IV. 研究成果の刊行物・別刷

THE INTERNATIONAL WEEKLY JOURNAL OF SCIENCE

nature

Pathogenic exon-trapping by SVA retrotransposon and rescue in Fukuyama muscular dystrophy

Mariko Taniguchi-Ikeda[1,2]*, Kazuhiro Kobayashi[1]*, Motoi Kanagawa[1], Chih-chieh Yu[1], Kouhei Mori[1], Tetsuya Oda[1], Atsushi Kuga[1], Hiroki Kurahashi[3], Hasan O. Akman[4], Salvatore DiMauro[4], Ryuji Kaji[5], Toshifumi Yokota[6], Shin'ichi Takeda[7] & Tatsushi Toda[1]

[1]Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan. [2]Division of General Pediatrics, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan. [3]Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, Aichi 470-1192, Japan. [4]Department of Neurology, Columbia University Medical Center,NewYork, NY10032, USA. [5]Department of Clinical Neuroscience, The University of Tokushima Graduate School, Tokushima 770-8503, Japan. [6]Department of Medical Genetics, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB T6G 2H7, Canada. [7]Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo 187-8502, Japan.

**These authors contributed equally to this work.*

Reprinted from Nature, Vol. 478, No. 7367, pp. 127–131, 6 October 2011

LETTER

doi:10.1038/nature10456

Pathogenic exon-trapping by SVA retrotransposon and rescue in Fukuyama muscular dystrophy

Mariko Taniguchi-Ikeda[1,2]*, Kazuhiro Kobayashi[1]*, Motoi Kanagawa[1], Chih-chieh Yu[1], Kouhei Mori[1], Tetsuya Oda[1], Atsushi Kuga[1], Hiroki Kurahashi[3], Hasan O. Akman[4], Salvatore DiMauro[4], Ryuji Kaji[5], Toshifumi Yokota[6], Shin'ichi Takeda[7] & Tatsushi Toda[1]

Fukuyama muscular dystrophy (FCMD; MIM253800), one of the most common autosomal recessive disorders in Japan, was the first human disease found to result from ancestral insertion of a SINE-VNTR-*Alu* (SVA) retrotransposon into a causative gene[1–3]. In FCMD, the SVA insertion occurs in the 3′ untranslated region (UTR) of the *fukutin* gene. The pathogenic mechanism for FCMD is unknown, and no effective clinical treatments exist. Here we show that aberrant messenger RNA (mRNA) splicing, induced by SVA exon-trapping, underlies the molecular pathogenesis of FCMD. Quantitative mRNA analysis pinpointed a region that was missing from transcripts in patients with FCMD. This region spans part of the 3′ end of the fukutin coding region, a proximal part of the 3′ UTR and the SVA insertion. Correspondingly, *fukutin* mRNA transcripts in patients with FCMD and SVA knock-in model mice were shorter than the expected length. Sequence analysis revealed an abnormal splicing event, provoked by a strong acceptor site in SVA and a rare alternative donor site in *fukutin* exon 10. The resulting product truncates the fukutin carboxy (C) terminus and adds 129 amino acids encoded by the SVA. Introduction of antisense oligonucleotides (AONs) targeting the splice acceptor, the predicted exonic splicing enhancer and the intronic splicing enhancer prevented pathogenic exon-trapping by SVA in cells of patients with FCMD and model mice, rescuing normal *fukutin* mRNA expression and protein production. AON treatment also restored fukutin functions, including *O*-glycosylation of α-dystroglycan (α-DG) and laminin binding by α-DG. Moreover, we observe exon-trapping in other SVA insertions associated with disease (hypercholesterolemia[4], neutral lipid storage disease[5]) and human-specific SVA insertion in a novel gene. Thus, although splicing into SVA is known[6–8], we have discovered in human disease a role for SVA-mediated exon-trapping and demonstrated the promise of splicing modulation therapy as the first radical clinical treatment for FCMD and other SVA-mediated diseases.

FCMD (incidence 1/34,000 births) shares phenotypic similarities with other severe muscular dystrophies, including muscle–eye–brain disease and Walker–Warburg syndrome. All show deficiencies in *O*-glycosylation of α-DG, an extracellular protein anchored on the plasma membrane. Insufficient *O*-glycosylation interferes with the ability of α-DG to interact with extracellular matrix proteins such as laminin[9,10]. For this reason, FCMD, muscle–eye–brain disease and Walker–Warburg syndrome are categorized as 'α-dystroglycanopathies (α-DGpathy)'[10]; so far, no effective treatments exist for these conditions. SVA is a hominid-specific, composite non-coding retrotransposon that contains SINE (short interspersed sequence), VNTR (variable number of tandem repeat), and *Alu* sequences. It is still active in humans, polymorphic and mobilized by the human LINE-1 *in trans*[6,11–15].

In previous work, we showed that *fukutin* mRNA (10 exons, 7.4- and 6.4-kilobase (kb) cDNAs in size with two poly-A sites, 461-amino-acid protein with calculated molecular mass of 53.7 kDa) was not detectable by northern blot analysis in patients with FCMD carrying the SVA insertion[2]. To investigate the aetiology of this decreased expression, we have now analysed whole *fukutin* mRNA in lymphoblasts from patients with FCMD using quantitative PCR with reverse transcription (qRT–PCR). PCR products corresponding to the protein-coding region of *fukutin*, as well as those including sequences in the distal part of the 3′ UTR (and thus downstream of the SVA insertion), were similar in abundance to those from an unaffected control (Fig. 1a). However, products located at sequence positions within the 3′ UTR were markedly decreased relative to the control. From these results and along with previous reports of many 3′ and 5′ splice sites within SVA elements[6–8], we hypothesized that abnormal splicing occurs somewhere between the end of the fukutin protein-coding region and the SVA insertion.

We then performed long-range RT–PCR using primers that flank the region corresponding to decreased expression. In patients with FCMD, we detected a single 3-kb PCR product, which is shorter than the 5-kb product seen in the normal control (Fig. 1b). This observation was consistent in several tissue types from patients with FCMD (Supplementary Fig. 1). PCR from genomic DNA produced an 8-kb product in patients with FCMD, compared with a 5-kb product in the control (Fig. 1b). Sequence analysis of the 3-kb product from FCMD cDNA revealed a splicing event (Supplementary Fig. 2). This event generates a new donor-side breakpoint within the final coding exon (exon 10), located 116 base pairs (bp) upstream from the authentic stop codon. A rare alternative donor site at that position is activated and trapped by an alternative acceptor site located within the inserted SVA, creating an additional and aberrant exonic sequence (exon 11) (Fig. 1c). The acceptor-side breakpoint is located 274 bp downstream from the start of the SVA insertion, between ag and TC (Fig. 1c). The acceptor site has not been described in the previous reports of SVA splicing[6,7]. This location is preceded by a pyrimidine-rich stretch, the SVA $(TCTCCC)_{41}$ hexamer at the 5′ end of the SVA element, with a possible favourable branch point. Predicted exonic splicing enhancer sites occur around 70 bp downstream from the new acceptor site. We confirmed that the aberrant splicing event can be abolished by replacing AG with GG at the acceptor junction in cultured cells transfected with a *fukutin* construct carrying SVA insertion (Supplementary Fig. 3). *Fukutin* expression was not altered by cycloheximide treatment, indicating that the transcript was not subject to nonsense-mediated mRNA decay, possibly because this exon-trapping occurred within the last

[1]Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan. [2]Division of General Pediatrics, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan. [3]Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, Aichi 470-1192, Japan. [4]Department of Neurology, Columbia University Medical Center, New York, NY 10032, USA. [5]Department of Clinical Neuroscience, The University of Tokushima Graduate School, Tokushima 770-8503, Japan. [6]Department of Medical Genetics, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB T6G 2H7, Canada. [7]Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo 187-8502, Japan.
*These authors contributed equally to this work.

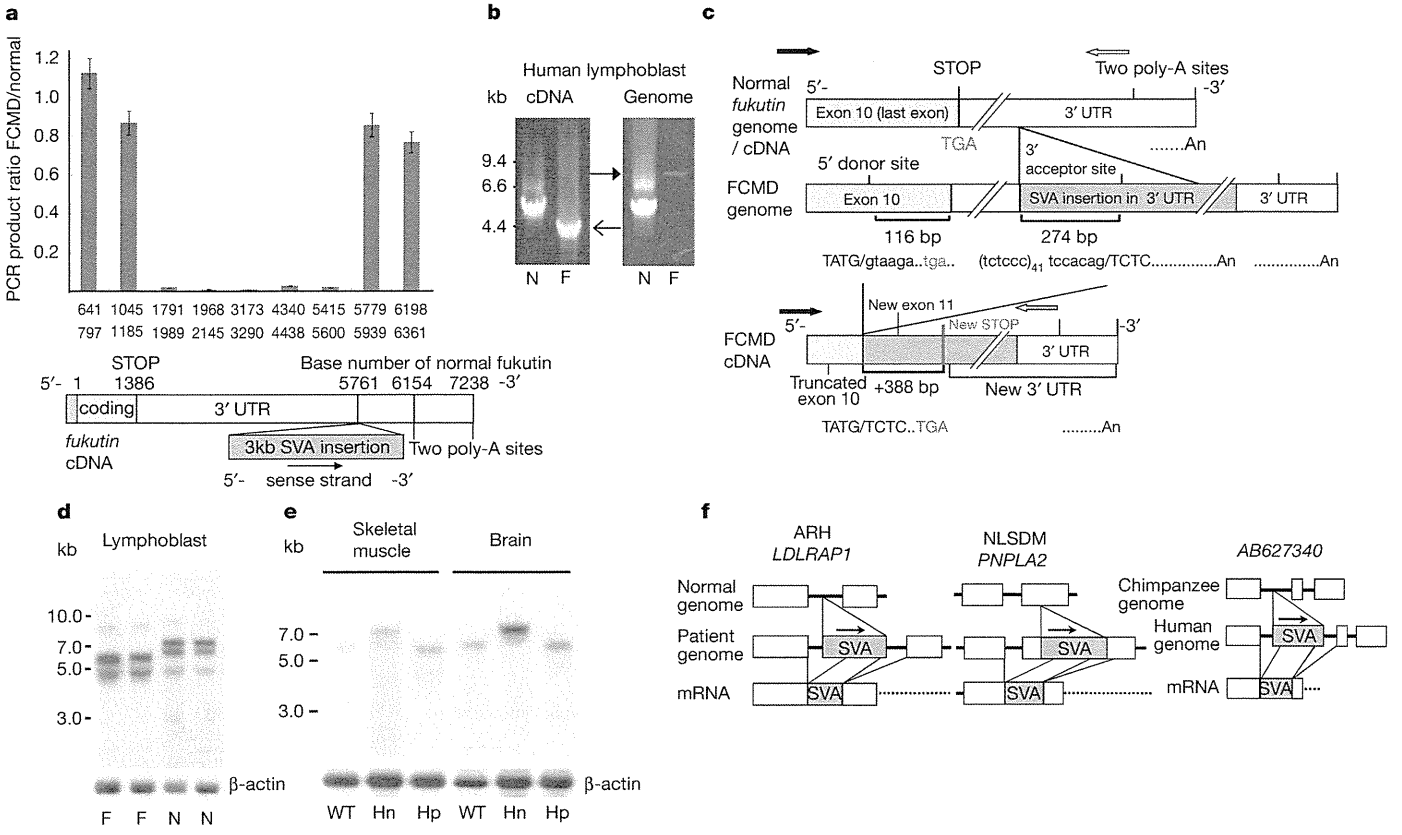

Figure 1 | An SVA retrotransposal insertion induces abnormal splicing in FCMD. **a**, Expression analysis of various regions of *fukutin* mRNA in lymphoblasts. Grey bar, the ratio of RT–PCR product in patients with FCMD relative to the normal control; numbers on the *x* axis, nucleotide positions of both forward and reverse primers in *fukutin*. Error bars, s.e.m. **b**, Long-range PCR using primers flanking the expression-decreasing area (nucleotide position 1,061–5,941) detected a 3-kb PCR product in FCMD lymphoblast cDNA (open arrow) and an 8-kb product in FCMD genomic DNA (filled arrow). In the normal control, cDNA and genomic DNA both showed 5-kb PCR products. The 8-kb band was weak, probably because VNTR region of SVA is GC-rich (82%). **c**, Representation of genomic DNA and cDNA in FCMD. Black and white arrows, forward and reverse sequencing primers. The intronic sequence in FCMD is indicated in lower case. The authentic stop codon is coloured red, and the new stop codon is coloured blue. **d, e**, Northern blot analysis of *fukutin* in human lymphoblasts (**d**) and model mice (**e**); F, FCMD; N, normal control. The wild-type mouse *fukutin* mRNA was detected at a size of 6.1 kb. Both skeletal muscle (left) and brain (right) showed smaller, abnormal bands in Hp/Hp mice. WT, wild type; Hn, Hn/Hn mice; Hp, Hp/Hp mice. **f**, Representation of genomic DNA and cDNA in ARH (*LDLRAP1*, left), NLSDM (*PNPLA2*, middle) and human (*AB627340*, right).

exon, and the new stop codon exists downstream of the new last exon–exon junction (Supplementary Fig. 4).

