protein, was originally found to be associated with the p120 ras GTPase-activating protein (GAP) and has been implicated as a component of the signal transduction pathway downstream of receptor tyrosine kinases.[42] No polymorphisms in the encoding gene were found.

SEMAW (Semaphorin W) consists of two isoforms, which are produced by alternative splicing. SemaW possesses one transmembrane domain and belongs to the class IV subgroup of the semaphorin family. SemaW is expressed at low levels in the developing embryo, but at high levels in the adult CNS and may be involved in axonal guidance.[43] One polymorphism was identified at position 375 in members of family B but not family C, which affects a *Nla*IV site and does not alter the translated sequence.

Discussion

Parkinson's disease (PD) is a complex multifactorial disease resulting from an interaction between environmental and genetic susceptibility factors.[44] The identification and study of associated mutant genes provides valuable tools for understanding the pathological progression of disease and the development of rational treatment strategies.

Previously, we mapped a susceptibility locus for autosomal dominant PD to a 3.2 cM region of chromosome 2p13.[17] In this study we utilised sequence data generated by the Human Genome Project (HGP) and PCR to construct a BAC-based physical map that covers the PARK3 locus. One gap of approximately 250 Kb remained in the contig, located between the last marker common to affected individuals from families B and C (CY7-PH3) and the first non-conserved marker (6544; Figure 2). This region will be filled, as sequence data becomes available via the HGP.[45,46] Alternatively, this gap may prove to be outside of the critical PARK3 region, as suggested by Single Nucleotide Polymorphism (SNP) analysis of candidate genes (see below). This contig allowed us to refine the minimum common haplotype for PARK3 to a region defined by the polymorphic markers 6461 and CY7-PH3, corresponding to a physical distance of approximately 2.5 Mb. Based on BAC insert size, we estimate the critical region containing PARK3, flanked by the first two non-conserved markers (PAC35-PH2 and 6544) to be approximately 4.5 Mb.

Candidate genes located within the 2p13 region (Genemap '99) were selected for sequence analysis in affected individuals from families B and C. Sequence analysis of the genes SLC1A4, TIA-1 and NP220 did not reveal any coding sequence mutations in affected individuals. Two silent polymorphisms, which segregated with disease in families B and C, were detected in the coding sequence of NP220. However, these alterations represent common SNPs as they were also observed in a normal control population. Subsequent to sequence analysis these three genes were mapped telomeric to the critical PARK3 region and can probably be excluded as candidates for PARK3. Similarly, SemaW was subsequently mapped out of the critical PARK3 region. The gene is located in the same BAC clone as the polymorphic marker 6544 (AC006544) but is nearer to the centromere. A silent polymorphism at nucleotide 375 was detected in affected individuals from family B but not family C (Table 1), confirming the common haplotype ends telomeric of the polymorphic marker 6544.

The genes KIAA0919, SPR, EGR4, NN84AG and Ccth are all located within the common PARK3 haplotype. KIAA0919 and SPR were considered attractive candidates for PARK3 as both are expressed in the brain and are involved in exocytosis and tyrosine hydroxylase synthesis, respectively.[31,32] The coding sequence and approximately 20 bp of each flanking intron was amplified from genomic DNA and sequenced. However, no sequence alterations were detected in either gene, suggesting they do not correspond to PARK3. Similarly, no changes were detected in the coding sequence of EGR4, NN84AG and CcTH. There was a polymorphism in the 3′ UTR of NN84AG and one in the 3′ UTR of Ccth. Both polymorphisms segregated with disease in affected indivi-

duals from families B and C, as expected given the genes are located within the common haplotype (Figure 2).

A further five candidate genes located in the PARK3 region were sequenced. PIR1, AMSH, GCS1, DCTN1 and DOK1 are all located centromeric to the last common polymorphic marker (CY7-PH3), but telomeric to the first non-conserved marker (6544) and hence fall within the approximately 4.5 Mb PARK3 interval. No sequence variants were detected in AMSH, DCTN1 or DOK1. A polymorphism was detected in the 5′ UTR of PIR1, which segregated with the disease in affected individuals from families B and C (Table 1). In contrast, two coding sequence alterations were detected in GCS1 in affected individuals from family B (Table 1). These resulted in the non-conservative amino acid substitutions N238D and P292S respectively. However, these sequence alterations were not observed in affected individuals from family C. These results suggest none of these five genes are likely to correspond to PARK3.

The observation of a polymorphism in PIR1 segregating with disease in both families, but coding sequence changes in GCS1 segregating with disease in family B only, suggest the haplotype shared by the two families ends between PIR1 and GCS1. If so, the critical PARK3 region will be reduced by a minimum of approximately 1 Mb, and the current contig gap located between AC073263 and NT002233 will be eliminated. Additional polymorphic markers located between CY7-PH3 and 6544 are currently being generated and typed in families B and C to further refine the PARK3 locus.

In summary, fourteen candidate genes for PARK3 were sequenced and no potentially pathogenic mutations were detected, suggesting none of these genes correspond to PARK3. However, this assumes that the autosomal dominant inheritance of PARK3 results from a heterozygous mutation in the coding sequence of the gene, analogous to the A30P and A53T alterations described for autosomal dominant PARK1 mutations. The possibility exists that mutations in the noncoding portion of a gene located within the critical PARK3 region, for example EGR4, NN8-4AG, Ccth, PIR1, AMSH, GCS1 or DOK1, may give rise to the syndrome. The majority of Fronto-Temporal Dementia with Parkinsonism (FTDP-17) cases result from non-coding mutations in the tau gene.[47] Future studies will examine the intron/exon junctions of genes within the critical PARK3 region.

No other currently described genes map within the critical PARK3 region, although the rapid progress in sequencing the human genome may result in the localisation of additional known genes within the region.[45,46] We have identified greater than 20 unigene clusters and hundreds of ESTs that map within the common haplotype, suggesting PARK3 may encode a previously uncharacterised gene. We are currently prioritising Unigene clusters and ESTs, based on what is known of their expression or function, for future cloning and sequence analysis. The physical map and refined haplotype reported here will contribute to the development of a detailed transcript map of the region and should result in the identification and analysis of PARK3.

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