We have recently generated knock-in mice that carry a humanized fukutin exon 10, which either includes (Hp allele) or excludes (Hn allele) the SVA insertion, and bred these strains with heterozygous fukutin knockout mice to obtain compound heterozygotes (Hp/−)[16]. Knock-in mice that are homozygous (Hp/Hp) and compound heterozygous (Hp/−) are representative of the human FCMD alleles. These mice exhibit hypoglycosylation of α-DG in skeletal muscle, which is the most significant characteristic in α-DGpathy[16]. Quantitative RT–PCR in various tissues from Hp/Hp mice revealed an aberrant splicing pattern identical to that seen in human patients (Supplementary Fig. 5). Northern blot analysis detected abnormally spliced *fukutin* mRNA species at the expected sizes of 5.6 and 4.6 kb in patients with FCMD, whereas the normal *fukutin* mRNAs appeared at 7.4 and 6.4 kb (Fig. 1d and Methods). We replicated these results in the knock-in model mice (Fig. 1e and Supplementary Fig. 6a). The consistent observations between patients with FCMD and knock-in model mice lead us to conclude that a splicing abnormality underlies the pathogenesis of FCMD.

Abnormal splicing excises the authentic stop codon and produces another stop codon located 388 bp downstream from the 5′ side of the new exon 11 (Fig. 1c). The predicted protein lacks the C-terminal 38 amino acids of fukutin, instead containing 129 amino acids derived from the SVA sequence (Supplementary Fig. 7). Endogenous fukutin is scarce and difficult to detect; however, we were able to identify both normal and aberrant forms of the protein in human and mouse using immunoprecipitation followed by western blot analysis. The abnormal fukutin protein in FCMD displayed the predicted mobility shift (Fig. 2a–c and Supplementary Fig. 6b).

We introduced normal and aberrantly spliced *fukutin* cDNA constructs into mammalian cell lines. Whereas normal fukutin localized to the Golgi apparatus, the aberrantly spliced fukutin protein is displaced completely from the Golgi to the endoplasmic reticulum (Fig. 2d and Supplementary Fig. 8). Further examination showed that a *fukutin* construct lacking the C-terminal 38 amino acids also mislocalized to the endoplasmic reticulum (Fig. 2d and Supplementary Fig. 8), suggesting that the C-terminal domain of fukutin is important for localization to the Golgi. Thus, impairment of this domain may lead to fukutin dysfunction in FCMD. The mislocalization is unlikely to be toxic because FCMD is an autosomal recessive disease and heterozygous carriers of the SVA insertion have no symptoms.

We next tested if exon-trapping occurs in other diseases with SVA insertion[6]. In a patient with autosomal recessive hypercholesterolemia (ARH), a 2.6-kb SVA was inserted within intron 1 of the *LDLRAP1* gene[4]. A patient with lipid storage disease with subclinical myopathy (NLSDM) also had a 1.9-kb SVA insertion in exon 3 of the *PNPLA2* gene[5]. We found abnormally spliced products induced by SVA exon-trapping in these patients' fibroblast (Fig. 1f left and middle panels, Supplementary Figs 9 and 10, and Supplementary Table 1). Cycloheximide treatment to fibroblasts from these patients increased expression of the genes (Supplementary Figs 9a and 10a), suggesting

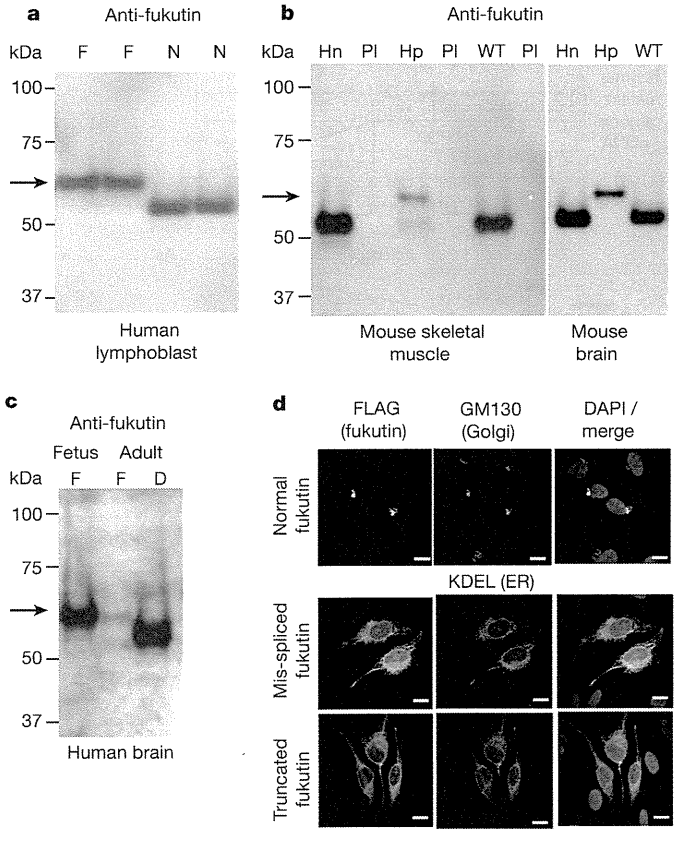

Figure 2 | Abnormal fukutin protein in FCMD. a–c, Immunoprecipitation analysis of fukutin protein in human lymphoblasts (**a**), both skeletal muscle and brain tissues from Hp/Hp mice (**b**) and brain tissue from patients with FCMD (**c**); filled arrow, abnormal fukutin; N, normal sample; F, sample from patient with FCMD; Hn, Hn/Hn mice; Hp, Hp/Hp mice; PI, pre-immune serum; D, patient with Duchenne muscular dystrophy. **d**, The subcellular localization of fukutin. Top, normal fukutin; middle, mis-spliced fukutin; bottom, truncated fukutin. Stained with anti-FLAG (left, to detect fukutin), anti-GM130 (middle, Golgi marker, top) and anti-KDEL (endoplasmic reticulum marker, middle and bottom), and merge (right, with DAPI stain). Scale bar, 10 μm.

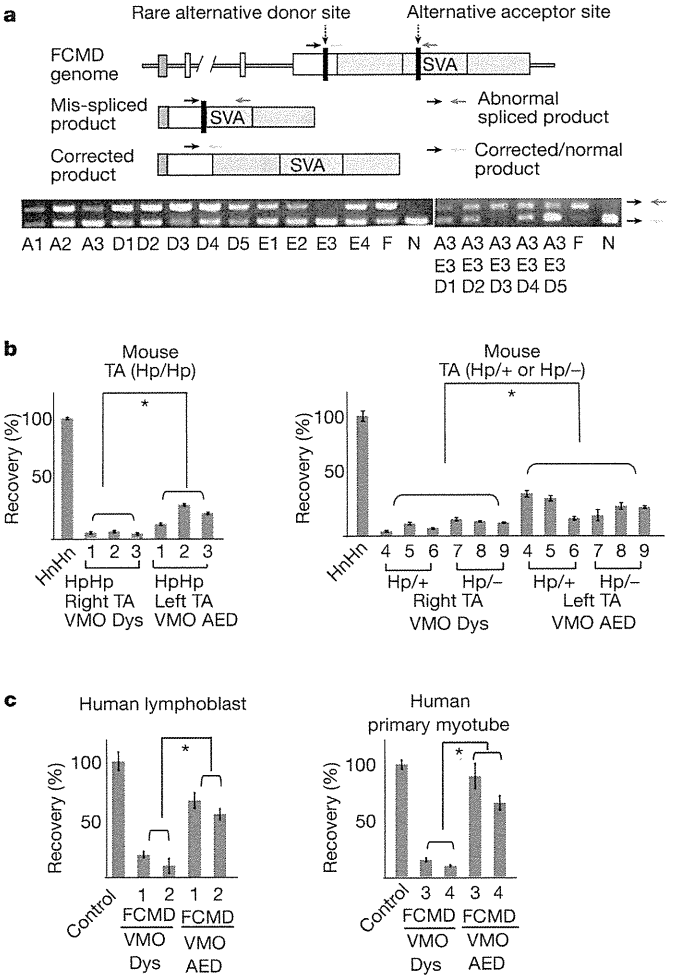

Figure 3 | AON cocktail rescues normal *fukutin* mRNA. a, RT–PCR diagram of three primers designed to assess normal *fukutin* mRNA recovery (upper). Black arrow, a common forward primer located on *fukutin* coding region; dark grey arrow, a reverse primer to detect the abnormal RT–PCR product (161 bp); light grey arrow, the other reverse primer to detect the restored normal RT–PCR product (129 bp). The effect on Hp/Hp ES cells treated with each single or a cocktail of AONs (lower). F, FCMD; N, normal sample. **b**, Rescue from abnormal splicing in VMO-treated Hp/Hp and Hp/− mice. Local injection of AED cocktail into tibialis anterior ($n = 3$). Dys, a negative control. **c**, Rescue from abnormal splicing in VMO-treated human FCMD lymphoblasts (left, $n = 2$) and myotubes (right, $n = 2$). The y axis shows the percentage recovery of normal mRNA (*$P < 0.01$ by Student's t-test). TA, tibialis anterior. Error bars, s.e.m.

that the SVA-trapped transcripts are likely to be subjected to nonsense-mediated mRNA decay[6,17]. In a search for the same events using the same acceptor site as FCMD in the human genome, we located two expressed sequence tags on human chromosome 4 (DA436529 and DA060755) that represent a spliced transcript induced by an SVA element. We found exonization in a human-specific insertion of SVA (AB627340) into a small gene (Fig. 1f right panel and Supplementary Fig. 11). The human-specific exon-trapping of SVA in the small gene might influence human evolution and development.

FCMD alleles of the *fukutin* gene contain a fully intact protein coding sequence, raising the possibility that FCMD could be treated by restoring translation of the full-length protein through splicing modulation with AONs. To identify promising target sequences in various cell lines, we produced 25-mer 2′-O-methyl phosphoramidite (2′OMePS) AONs targeted to the acceptor (A1–A3), donor (D1–D5) and exonic splicing enhancer sites (E1–E4) in *fukutin* pre-mRNA (Supplementary Fig. 12). We introduced the AONs into various cell types and assessed the recovery of normal processing and restoration of the authentic stop codon (Fig. 3a). Cells with A3 and E3 showed strong suppression of SVA-derived splicing. The greatest recovery of *fukutin* mRNA, to levels of more than 40% of the normal control, was achieved with a combination of A3, E3 and D5 (AED) (Fig. 3a). The D5 sequence overlaps with a predicted intronic splicing enhancer site within the aberrant intronic sequence; in normal *fukutin*, this sequence resides in exon 10 (Supplementary Fig. 12).

We injected octa-guanidine morpholino oligonucleotide (vivomorpholino, VMO)[18] AED cocktail locally into skeletal muscle of knock-in mice and evaluated the therapeutic effect by calculating the percentage recovery of normally processed mRNA. In the AED-treated tibialis anterior and gastrocnemius of Hp/Hp and Hp/− mice, the amount of corrected *fukutin* mRNA increased significantly relative to mice treated with control VMO (Fig. 3b and Supplementary Fig. 13). We assessed fukutin protein recovery in injected skeletal muscle tissue from Hp/Hp mice. Consistent with the significant increase of restored normal mRNA, normal fukutin protein was rescued (Fig. 4a). We examined α-DG glycosylation in AED-treated Hp/− mice. Deficiently glycosylated α-DG, at the predicted smaller size, was reduced in abundance, whereas normal-sized α-DG increased after AED treatment (Fig. 4b). The signal intensity for glycosylated α-DG was clearly increased, and a shift in the α-DG core was observed, indicating that the rescued fukutin is functional. Laminin overlay assays revealed a marked increase in α-DG laminin-binding ability, indicating that α-DG

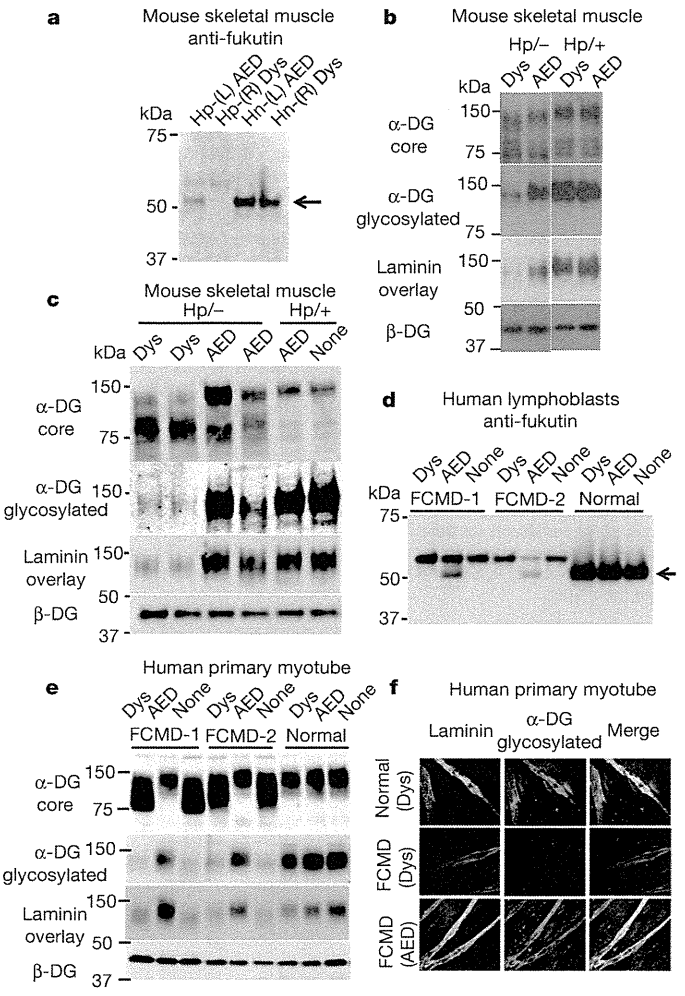

Figure 4 | AON cocktail treatment rescues normal fukutin protein and functional α-DG. a, d, Immunoprecipitation analysis of fukutin protein after local treatment with VMO (AED) in FCMD model mice (**a**) and human FCMD lymphoblasts (**d**). Arrow, normal fukutin protein. L, left tibialis anterior; R, right tibialis anterior; Dys, negative control. **b, c, e,** Tibialis anterior muscle after local (**b**) or systemic (**c**) treatment with AED and human FCMD lymphoblasts treated with the AED (**e**) were analysed by western blot using antibodies against α-DG core protein (top panel) and glycosylated α-DG (second), and by a laminin overlay assay (third). Bottom, β-DG (internal control). **f,** Laminin clustering assay. Left, anti-laminin; middle, anti-glycosylated α-DG; right, merged images. Upper, normal myotubes treated with control VMO; middle, FCMD patient myotubes treated with control VMO; bottom, FCMD patient myotubes treated with AED.

function also is recovered (Fig. 4b). We next tested systemic AED treatment by intravenous injection of Hp/− mice. This treatment also showed the recovery of normally glycosylated α-DG in AED-treated mice (Fig. 4c).

We administered the VMO AED cocktail to human lymphoblasts and myotubes. As in knock-in mice, we observed successful correction of the splicing abnormality. The corrected *fukutin* mRNA was restored to 50% or more of the levels seen in normal controls (Fig. 3c). We believe this to be sufficient recovery, considering that unaffected FCMD carriers have only 50% of normal *fukutin* mRNA. Finally, we tested recovery of the fukutin protein and the glycosylation of α-DG in the cells of patients with FCMD. Not only was normal fukutin protein expression significantly rescued in AED-treated lymphoblasts (Fig. 4d), but also we observed recovery of normally glycosylated α-DG in AED-treated myotubes (Fig. 4e). Immunofluorescence staining also showed immensely increased glycosylated α-DG (Fig. 4f). A laminin clustering assay showed increased laminin clustering ability, which is characteristically absent in α-DGpathy[19] (Fig. 4f). These data show that AED treatment effectively rescues normal fukutin, confirming our observation of abnormal *fukutin* splicing and raising the possibility of splicing modulation therapy as the first treatment for FCMD. To treat neuronal migration disorder of FCMD, prenatal treatment may be necessary, but it is currently difficult for ethical and technical reasons. Nevertheless, improving even only the muscular symptoms would greatly ameliorate quality of life of the patients as well as their families.

Retrotransposons account for nearly half of the human genome[20]. Increased numbers of reports have highlighted positive and negative contributions of retrotransposons to human health and disease[21,22]. In addition to being the causative factor for FCMD, ARH and NLSDM, SVA insertions have also been implicated in hereditary elliptocytosis, X-linked agammaglobulinemia, neurofibromatosis type 2 and X-linked dystonia-Parkinsonism[12,23–26]. It has been suggested that SVA insertions cause such diseases through genomic deletion, reduced mRNA expression or skipping of neighbouring exons[17,22]. Recently, SVA splicing has been suggested to generate variation within and across species by activating functional 3′ splice sites within SVAs across the human genome, controlling gene transcription, creating alternative splicing by exon-trapping, or inducing premature stop codons, and was experimentally demonstrated[6]. Our findings emphasize the importance of SVA functions in human disease and support the possibility of radical treatment against SVA-induced disease by splicing modulation therapy. AONs have become one of the most promising and practical candidate chemicals for splicing modulation therapy in cancer[27], infectious diseases[28] and Duchenne muscular dystrophy[29,30]. In demonstrating the ability of AONs to rescue fukutin function in FCMD, we introduce a novel clinical role for them in treating FCMD and other SVA-mediated diseases, while providing new insights about the influence of SVAs on human evolution, development and disease.

METHODS SUMMARY

For AON treatment, 25-mer 2′OMePS (GeneDesign and Invitrogen) and octaguanidine morpholino (VMO; Gene-Tools) were used. The knock-in mouse was produced as described previously[16].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 16 September 2010; accepted 12 August 2011.

1. Toda, T. *et al.* Localization of a gene for Fukuyama type congenital muscular dystrophy to chromosome 9q31–33. *Nature Genet.* **5,** 283–286 (1993).
2. Kobayashi, K. *et al.* An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. *Nature* **394,** 388–392 (1998).
3. Watanabe, M. *et al.* Founder SVA retrotransposal insertion in Fukuyama-type congenital muscular dystrophy and its origin in Japanese and Northeast Asian populations. *Am. J. Med. Genet. A.* **138,** 344–348 (2005).
4. Wilund, K. R. *et al.* Molecular mechanisms of autosomal recessive hypercholesterolemia. *Hum. Mol. Genet.* **11,** 3019–3030 (2002).
5. Akman, H. O. *et al.* Neutral lipid storage disease with subclinical myopathy due to a retrotransposal insertion in the *PNPLA2* gene. *Neuromuscul. Disord.* **20,** 397–402 (2010).
6. Hancks, D. C. *et al.* Exon-trapping mediated by the human retrotransposon SVA. *Genome Res.* **19,** 1983–1991 (2009).
7. Damert, A. *et al.* 5′-Transducing SVA retrotransposon groups spread efficiently throughout the human genome. *Genome Res.* **19,** 1992–2008 (2009).
8. Bantysh, O. B. & Buzdin, A. A. Novel family of human transposable elements formed due to fusion of the first exon of gene MAST2 with retrotransposon SVA. *Biochemistry (Mosc.)* **74,** 1393–1399 (2009).
9. Michele, D. E. *et al.* Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. *Nature* **418,** 417–422 (2002).
10. Barresi, R. & Campbell, K. P. Dystroglycan: from biosynthesis to pathogenesis of human disease. *J. Cell Sci.* **119,** 199–207 (2006).
11. Strichman-Almashanu, L. Z. *et al.* Retroposed copies of the HMG genes: a window to genome dynamics. *Genome Res.* **13,** 800–812 (2003).
12. Ostertag, E. M. *et al.* SVA elements are nonautonomous retrotransposons that cause disease in humans. *Am. J. Hum. Genet.* **73,** 1444–1451 (2003).
13. Bennett, E. A. *et al.* Natural genetic variation caused by transposable elements in humans. *Genetics* **168,** 933–951 (2004).
14. Wang, H. *et al.* SVA elements: a hominid-specific retroposon family. *J. Mol. Biol.* **354,** 994–1007 (2005).

15. Hancks, D. C. *et al.* Retrotransposition of marked SVA elements by human L1s in cultured cells. *Hum. Mol. Genet.* **20,** 3386–3400 (2011).
16. Kanagawa, M. *et al.* Residual laminin-binding activity and enhanced dystroglycan glycosylation by LARGE in novel model mice to dystroglycanopathy. *Hum. Mol. Genet.* **18,** 621–631 (2009).
17. Hancks, D. C. & Kazazian, H. H. Jr. SVA retrotransposons: evolution and genetic instability. *Semin. Cancer Biol.* **20,** 234–245 (2010).
18. Wu, B. *et al.* Octa-guanidine morpholino restores dystrophin expression in cardiac and skeletal muscles and ameliorates pathology in dystrophic mdx mice. *Mol. Ther.* **17,** 864–871 (2009).
19. Barresi, R. *et al.* LARGE can functionally bypass α-dystroglycan glycosylation defects in distinct congenital muscular dystrophies. *Nature Med.* **10,** 696–703 (2004).
20. Lander, E. S. *et al.* Initial sequencing and analysis of the human genome. *Nature* **409,** 860–921 (2001).
21. Kazazian, H. H. Jr. Mobile elements: drivers of genome evolution. *Science* **303,** 1626–1632 (2004).
22. Cordaux, R. & Batzer, M. A. The impact of retrotransposons on human genome evolution. *Nature Rev. Genet.* **10,** 691–703 (2009).
23. Hassoun, H. *et al.* A novel mobile element inserted in the α spectrin gene: spectrin dayton. A truncated α spectrin associated with hereditary elliptocytosis. *J. Clin. Invest.* **94,** 643–648 (1994).
24. Rohrer, J. *et al.* Unusual mutations in Btk: an insertion, a duplication, an inversion, and four large deletions. *Clin. Immunol.* **90,** 28–37 (1999).
25. Legoix, P. *et al.* Molecular characterization of germline NF2 gene rearrangements. *Genomics* **65,** 62–66 (2000).
26. Makino, S. *et al.* Reduced neuron-specific expression of the TAF1 gene is associated with X-linked dystonia-parkinsonism. *Am. J. Hum. Genet.* **80,** 393–406 (2007).
27. O'Brien, S. *et al.* Randomized phase III trial of fludarabine plus cyclophosphamide with or without oblimersen sodium (Bcl-2 antisense) in patients with relapsed or refractory chronic lymphocytic leukemia. *J. Clin. Oncol.* **25,** 1114–1120 (2007).
28. Crooke, S. T. *et al.* Vitravene—another piece in the mosaic. *Antisense Nucleic Acid Drug Dev.* **8,** vii–viii (1998).
29. Lu, Q. L. *et al.* Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. *Nature Med.* **9,** 1009–1014 (2003).
30. Alter, J. *et al.* Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. *Nature Med.* **12,** 175–177 (2006).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank S. Nakagawa, K. Ohno, S. Tsujino, N. Taniguchi, and I. Nonaka for comments; M. Okabe and A. Kawai for generating the ES cell line from knock-in model mice; Y. Motoyoshi and J. C. Cohen for providing patients' samples; W. Sako and Y. Izumi for sending patients' samples; I. Mizuta, T. Mure, M. Furukawa, K. Kaneshiro, Y. Dainin and all laboratory members for technical support; and J. Logan for editing the manuscript. We thank the GAIN for providing chimpanzee brain samples. This work was supported by an Intramural Research Grant (20B-13) for Neurological and Psychiatric Disorders from the National Center of Neurology and Psychiatry (to T.T.), the Global COE Program (Frontier Biomedical Science Underlying Organelle Network Biology) (to T.T., M.T.-I. and M.K.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Grants-in-Aid for Scientific Research (A) (23249049 to T.T.), and Young Scientists (A) (21689030 to K.K.) and (B) (20790980 to M.T.-I.) from the Japan Society for the Promotion of Science, and the Takeda Science Foundation (to K.K.).

Author Contributions M.T.-I., K.K., M.K. and T.T. designed the study. M.T.-I. performed most of the experiments. K.K. developed a system to detect endogenous fukutin protein. M.K. performed biochemical analysis of VMO-injected mice. C.Y. produced the *fukutin* cDNA constructs for transfection experiments. K.M., T.O., and A.K. performed analyses of AON treatment in mice and various cell types. H.K., T.Y. and S.T. provided intellectual input. H.O.A., S.D. and R.K., provided patients' samples. M.T.-I., K.K. and T.T. wrote the paper.

Author Information The patient *fukutin* and a chimpanzee mRNA sequences are deposited in GenBank/European Molecular Biology Laboratory/DNA Data Bank of Japan under accession numbers AB609007 and AB627340, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to T.T. (toda@med.kobe-u.ac.jp).

Journal of Human Genetics (2011) 56, 330–334

www.nature.com/jhg

ORIGINAL ARTICLE

Genetic and clinical analysis in a Chinese parkinsonism-predominant spinocerebellar ataxia type 2 family

Hao Sun[1], Wataru Satake[2], Changjun Zhang[3], Yoshitaka Nagai[2], Youyong Tian[4], Shouzhi Fu[4], Jiankun Yu[1], Yaping Qian[1], Yuan Qian[1], Jiayou Chu[1] and Tatsushi Toda[2]

Parkinson's disease is a degenerative central nervous system disorder that often impairs motor skills, speech and other functions. We discovered a large Chinese family showing primarily parkinsonism symptoms with autosomal dominant inheritance. Six affected individuals in the family showed typical parkinsonism symptoms, including pill-rolling tremor. Two other affected individuals showed cerebellar ataxia symptoms. A whole-genome scan using the 50K single nucleotide polymorphism array with three different linkage methods detected two positive regions on chromosome 12q24.1 and 5q13.3. The *ATXN2* gene, responsible for spinocerebellar ataxia type 2 (SCA2) was located precisely in the center of the positive region on chromosome 12. Further analysis of SCA2 revealed heterozygous pathological CAG expansions in the family. The affected individuals' symptoms were typical of parkinsonism, but complex. Inverse correlation between CAG repeat size and age of onset is not obvious in this pedigree. This parkinsonism-predominant SCA2 family shared the same disease gene locus with other 'standard' SCA2 families, but it is possible that variations in one or more modifier genes might account for the parkinsonism-predominant SCA2 predisposition observed in this pedigree.
Journal of Human Genetics (2011) **56,** 330–334; doi:10.1038/jhg.2011.14; published online 10 February 2011

Keywords: 5q13; genome-wide scan; linkage study; single nucleotide polymorphism chip

INTRODUCTION

Parkinson's disease (PD), a degenerative central nervous system disorder, often impairs motor skills, speech and other functions.[1] It is found worldwide, with incidence rates varying from country to country. The prevalence of PD increases with age. In Europe, PD affects about 1–2% of individuals over 60 years of age.[2] Although there is no cure for PD, further understanding of its genetic risks can improve neuroprotective or preventive approaches. Causative genes for Mendelian-inherited parkinsonism have been identified. Point mutations and multiplications in the *SNCA* gene have been found in some families with autosomal dominant inheritance.[3,4] To date, mutations in the *LRRK2* gene are the most common cause of Mendelian PD. In studies across several populations, 5–15% of autosomal dominant PD families carried mutations in *LRRK2* (see refs 5, 6). Mutations in three genes, *PARK2* (encoding parkin), *PINK1* (*PARK6*) and *DJ-1* (*PARK7*), have been identified in autosomal recessive PD, which is characterized by an early age at onset and therefore referred to as autosomal recessive juvenile parkinsonism.[7–9] The expanded *ATXN2* gene, which causes spinocerebellar ataxia type 2 (SCA2), was found in some families with only or mainly typical parkinsonism.[10,11] Although some parkinsonism clinical signs such as dystonia and tremor have been described in SCA2, dopamine-responsive parkinsonism has been infrequently described in SCA2 (see ref. 12). The sign of dopamine-responsive just has been seen in some Chinese families[13,14] and some white families.[15,16]

We described here a large family from Hubei, China, that showed primarily autosomal dominant inheritance of parkinsonism symptoms acrossing four generations. Affected family members exhibited typical clinical features of PD, such as pill-rolling tremors and levodopa responsiveness. However, some family members showed cerebellar symptoms. The patients who showed the atypical phenotypes opposed to the typical cerebellar ataxia maybe have more complex genetic causes than normal SCA2 patients. So, we performed a whole-genome linkage study to identify possible genetic causes in

[1]The Department of Medical Genetics, Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, Yunnan, China; [2]Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe, Japan; [3]Institute of Eugenics and Genetics, Hubei, China and [4]Emergency Department, the Peoples Hospital of Shiyan, Hubei, China
Correspondence: Professor T Toda, Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-chou, Chuo-ku, Kobe 650-0017, Japan.
E-mail: toda@med.kobe-u.ac.jp
or Professor J Chu, The Department of Medical Genetics, Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, 379 Jiaolin Road, Kunming, Yunnan 650118, China.
E-mail: chujy@imbcams.com.cn
Received 29 November 2010; revised 10 January 2011; accepted 13 January 2011; published online 10 February 2011

this family. At same time, the molecular and clinical features of this family were analyzed.

MATERIALS AND METHODS

Clinical information

The proband (IV-3) was initially diagnosed as PD in 2001, and therefore the family was classified as a PD pedigree. In 2007, we performed neurological examinations for eight patients in the family, and we examined four patients using magnetic resonance imaging (MRI). Blood samples were obtained from patients and unaffected relatives with informed consent. Approval for the study was obtained from the Ethical Committees of participating institutions.

Whole-genome linkage analysis

Genomic DNA was isolated from blood using QIAamp DNA Blood Mini Kits (Qiagen, Shanghai, China). Single nucleotide polymorphism genotyping was performed for 27 individuals from the family (Figure 1) using the Human Mapping 50K Xba 240 SNP array (Affymetrix, Santa Clara, CA, USA). Signal intensity data were analyzed using GeneChip DNA analysis software GDAS v.3.0.2.8 (Affymetrix). The genotype data were converted to linkage format using ALOHOMORA software[17] and subjected to quality control routines, including gender check and graphical representation of relationship errors.[18] Mendelian errors were detected with PedCheck,[19] and non-informative markers were deleted before further analysis. Genome-wide non-parametric multipoint linkage, single parametric and single non-parametric linkage analysis were performed using GeneSpring GT software (Agilent, Santa Clara, CA, USA).

Trinucleotide repeat analysis

We screened for mutations in the *ATXN2* gene using PCR amplification with previously published SCA-2A and SCA-2B oligonucleotide primers.[20] PCR products were sized precisely using capillary electrophoresis with an ABI 3730xL DNA analyzer (Applied Biosystems, Foster city, CA, USA) and compared with known samples using GeneMapper V3.5 (Applied Biosystems, Foster city, CA, USA). Some samples were isolated from agarose gels and used as DNA templates for sequencing with the Big-dye terminator kit (Applied Biosystems) on the ABI 3730xL analyzer.

RESULTS

Clinical information

The family (Figure 1) consisted of 39 members in four generations, with 16 affected members. All family members reside in Hubei Province, China. The inheritance pattern is autosomal dominant. We collected peripheral blood from 27 family members, including 10 affected members. Clinical data were shown in Table 1 for 8 of 10 patients collected in 2007. Detailed clinical data were unavailable for

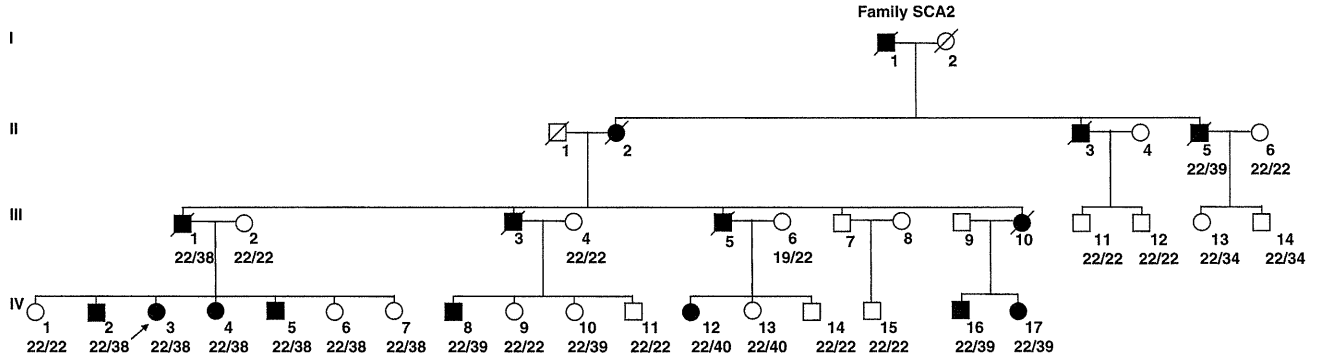

Figure 1 Pedigree of a Chinese family ascertained with parkinsonism-predominant spinocerebellar ataxia type 2 (SCA2). Squares indicate males; circles, females. A slash through the symbol indicates deceased and an arrow points to the proband. The pedigree contains 16 known affected individuals; eight patients are living. SCA2 CAG repeat allele sizes are listed below the pedigree symbols of the 27 individuals who have been genotyped.

Table 1 Clinical and genetic features of the SCA2 family

No.	*IV-2*	*IV-3*	*IV-4*	*IV-5*	*IV-8*	*IV-12*	*IV-16*	*IV-17*
Age at onset	33	36	37	22	20	37	39	35
Age at examination	46	44	42	39	50	45	51	38
Resting tremor	2	1	0	2	0	0	2[a]	0
Bradykinesia	3	2	0	3	0	1	2	2
Rigidity	3	2	0	2	0	1	2	1
Postural instability	2	1	0	2	0	2	2	1
Masked face	3	2	0	3	1	2	2	2
Levodopa response	+	+	–	NT	NT	+	+	NT
Gait ataxia	0	0	2	1	0	0	0	0
Limb ataxia	1	1	2	1	0	0	0	0
Slow saccade	2	1	1	2	3[b]	1	1	0
Vertical gaze palsy	2	0	0	2	3[b]	0	0	0
Hyporeflexia	3	3	1	0	1	0	0	0
Cerebellar atrophy on MRI	2	1	3	1	NT	NT	NT	NT
CAG repeats	22/38	22/38	22/38	22/38	22/39	22/40	22/39	22/39

Abbreviations: MRI, magnetic resonance imaging; SCA2, spinocerebellar ataxia type 2.
[a]Pill-rolling tremor.
[b]difficulty initiating pursuit movements.
0 indicates that the individual was tested and the symptom was absent. 1, mild; 2, moderate; and 3, marked. NT indicates that the individual could not be tested. A (+) indicates that the finding was present; a (–) indicates absent.

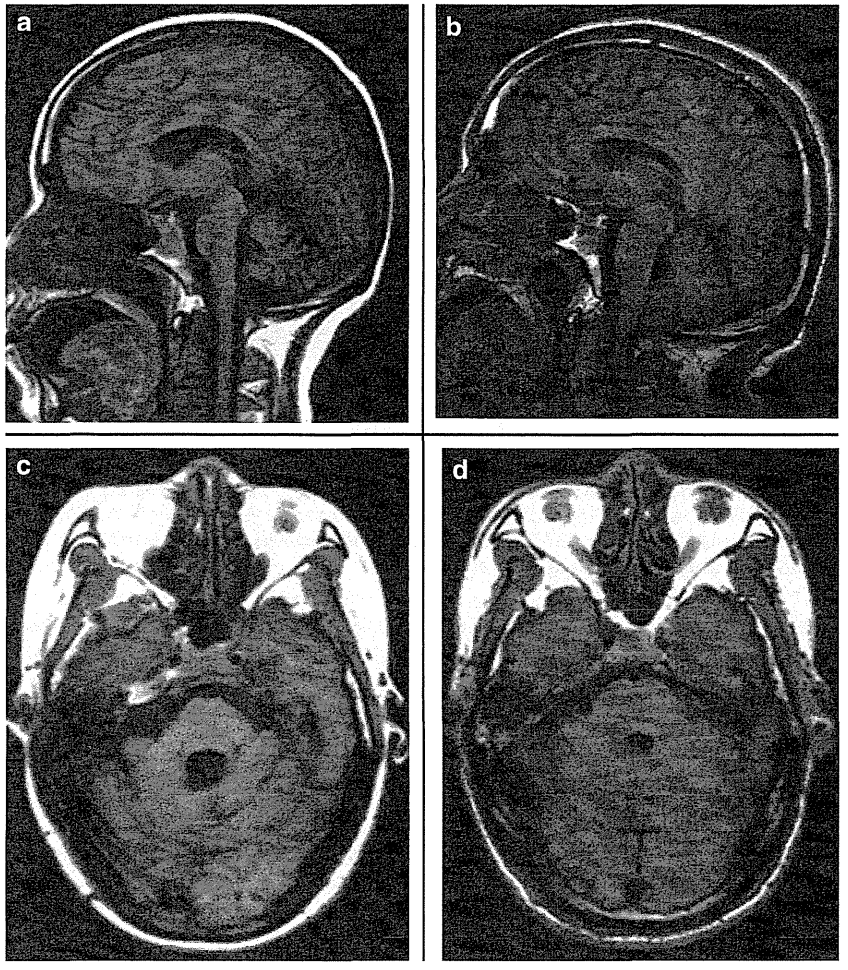

Figure 2 T1-weighted magnetic resonance imaging (MRI) of IV-4 (**a**, **c**) and IV-5 (**b**, **d**). Patient IV-4 showed marked cerebellar atrophy, and patient IV-5 showed no cerebellar atrophy.

the other two deceased patients (II-5 and III-1). Family members of the two patients provided ambiguous clinical data, and their preliminary diagnosis indicated that the two patients had parkinsonism symptoms. Blood samples, collected in 2001, were provided by their neurologist. Age of symptomatic disease onset varied from 20 to 39 years, with an average age at onset of 32.4 years.

Most patients showed typical parkinsonism symptoms, such as resting tremor, bradykinesia, rigidity and postural instability (IV-2, IV-3, IV-5, IV-12, IV-16 and IV-17). Patient IV-16 had pill-rolling tremors. However, two patients (IV-4 and IV-8) showed cerebellar symptoms such as limb ataxia and slow saccade, but no typical parkinsonism symptoms.

Five of eight patients were treated with levodopa. Only one (Patient IV-4) of them showed no response. This patient also lacked typical parkinsonism symptoms. Patients IV-2, IV-3, IV-4 and IV-5 were examined using MRI analysis. MRI images from Patients IV-4 and IV-5 were shown in Figure 2. Marked cerebellar atrophy was found in Patient IV-4, and no cerebellar atrophy was found in Patient IV-5.

Whole-genome linkage analysis

Genome-wide analysis revealed two positive regions for linkage, on chromosomes 12 and 5. On chromosome 12, the highest single-point parametric log of odds (LOD) score (2.59) was detected at rs2695281 (100.5 Mb, NCBI Build 36). The highest multi-point non-parametric LOD score on chromosome 12 was 3.5, with a multi-point non-parametric LOD score >3 at 94.9–115.6 Mb (NCBI Build 36). On chromosome 5, the highest single-point parametric LOD score (2.73) was detected at rs10491487 (80.4 Mb, NCBI Build 36). The highest multi-point non-parametric LOD score on chromosome 5 was 3.5 with a multi-point non-parametric LOD score >3 at 79.8–81.2 Mb (NCBI Build 36). Multi-point non-parametric linkage results were compatible with the single-point parametric linkage results on both chromosomes 5 and 12. Single-point non-parametric linkage analysis detected no positive result. Most of single-point non-parametric LOD scores were smaller than 2, and the highest was 2.1, on chromosome 21 (Figure 3). Gender and relationships in the single nucleotide polymorphism array data were accurate, and PedCheck detected no Mendelian errors.

The *ATXN2* gene, responsible for SCA2, was located at 110.3 Mb (NCBI Build 36) on chromosome 12, within the linkage-positive region of 94.9–115.6 Mb.

Trinucleotide repeat analysis

We performed SCA2 molecular analysis in all 27 family members. Three types of heterozygous pathological CAG expansion (38, 39 and 40 repeats) and two types of normal repeats (19 and 22) were identified. A borderline 34 CAG repeat was found in the two family members. Genotyopes with repeat numbers for individual family

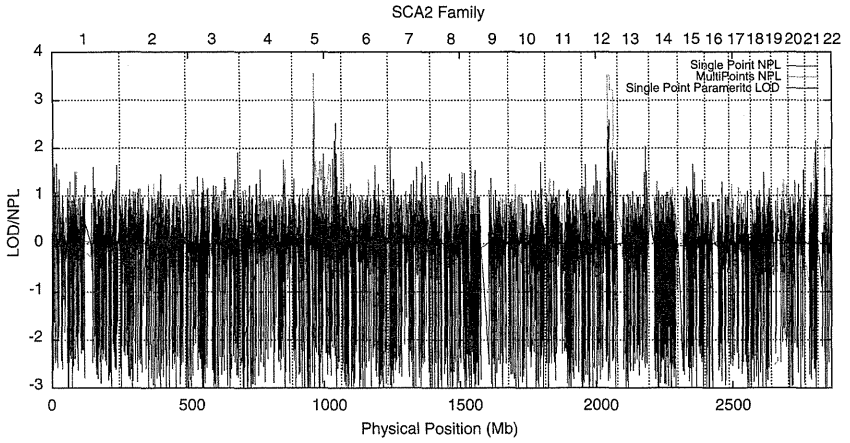

Figure 3 Three types of log of odds (LOD) scores were calculated from the genome-wide scan. The LOD score plot was created with GNUPLOT 4.0 (http://www.gnuplot.info) using the LOD plot-drawing Perl script included in the ALOHOMORA package. On chromosome 12, the highest MultiPoint non-parametric LOD score was 3.5 and the highest SinglePoint parametric LOD score was 2.59. On chromosome 5, the highest MultiPoint non-parametric LOD score was 3.5 and the highest SinglePoint parametric LOD score was 2.73. The MultiPoint non-parametric linkage results were compatible with the SinglePoint parametric linkage results on chromosomes 5 and 12. LOD scores <-3 are not shown.

members were shown in Figure 1. Sequence analysis of expanded alleles from IV-5, IV-8, IV-12 and III-14 revealed interruptions of CAG base pair repeats with CAA. The 38-repeat expansion in IV-5 contained 29 CAGs, followed by one CAA and eight CAGs. The 39-repeat expansion IV-8 contained 30 CAGs, one CAA and eight CAGs. The 40-repeat expansion in IV-12 contained 31 CAGs, one CAA and eight CAGs. The normal 34-repeat CAG expansion in III-14 contained 25 CAGs, one CAA and eight CAGs.

All affected individuals in the branch of III-1 carried 38 trinucleotide repeats. IV-6 and IV-7 also carried the 38 trinucleotide repeats, but as of 2007 no parkinsonism or ataxia had been observed in these individuals. The 39 trinucleotide repeats occurred in the branches of II-5, III-3 and III-10. Similar to IV-6 and IV-7, individual IV-10 carried the 39 trinucleotide repeats, but had shown no parkinsonism or ataxia symptoms in 2007. In the branch of II-5, the 39 repeats were shortened to 34 in transmission. III-13 and III-14, who carried 34 trinucleotide repeats, showed no symptoms.

DISCUSSION

We described a levodopa-responsive parkinsonism Chinese family with SCA2 trinucleotide expansions. The symptoms observed in this family were primarily parkinsonism, but complex. Some affected family members showed typical clinical manifestations of parkinsonism. Four of five patients responded to levodopa treatment. One affected individual (IV-4) lacking parkinsonism symptoms was unresponsive to levodopa; MRI analysis of this patient showed marked cerebellar atrophy. Patient IV-8 showed just mild masked face, but his cerebellar symptoms were severe. In other patients (IV-12, IV-16 and IV-17), cerebellar symptoms were minor or absent. Overall, the clinical signs in this family appear most similar to parkinsonism.

Molecular analysis of SCA2 expansion in the family revealed three types of expanded CAG repeats. An inverse correlation has been established between age of SCA2 onset and CAG repeat length, with repeat length accounting for 54–80% of variance.[21,22] However, such inverse correlation was not observed in our pedigree. In the branches of III-1 and III-2, the age of onset in affected individuals who carried the 38 CAG repeats ranged from 22 to 37. Two other individuals with 38 CAG repeats showed no clinical signs at the time of examination (IV-6, age 37 years in 2007; and IV-7, age 35 years in 2007). The age of onset in the three affected individuals who carried 39 CAG repeats (II-5, IV-16 and IV-17) ranged from 20 to 39 years. Another carrier of 39 CAG repeats (IV-10) showed no clinical signs in 2007, at age 43 years. One of the two individuals who carried 40 CAG repeats had an age at onset of 37 years (IV-12). The other (IV-13) had no clinical signs in 2007, at age 43 years. These observations showed that repeat length alone cannot account for age of onset in this family. Conversely, it is not possible to predict when, or if, the unaffected carriers of expanded CAG repeats will eventually show clinical signs.

Some researches suggested that CAA interruption can lead to phenotypical variation.[23,24] The results of Sobczak *et al.*[25] showed that the CAA interruptions are major determinants of the CAG repeat folding in the SCA2 transcripts. The SCA2 transcripts interrupted by the CAA should generate shorter branched hairpins and the uninterrupted repeats transcripts form single slippery hairpins. The patients who carried SCA2 expansions with and without interruptions show two different phenotypes.[23,24] It may be caused by the different CAG repeat folding that would interact differently with double-stranded RNA binding proteins and interfere with mRNA transcription or translation.[24] That structural organization of CAG expansions with interruption associate with phenotypic variation has been also reported in other neurodegenerative disorders such as SCA1 (see ref. 26). In our family, all patients carried the CAG expansions with one CAA interruption, but showed two different phenotypes. The patients IV-4 and IV-8 showed more ataxia symptoms than the other patients. Especially, patient IV-4 had no response to the levodopa treatment and had marked cerebellar atrophy on MRI. The symptoms of IV-4 made him look more like the typical SCA2. Therefore, the phenotypic variation in our family may be caused by other unknown reason such as co-effect of *SCA2* gene and some modifier gene, rather than the different CAA interruption.

The SCA2 CAG repeat is highly unstable through intergenerational transmission, with a tendency to expand. One study reported that there are 27 families' SCA2 CAG repeats changed in length among 32 SCA2 families, with a mean increase of 2.2 repeat units.[27] In this family, we observed eight transmissions of an expanded SCA2 CAG repeat with no increase in repeat length. Six transmissions yielded no change, whereas two transmissions yielded contractions. As blood samples from several older patients were not collected, we cannot know exactly which SCA2 CAG repeat increased through intergenerational transmission. The explanation for the relative instability in the

family may include genetic or epigenetic factors. A previous study defined the range of the normal SCA2 allele size as 17–31 CAG repeats, whereas full pathogenic mutations had 36–64 repeats.[28] Two unaffected family members who carried the contracted CAG repeat had borderline mutations of 34 CAG repeats (III-13, 45 years of age in 2007; and III-14, 38 years of age in 2007), although 34 CAG repeats were found in some SCA2 patients.[29]

Our linkage analysis revealed two positive regions, one at chromosome 12q24.1 and one at chromosome 5q13.3. On chromosome 12, the mutated *ATXN2* gene, which causes SCA2, is located in the middle of the linkage-positive region. This demonstrates the accuracy of the detection methods and shows that the positive regions are reliable. As the disease gene of SCA2 was located to chromosome 12q23-24.1 (see ref. 30), most of the reports about parkinsonism-predominant SCA2 was based on case cohorts. The linkage study in our family strengthened that the parkinsonism-predominant SCA2 carried the same pathogenic gene as the typical SCA2 from the whole genome perspective. We assume that this parkinsonism-predominant SCA2 family shares a disease locus with other standard SCA2 families, but it is possible that one or more modifier genes interact with *ATXN2* to produce clinical signs more similar to parkinsonism. It is difficult to predict whether the other positive region at 5q13.3 is a real positive region or an artifact. If it is not an artifact, perhaps there would be a modifier gene harbored in the region. To prove it, more detailed gene mutation analyses in the region or other linkage analyses for additional parkinsonism-predominant SCA2 families need to be performed.

The prevalence of SCA2 among patients with familial parkinsonism ranged from 1.5 to 10% (ref. 12). It is seen occasionally in German[28] and Japanese[10] populations. After Gwinn-Hardy *et al.* described a Chinese American family with only or mainly typical parkinsonism in 2000 (see ref. 11), similar families have been reported. Surprisingly, Lu *et al.*[23] reported that four families with SCA2 were identified among 41 families with familial parkinsonism, about 10% of familial parkinsonism carried the expanded SCA2 CAG repeats in Taiwan people. Therefore, it is possible that the mutation rate of potential modifiers might account for the ethnic differences in the predisposition of parkinsonism-predominant SCA2. Better understanding of factors that determine a predominant parkinsonism phenotype in SCA2 may shed light on the pathogenesis of PD.

ACKNOWLEDGEMENTS

This work was supported by the Chinese National Natural Science Foundation (NO. 30400264) and the Yunnan Science and Technology Program (NO. 2008ZC068M). The authors thank the members of the Chinese family for their interest, support and cooperation in this study.

1 Jankovic, J. Parkinson's disease: clinical features and diagnosis. *J. Neurol. Neurosurg. Psychiatry* **79,** 368–376 (2008).
2 de Rijk, M. C., Tzourio, C., Breteler, M. M., Dartigues, J. F., Amaducci, L., Lopez Pousa, S. *et al.* Prevalence of parkinsonism and Parkinson's disease in Europe: the EUROPARKINSON Collaborative Study. European community concerted action on the epidemiology of Parkinson's disease. *J. Neurol. Neurosurg. Psychiatry* **62,** 10–15 (1997).
3 Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A. *et al.* Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* **276,** 2045–2047 (1997).
4 Singleton, A. B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J. *et al.* alpha-Synuclein locus triplication causes Parkinson's disease. *Science* **302,** 841 (2003).
5 Di Fonzo, A., Tassorelli, C., De Mari, M., Chien, H. F., Ferreira, J., Rohe, C. F. *et al.* Comprehensive analysis of the LRRK2 gene in sixty families with Parkinson's disease. *Europ. J. Hum. Genet.* **14,** 322–331 (2006).
6 Berg, D., Schweitzer, K., Leitner, P., Zimprich, A., Lichtner, P., Belcredi, P. *et al.* Type and frequency of mutations in the LRRK2 gene in familial and sporadic Parkinson's disease*. *Brain* **128,** 3000–3011 (2005).
7 Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S. *et al.* Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* **392,** 605–608 (1998).
8 Valente, E. M., Abou-Sleiman, P. M., Caputo, V., Muqit, M. M., Harvey, K., Gispert, S. *et al.* Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* **304,** 1158–1160 (2004).
9 Bonifati, V., Rizzu, P., van Baren, M. J., Schaap, O., Breedveld, G. J., Krieger, E. *et al.* Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science* **299,** 256–259 (2003).
10 Sasaki, H., Wakisaka, A., Sanpei, K., Takano, H., Igarashi, S., Ikeuchi, T. *et al.* Phenotype variation correlates with CAG repeat length in SCA2–a study of 28 Japanese patients. *J. Neurol. Sci.* **159,** 202–208 (1998).
11 Gwinn-Hardy, K., Chen, J. Y., Liu, H. C., Liu, T. Y., Boss, M., Seltzer, W. *et al.* Spinocerebellar ataxia type 2 with parkinsonism in ethnic Chinese. *Neurology* **55,** 800–805 (2000).
12 Furtado, S., Payami, H., Lockhart, P. J., Hanson, M., Nutt, J. G., Singleton, A. A. *et al.* Profile of families with parkinsonism-predominant spinocerebellar ataxia type 2 (SCA2). *Mov. Disord.* **19,** 622–629 (2004).
13 Shan, D. E., Soong, B. W., Sun, C. M., Lee, S. J., Liao, K. K. & Liu, R. S. Spinocerebellar ataxia type 2 presenting as familial levodopa-responsive parkinsonism. *Ann. Neurol.* **50,** 812–815 (2001).
14 Lu, C. S., Wu Chou, Y. H., Yen, T. C., Tsai, C. H., Chen, R. S. & Chang, H. C. Dopa-responsive parkinsonism phenotype of spinocerebellar ataxia type 2. *Mov. Disord.* **17,** 1046–1051 (2002).
15 Furtado, S., Farrer, M., Tsuboi, Y., Klimek, M. L., de la Fuente-Fernandez, R., Hussey, J. *et al.* SCA-2 presenting as parkinsonism in an Alberta family: clinical, genetic, and PET findings. *Neurology* **59,** 1625–1627 (2002).
16 Modoni, A., Contarino, M. F., Bentivoglio, A. R., Tabolacci, E., Santoro, M., Calcagni, M. L. *et al.* Prevalence of spinocerebellar ataxia type 2 mutation among Italian Parkinsonian patients. *Mov. Disord.* **22,** 324–327 (2007).
17 Ruschendorf, F. & Nurnberg, P. ALOHOMORA: a tool for linkage analysis using 10 K SNP array data. *Bioinformatics* **21,** 2123–2125 (2005).
18 Abecasis, G. R., Cherny, S. S., Cookson, W. O. & Cardon, L. R. GRR: graphical representation of relationship errors. *Bioinformatics* **17,** 742–743 (2001).
19 O'Connell, J. R. & Weeks, D. E. PedCheck: a program for identification of genotype incompatibilities in linkage analysis. *Am. J. Hum. Genet.* **63,** 259–266 (1998).
20 Pulst, S. M., Nechiporuk, A., Nechiporuk, T., Gispert, S., Chen, X. N., Lopes-Cendes, I. *et al.* Moderate expansion of a normally biallelic trinucleotide repeat in spinocerebellar ataxia type 2. *Nat. Genet.* **14,** 269–276 (1996).
21 Giunti, P., Sabbadini, G., Sweeney, M. G., Davis, M. B., Veneziano, L., Mantuano, E. *et al.* The role of the SCA2 trinucleotide repeat expansion in 89 autosomal dominant cerebellar ataxia families. Frequency, clinical and genetic correlates. *Brain* **121** (Part 3), 459–467 (1998).
22 Geschwind, D. H., Perlman, S., Figueroa, C. P., Treiman, L. J. & Pulst, S. M. The prevalence and wide clinical spectrum of the spinocerebellar ataxia type 2 trinucleotide repeat in patients with autosomal dominant cerebellar ataxia. *Am. J. Hum. Genet.* **60,** 842–850 (1997).
23 Lu, C. S., Wu Chou, Y. H., Kuo, P. C., Chang, H. C. & Weng, Y. H. The parkinsonian phenotype of spinocerebellar ataxia type 2. *Arch. Neurol.* **61,** 35–38 (2004).
24 Charles, P., Camuzat, A., Benammar, N., Sellal, F., Destee, A., Bonnet, A. M. *et al.* Are interrupted SCA2 CAG repeat expansions responsible for parkinsonism? *Neurology* **69,** 1970–1975 (2007).
25 Sobczak, K. & Krzyzosiak, W. J. CAG repeats containing CAA interruptions form branched hairpin structures in spinocerebellar ataxia type 2 transcripts. *J. Biol. Chem.* **280,** 3898–3910 (2005).
26 Lin, J. X., Ishikawa, K., Sakamoto, M., Tsunemi, T., Ishiguro, T., Amino, T. *et al.* Direct and accurate measurement of CAG repeat configuration in the ataxin-1 (ATXN-1) gene by 'dual-fluorescence labeled PCR-restriction fragment length analysis'. *J. Hum. Genet.* **53,** 287–295 (2008).
27 Cancel, G., Durr, A., Didierjean, O., Imbert, G., Burk, K., Lezin, A. *et al.* Molecular and clinical correlations in spinocerebellar ataxia 2: a study of 32 families. *Hum. Mol. Genet.* **6,** 709–715 (1997).
28 Riess, O., Laccone, F. A., Gispert, S., Schols, L., Zuhlke, C., Vieira-Saecker, A. M. *et al.* SCA2 trinucleotide expansion in German SCA patients. *Neurogenetics.* **1,** 59–64 (1997).
29 Almaguer-Mederos, L. E., Falcon, N. S., Almira, Y. R., Zaldivar, Y. G., Almarales, D. C., Gongora, E. M. *et al.* Estimation of the age at onset in spinocerebellar ataxia type 2 Cuban patients by survival analysis. *Clin. Genet.* **78,** 169–174.
30 Gispert, S., Twells, R., Orozco, G., Brice, A., Weber, J., Heredero, L. *et al.* Chromosomal assignment of the second locus for autosomal dominant cerebellar ataxia (SCA2) to chromosome 12q23-24.1. *Nat. Genet.* **4,** 295–299 (1993).

ELSEVIER

Neurobiology of Aging 32 (2011) 2108.e1–2108.e5

NEUROBIOLOGY OF AGING

www.elsevier.com/locate/neuaging

Genetic Reports Abstracts

Role of sepiapterin reductase gene at the PARK3 locus in Parkinson's disease

Manu Sharma[a,b,*], Demetrius M. Maraganore[c], John P.A. Ioannidis[d,e], Olaf Riess[f], Jan O. Aasly[g], Grazia Annesi[h], Nadine Abahuni[i], Anna Rita Bentivoglio[j], Alexis Brice[k], Christine Van Broeckhoven[l,m], Marie-Christine Chartier-Harlin[n], Alain Destée[n], Ana Djarmati[o], Alexis Elbaz[p], Matthew Farrer[q], Carlo Ferrarese[r], J. Mark Gibson[s,t], Suzana Gispert[i], Nobutaka Hattori[u], Barbara Jasinska-Myga[v], Christine Klein[o], Suzanne Lesage[k], Timothy Lynch[s,t], Peter Lichtner[w], Jean-Charles Lambert[x], Anthony E. Lang[y], George D. Mellick[z,aa], Francesa De Nigris[j], Grzegorz Opala[v], Aldo Quattrone[bb], Chiara Riva[r], Ekaterina Rogaeva[cc], Owen A. Ross[dd], Wataru Satake[ee], Peter A. Silburn[z,aa], Jessie Theuns[l,m], Tatsushi Toda[dd], Hiroyuki Tomiyama[t], Ryan J. Uitti[ff], Karin Wirdefeldt[gg], Zbigniew Wszolek[ff], Thomas Gasser[a,b], Rejko Krüger[a,b], for the Genetic Epidemiology of Parkinson's Disease Consortium

[a] *Center of Neurology and Hertie-Institute for Clinical Brain Research, University of Tübingen, Tuebingen, Germany*
[b] *DZNE-Geman Centre for Neurodegenerative Diseases, Tuebingen, Germany*
[c] *Department of Neurology North Shore University Health System, IL, USA*
[d] *Department of Hygiene and Epidemiology, University of Ioannina School of Medicine, Ioannina, Greece*
[e] *Stanford Prevention Research Centre, Stanford, CA, USA*
[f] *Department of Medical Genetics, University of Tübingen, Germany*
[g] *Department of Neurology, St. Olav's hospital and NTNU, Trondheim, Norway*
[h] *Institute of Neurological Sciences, National Research Council, Cosenza, Italy*
[i] *Department of Neurology, Goethe University, Frankfurt am Main, Germany*
[j] *Department of Neurology, Catholic University, Rome, Italy*
[k] *INSERM, UMR_S975, Université Pierre et Marie Curie-Paris, CNRS, UMR 7225, AP-HP, Pitié-Salpêtrière Hospital, Paris, France*
[l] *Laboratory of Neurogenetics, Institute Born-Bunge, University of Antwerp, Antwerpen, Belgium*
[m] *Neurodegenerative Brain Diseases Group, Department of Molecular Genetics, VIB, Antwerpen, Belgium*
[n] *Inserm UMR837, Centre de Recherche JPArc, Service de Neurologie et de Pathologie du Mouvement CHRU de Lille, University Lille Nord de France*
[o] *Section of Clinical and Molecular Neurogenetics at the Department of Neurology, University of Lübeck, Germany*
[p] *INSERM, Unit 708, F-75013, Paris, France and UPMC Univ Paris 06, U708, Neuroepidemiology, F-75005, Paris, France*
[q] *Department of Genetics, University of British Columbia, Vancouver, BC, Canada*
[r] *Department of Neuroscience-Section of Neurology, University of Milano-Bicocca San Gerardo Hospital, Monza, Italy*
[s] *The Dublin Neurological Institute at the Mater Misericordiae University Hospital, and Conway Institute, University College Dublin, Ireland*
[t] *Department of Neurology, Royal Victoria Hospital, Belfast, Ireland*
[u] *Department of Neurology, Juntendo University School of Medicine, Tokyo, Japan*
[v] *Department of Neurology, Medical University of Silesia, Katowice, Poland*
[w] *Helmholtz Zentrum München, German Research Centre for Environmental Health (Gmbh), Neuherberg, Germany*
[x] *INSERM U744, Institut Pasteur de Lille, Université Lille Nord de France, UDSL, Lille, France*
[y] *Movement Disorders Centre, Toronto Western Hospital, University of Toronto, Toronto, Canada*
[z] *Eskitis Institute for Cell and Molecular Therapies, Griffith University, Nathan, QLD, Australia*
[aa] *University of Queensland, Centre for Clinical Research, Royal Brisbane Hospital*
[bb] *Institute of Neurology, University Magna Graecia, Catanzaro, Italy*
[cc] *Tanz Centre for Research in Neurodegenerative Diseases, Department of Medicine, University of Toronto, Toronto, Canada*
[dd] *Department of Neuroscience, Mayo Clinic, Jacksonville, FL, USA*
[ee] *Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe, Japan*
[ff] *Department of Neurology, Mayo Clinic, Jacksonville, FL, USA*
[gg] *Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden*

Received 23 December 2010; received in revised form 15 April 2011; accepted 30 May 2011

doi:10.1016/j.neurobiolaging.2011.05.024

Abstract

Sepiapterin reductase (*SPR*) gene is an enzyme which catalyses the final step of tetrahydrobiopterin synthesis (BH4) and was implicated in Parkinson's disease (PD) pathogenesis as a candidate gene for PARK3 locus. A number of studies yielded association of the PARK3 locus with PD, and *SPR* knockout mice were shown to display parkinsonian features. To evaluate the role of *SPR* gene polymorphisms in diverse populations in PD, we performed collaborative analyses in the Genetic Epidemiology of Parkinson Disease (GEO-PD) Consortium. A total of 5 single nucleotide polymorphisms (3 in the promoter region and 2 in the 3′ untranslated region [UTR]) were genotyped. Fixed as well as random effect models were used to provide summary risk estimates of *SPR* variants. A total of 19 sites provided data for 6547 cases and 9321 controls. Overall odds ratio estimates varied from 0.92 to 1.01. No overall association with the *SPR* gene using either fixed effect or random effect model was observed in the studied population. I^2 Metric varied from 0% to 36.2%. There was some evidence for an association for participants of North European/Scandinavian descent with the strongest signal for rs1876487 (odds ratio = 0.82; p value = 0.003). Interestingly, families which were used to map the PARK3 locus, have Scandinavian ancestry suggesting a founder effect. In conclusion, this large association study for the *SPR* gene revealed no association for PD worldwide. However, taking the initial mapping of the PARK3 into account, the role of a population-specific effect warrants consideration in future studies.

Keywords: Parkinson disease; *SPR*; PARK3; PD genetic studies; PD-GWAS

1. Introduction

We performed a large multicenter collaborative study among the Genetic Epidemiology of Parkinson's Disease (GEO-PD) Consortium sites to assess the world-wide the role of common variation in the *SPR* gene in Parkinson's disease (PD). This large study includes over 15,868 subjects from 19 sites representing 14 countries from 4 continents (supplementary material).

2. Methods

A total of 19 teams representing 14 countries and 4 continents agreed to participate and contributed clinical and genotypic data for a total of 15,868 individuals (6547 cases and 9321 controls). A total of 5 single nucleotide polymorphisms (SNPs) were selected for genotyping: rs1396107, rs1567230, rs2421095, rs1876487, and rs1561244 listed in order from 5′ to 3′ end of the gene (Karamohamed et al., 2003; Sharma et al., 2006) (supplementary material).

3. Results

Nineteen sites contributed 6547 cases and 9321 controls. Characteristics of all participating sites are shown in Table 1 (supplementary material). The distribution of allele frequencies of each SNP per site is shown in Supplementary Table 2. The meta-analysis did not reveal nominal significant associations either by random or fixed effect models, with the tentative exception of rs1876487. The summary odds ratio (OR) for rs1876487 was 0.95 (95% confidence interval, 0.89–1.00) with a p value of 0.05, uncorrected for multiple testing (Table 1 and supplementary material).

4. Discussion

This very large association study of common variants in the *SPR* gene with PD has revealed no evidence of association world-wide and it excludes large effects for any of the tested variants (supplementary material). Although most genetic association studies typically consider all European populations to share some common ancestry, a recent study established direct correlation between genetic makeup and the geographic location from which samples are ascertained within the European continent. This has also been shown in PD genetics, where in a recently published Genome-Wide Association Study (GWAS) on PD the authors observed a frequency gradient and differential genetic impact for SNP rs3129882 within European population for human leukocyte antigen (HLA) locus (supplementary material). Thus it is conceivable that rs1876487 and/or rs1567230 (D' = 1.0; r^2 = 0.29) may modulate the disease susceptibility only in populations from Northern European descent. Furthermore, haploytpe analysis restricted to North European population showed suggestive evidence of association for haplotype (rs2421095-rs1876487-rs1561244; odds ratio, 0.57; p-value 0.07), again suggesting the role of founder effect for PARK3 locus in North European/Scandinavian populations. Acknowledging these caveats, our study is large enough to suggest that these variants are unlikely to be a clinically important determinant of PD risk world-wide and future efforts should focus specifically on Northern European populations.

Disclosure statement

All authors have reported no actual or potential conflict of interest.

* Corresponding author at: Hertie-Insitute of Clinical Brain Research, Department of Neurology, University of Tuebingen, Hoppe-Seyler-Str. 3, 72076 Tübingen, Germany. Tel.: +49 7071 29 81 968; fax: +49 7071 29 4620.

E-mail address: manu.sharma@uni-tuebingen.de (M. Sharma).

Table 1
Summary effect estimates and confidence interval for *SPR* gene

		Overall			North European/Scandinavian		
SNP	Site	RE OR (95% CI)	FE OR (95% CI)	Het p (I^2)	RE OR (95% CI)	FE OR (95% CI)	Het p (I^2)
rs1396107	17	0.97 (0.90–1.03)	0.97 (0.90–1.03)	0.80 (0%)	0.87 (0.76–0.99)	0.87 (0.76–0.99)	0.42 (0%)
rs1567230	18	0.93 (0.82–1.04)	0.92 (0.83–1.03)	0.29 (14%)	0.75 (0.59–0.94)	0.74 (0.60–0.91)*	0.31 (13%)
rs2421095	19	0.93 (0.84–1.04)	0.93 (0.85–1.01)	0.18 (21%)	0.78 (0.63–0.96)	0.78 (0.63–0.96)	0.68 (0%)
rs1876487	18	0.94 (0.89–1.00)	0.94 (0.89–1.00)	0.46 (0%)	0.83 (0.72–0.96)	0.82 (0.72–0.93)*	0.33 (12%)
rs1561244	17	1.01 (0.91–1.12)	1.00 (0.92–1.08)	0.07 (36%)	0.88 (0.72–1.08)	0.85 (0.73–1.00)	0.23 (30%)

Key: CI, confidence interval; FE, fixed effects; Het, heterogeneity (Q statistic); OR, odds ratio; RE, random effects.
* $p < 0.01$.

Appropriate approval and procedures were used concerning human subjects.

Acknowledgements

Australia: From the Queensland Parkinson's Project: R.S. Boyle and A. Sellbach (Princess Alexandra Hospital, Brisbane), J.D. O'Sullivan (Royal Brisbane and Women's Hospital, Brisbane), G.T. Sutherland, G.A. Siebert and N.N.W. Dissanayaka (Eskitis Institute for Cell and Molecular Therapies, Griffith University, Nathan, QLD).

Belgium: Christine Van Broeckhoven, Ph.D., D.Sc. (Neurodegenerative Brain Diseases Group, Department of Molecular Genetics, VIB; and Laboratory of Neurogenetics, Institute Born-Bunge, University of Antwerp; Antwerpen), Jessie Theuns, PhD (Neurodegenerative Brain Diseases Group, Department of Molecular Genetics, VIB; and Laboratory of Neurogenetics, Institute Born-Bunge, University of Antwerp; Antwerpen), David Crosiers, M.D. (Neurodegenerative Brain Diseases Group, Department of Molecular Genetics, VIB; and Laboratory of Neurobiology, Institute Born-Bunge, University of Antwerp; and Department of Neurology, University Hospital Antwerp; Antwerpen), Barbara Pickut, M.D. (Department of Neurology, University Hospital Antwerp; Antwerpen), Sebastiaan Engelborghs, M.D., Ph.D. (Laboratory of Neurochemistry and Behavior, Institute Born-Bunge, University of Antwerp; and Department of Neurology and Memory Clinic, Hospital Network Antwerp Middelheim and Hoge Beuken; Antwerpen), Bram Meeus (Neurodegenerative Brain Diseases Group, Department of Molecular Genetics, VIB; and Laboratory of Neurogenetics, Institute Born-Bunge, University of Antwerp; Antwerpen), Peter P. De Deyn, M.D., Ph.D. (Laboratory of Neurochemistry and Behavior, Institute Born-Bunge, University of Antwerp; and Department of Neurology and Memory Clinic, Hospital Network Antwerp Middelheim and Hoge Beuken; Antwerpen), Patrick Cras, M.D., Ph.D. (Laboratory of Neurobiology, Institute Born-Bunge, University of Antwerp; and Department of Neurology, University Hospital Antwerp; Antwerpen). Funding: The research at this site was in part supported by the Special Research Fund of the University of Antwerp, the Medical Research Foundation Antwerp and Neurosearch, the Research Foundation Flanders (FWO), the Flanders government agency for Innovation by Science and Technology (IWT), the Foundation for Alzheimer Research (SAO/FRMA), the Belgian Science Policy Office Interuniversity Attraction Poles (IAP) Program P6/43, a Methusalem Excellence Grant of the Flanders Government and, and the Alzheimer's Association USA,. B.M. received a Ph.D. fellowship of the IWT and D.C. of the FWO; J.T. received a FWO postdoctoral fellowship.

Canada: Ekaterina Rogaeva, Ph.D. (Tanz Centre for Research in Neurodegenerative Diseases, Department of Medicine, Division of Neurology, University of Toronto; ON, Canada); Anthony E. Lang, M.D., Movement Disorders Centre, Toronto Western Hospital, University of Toronto, Toronto, ON, Canada.

France: From the French Parkinson's Disease Genetics Study Group: Y. Agid, M. Anheim, A.-M. Bonnet, M. Borg, A. Brice, E. Broussolle, J.C. Corvol, P. Damier, A. Destée, A. Dürr, F. Durif, S. Lesage, E. Lohmann, P. Pollak, O. Rascol, F. Tison, C. Tranchant, F. Viallet, and M. Vidailhet. Also, Christophe Tzourio (Inserm U708, Paris), Philippe Amouyel (Inserm U744, Lille), Marie-Anne Loriot (Inserm UMRS775, Paris). Eugénie Mutez (Inserm UMR837, Service de Neurologie et de Pathologie du Mouvement CHRU de Lille, University Lille Nord de France), Aurélie Duflot, (UMR837 Inserm-University Lille 2, CHRU de Lille), Jean-Philippe Legendre (Service de Neurologie et Pathologie du Mouvement, Clinique de Neurologie du CHU de Lille), Nawal Waucquier (Service de Neurologie et Pathologie du Mouvement, Clinique de Neurologie du CHU de Lille), the 2 Centres de Ressources Biologiques (IPL-Lille, CHRU-Lille) and its scientific committee (AD, MCCH, P. Amouyel, F. Pasquier, R. Bordet). Funding: This work is supported by the French National Agency of Research (ANR-08-MNP-012). Lille: Financial support from CHRU de Lille, University Lille 2, Inserm, French Ministry PHRCs (1994/, 2002/1918, 2005/1914), Association France Parkinson (2005), Fondation de France 2004-013306, Fondation de la Recherche Médicale (2006), P.P.F. (Synucléothèque 2005-2009),

Germany: Thomas Gasser, M.D., (Department of Neurology, University Hospital Tuebingen), Olaf Riess, M.D. (Department of Neurology, University Hospital Tuebingen),

Daniela Berg, M.D., (Department of Neurology, University Hospital Tuebingen), Claudia Schulte, M.Sc. (Department of Neurology, University Hospital Tuebingen), Christine Klein, M.D. (Section of Clinical and Molecular Neurogenetics at the Department of Neurology, University of Lübeck), Ana Djarmati, Ph.D. (Department of Neurology, University of Lübeck), Johann Hagenah, M.D. (Department of Neurology, University of Lübeck), Katja Lohmann, Ph.D. (Section of Clinical and Molecular Neurogenetics at the Department of Neurology, University of Lübeck), Georg Auburger, M.D. (Department of Neurology, Goethe University Frankfurt am Main, Germany), Rüdiger Hilker, M.D. (Department of Neurology, Goethe University Frankfurt am Main, Germany), Simone van de Loo, M.D. (Department of Neurology, Goethe University Frankfurt am Main, Germany).

Greece: Efthimios Dardiotis, M.D. (Department of Neurology, Faculty of Medicine, University of Thessaly and Institute of Biomedical Research & Technology, CERETETH, Larissa), Vaia Tsimourtou (Department of Neurology, Faculty of Medicine, University of Thessaly, Larissa), Styliani Ralli (Department of Neurology, Faculty of Medicine, University of Thessaly, Larissa), Persa Kountra, M.D. (Department of Neurology, Faculty of Medicine, University of Thessaly, Larissa), Konstantinos Aggelakis (Institute of Biomedical Research & Technology, CERETETH, Larissa).

Japan: Nobutaka Hattori, M.D., Ph.D. (Department of Neurology, Juntendo University School of Medicine, Tokyo), Hiroyuki Tomiyama, M.D., Ph.D. (Department of Neurology, Juntendo University School of Medicine, Tokyo), Manabu Funayama, Ph.D. (Department of Neurology, and Research Institute for Diseases of Old Age, Graduate School of Medicine, Juntendo University, Tokyo), Hiroyo Yoshino, B.S. (Research Institute for Diseases of Old Age, Graduate School of Medicine, Juntendo University, Tokyo), Yuanzhe Li, M.D., Ph.D. (Department of Neurology, Juntendo University School of Medicine, Tokyo), Yoko Imamichi (Department of Neurology, Juntendo University School of Medicine, Tokyo), Tatsushi Toda, M.D. (Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe, Japan), Wataru Satake (Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe, Japan). Funding: This work was supported by a grant from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Grants-in-Aid for Scientific Research (to HT: 21591098; to NH: 090052131), for Scientific Research on Priority Areas (to NH: 08071510), and for Young Scientists (to MF: 22790829).

Ireland: Prof. Tim Lynch, FRCPI, FRCP (The Dublin Neurological Institute at the Mater Misericordiae University Hospital, Clinical Investigator at the Conway Institute, University College Dublin, Ireland), and Dr J. Mark Gibson, M.D. (Department of Neurology, Royal Victoria Hospital, Belfast, Ireland).

Italy: Enza Maria Valente, M.D., Ph.D. (IRCCS, Casa Sollievo della Sofferenza Hospital, Mendel Institute, San Giovanni Rotondo), Alessandro Ferraris, M.D., (IRCCS, Casa Sollievo della Sofferenza Hospital, Mendel Institute, San Giovanni Rotondo), Bruno Dallapiccola (Mendel Institute, Casa Sollievo della Sofferenza Hospital, Rome), Tamara Ialongo, M.D., Ph.D. (Institute of Neurology, Catholic University, Rome), Laura Brighina, M.D., Ph.D. (Department of Neurology, Ospedale San Gerarado, Monza, Italy), Barbara Corradi, Ph.D. (Department of Paediatrics, University of Milano-Bicocca, Monza), Roberto Piolti, M.D. (Department of Neurology, Ospedale San Gerardo, Monza, Italy), Patrizia Tarantino, Ph.D. (Institute of Neurological Sciences, National Research Council), and Ferdinanda Annesi, Ph.D. (Institute of Neurological Sciences, National Research Council).

Norway: J. Aasly, M.D. (Department of Neurology, University of Trondheim, Norway).

Poland: Grzegorz Opala, M.D., Ph.D. (Department of Neurology, Aging, Degenerative and Cerebrovascular Disorders, Medical University of Silesia, Katowice), Barbara Jasinska-Myga, M.D., Ph.D. (Department of Neurology, Aging, Degenerative and Cerebrovascular Disorders, Medical University of Silesia, Katowice), Gabriela Klodowska-Duda, M.D., Ph.D. (Department of Neurology, Aging, Degenerative and Cerebrovascular Disorders, Medical University of Silesia, Katowice), Magdalena Boczarska-Jedynak, M.D., Ph.D. (Department of Neurology, Aging, Degenerative and Cerebrovascular Disorders, Medical University of Silesia, Katowice).

Singapore: Eng King Tan, M.D., Ph.D. (National Medical and Biomedical Research Councils, and the Duke-NUS Graduate Medical School, Singapore Millenium Foundation).

Sweden: Andrea Carmine Belin, Ph.D. (Department of Neuroscience, Karolinska Institutet, Stockholm), Lars Olson, Professor (Department of Neuroscience, Karolinska Institutet, Stockholm), Dagmar Galter, Ph.D. (Department of Neuroscience, Karolinska Institutet, Stockholm), Marie Westerlund, Ph.D. (Department of Neuroscience, Karolinska Institutet, Stockholm), Olof Sydow, Ph.D., (Department of Clinical Neuroscience, Karolinska University Hospital, Stockholm). Also Christer Nilsson, M.D., Ph.D. (Department of Geriatric Psychiatry, Lund University), Andreas Puschmann, M.D. (Department of Neurology, Lund University Hospital, Department of Geriatric Psychiatry, Lund University).

Taiwan: J.J. Lin, M.D., Department of Neurology, Cushang Show-Chwan Hospital, Taiwan.

USA: Demetrius M. Maraganore, M.D. (Department of Neurology, Mayo Clinic, Rochester, MN, USA), J. Eric Ahlskog, Ph.D., M.D. (Department of Neurology, Mayo Clinic, Rochester, MN, USA), Mariza de Andrade, Ph.D.

(Department of Health Sciences Research, Mayo Clinic, Rochester, MN, USA), Timothy G. Lesnick, M.S. (Department of Health Sciences Research, Mayo Clinic, Rochester, MN, USA), and Walter A. Rocca, M.D., M.P.H. (Departments of Neurology and Health Sciences Research, Mayo Clinic, Rochester, MN, USA). Also, Harvey Checkoway, Ph.D. (Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA, USA), Owen A. Ross, Ph.D. (Division of Neuroscience, Mayo Clinic, Jacksonville, FL, USA), Zbigniew K. Wszolek, M.D., and Ryan J. Uitti, M.D. (Department of Neurology, Mayo Clinic, Jacksonville, FL, USA). Funding: Z.K.W. is partially supported by the NIH/NINDS 1RC2NS070276, NS057567, P50NS072187, Mayo Clinic Florida (MCF) Research Committee CR programs (MCF #90052018 and MCF #90052030), and the gift from Carl Edward Bolch, Jr., and Susan Bass Bolch (MCF #90052031/PAU #90052).

Funding to investigators was provided by the German Federal Ministry for Education and Research (BMBF, NGFNplus; 01GS08134) to T.G., O.R., and R.K., and Rapid Response Innovation Award from the Michael J. Fox Foundation to M.S.; Grants-in-Aid for Scientific Research (to HT: 21591098), and Grants-in-Aid from the Research Committee of CNS Degenerative Diseases and Perry syndrome (to HT: 22140901) from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Appendix: A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2011.05.024.

References

Karamohamed, S., DeStefano, A.L., Wilk, J.B., Shoemaker, C.M., Golbe, L.I., Mark, M.H., Lazzarini, A.M., Suchowersky, O., Labelle, N., Guttman, M., Currie, L.J., Wooten, G.F., Stacy, M., Saint-Hilaire, M., Feldman, R.G., Sullivan, K.M., Xu, G., Watts, R., Growdon, J., Lew, M., Waters, C., Vieregge, P., Pramstaller, P.P., Klein, C., Racette, B.A., Perlmutter, J.S., Parsian, A., Singer, C., Montgomery, E., Baker, K., Gusella, J.F., Fink, S.J., Myers, R.H., Herbert, A., GenePD study, 2003. A haplotype at the PARK3 locus influences onset age for Parkinson's disease: the GenePD study. Neurology 61, 1557–1561.

Sharma, M., Mueller, J.C., Zimprich, A., Lichtner, P., Hofer, A., Leitner, P., Maass, S., Berg, D., Dürr, A., Bonifati, V., De Michele, G., Oostra, B., Brice, A., Wood, N.W., Muller-Myhsok, B., Gasser, T., European Consortium on Genetic Susceptibility in Parkinson's Disease (GSPD), 2006. The sepiapterin reductase gene region reveals association in the PARK3 locus: analysis of familial and sporadic Parkinson's disease in European populations. J. Med. Genet. 43, 557–562.

SAGE-Hindawi Access to Research
Journal of Amino Acids
Volume 2011, Article ID 265084, 10 pages
doi:10.4061/2011/265084

Review Article

The Aggregation Inhibitor Peptide QBP1 as a Therapeutic Molecule for the Polyglutamine Neurodegenerative Diseases

H. Akiko Popiel,[1] James R. Burke,[2] Warren J. Strittmatter,[2] Shinya Oishi,[3] Nobutaka Fujii,[3] Toshihide Takeuchi,[1] Tatsushi Toda,[4] Keiji Wada,[1] and Yoshitaka Nagai[1,5]

[1] *Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi, Kodaira, Tokyo 187-8502, Japan*
[2] *Department of Medicine (Neurology) and Deane Laboratory, Duke University Medical Center, Durham, NC 27710, USA*
[3] *Department of Bioorganic Medicinal Chemistry, Kyoto University Graduate School of Pharmaceutical Sciences, Kyoto 606-8501, Japan*
[4] *Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan*
[5] *Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Saitama 332-0012, Japan*

Correspondence should be addressed to Yoshitaka Nagai, nagai@ncnp.go.jp

Received 31 January 2011; Accepted 4 May 2011

Academic Editor: Andreas Wyttenbach

Misfolding and abnormal aggregation of proteins in the brain are implicated in the pathogenesis of various neurodegenerative diseases including Alzheimer's, Parkinson's, and the polyglutamine (polyQ) diseases. In the polyQ diseases, an abnormally expanded polyQ stretch triggers misfolding and aggregation of the disease-causing proteins, eventually resulting in neurodegeneration. In this paper, we introduce our therapeutic strategy against the polyQ diseases using polyQ binding peptide 1 (QBP1), a peptide that we identified by phage display screening. We showed that QBP1 specifically binds to the expanded polyQ stretch and inhibits its misfolding and aggregation, resulting in suppression of neurodegeneration in cell culture and animal models of the polyQ diseases. We further demonstrated the potential of protein transduction domains (PTDs) for *in vivo* delivery of QBP1. We hope that in the near future, chemical analogues of aggregation inhibitor peptides including QBP1 will be developed against protein misfolding-associated neurodegenerative diseases.

1. Introduction

Neurodegenerative diseases are a group of disorders, which are caused by progressive degeneration of neurons in various areas of the brain specific for each disorder, resulting in various neurological and psychiatric symptoms corresponding to each affected brain area. Few effective therapies have been established to date for these diseases, largely due to the fact that the underlying cause of the neurodegeneration long remained unknown. However, accumulating evidence now indicates that many of these neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), the polyglutamine (polyQ) diseases, amyotrophic lateral sclerosis, and the prion diseases, share a common pathomechanism (Figure 1). Pathological and biochemical studies have revealed that various protein inclusions accumulate inside and outside of neurons in the diseased brains, such as senile plaques composed of amyloid-β and neurofibrillary tangles composed of tau in AD, and Lewy bodies composed of α-synuclein in PD. Although the significance of these protein inclusions on disease pathology long remained controversial, recent molecular genetics studies revealed that the mutations responsible for the inherited forms of these diseases render the proteins to be prone to misfold and aggregate, or lead to the overproduction of aggregation-prone proteins. Furthermore, not only such genetic mutations, but also multiple environmental factors are thought to trigger the misfolding of otherwise normal proteins, and indeed the sporadic cases of these diseases also exhibit similar protein inclusions in the brain. It is noteworthy that the aggregates composed of different proteins accumulated in the different diseases all have a similar structure, namely, that they are β-sheet-rich amyloid. In addition, genetic animal models

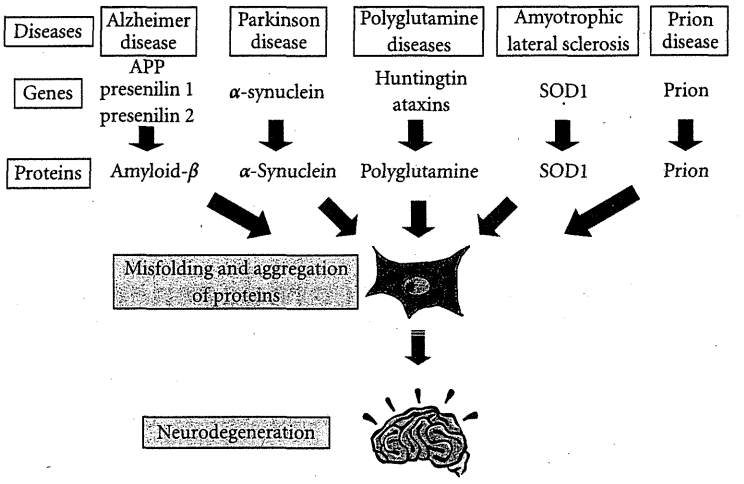

FIGURE 1: Misfolding and abnormal aggregation of proteins as a common molecular pathogenesis of the protein misfolding diseases. The genetic mutations responsible for the inherited forms of various neurodegenerative diseases render the proteins prone to misfold and aggregate, or lead to the overproduction of aggregation-prone proteins, which accumulate as inclusions inside and outside neurons in the diseased brains, and eventually cause neurodegeneration. These facts indicate that the misfolding and abnormal aggregation of proteins are crucial in the pathogenesis of these diseases, which are known as the "protein misfolding diseases."

expressing these aggregation-prone mutant proteins have been found to develop similar protein inclusions as well as neurodegeneration. These facts, taken together, have strongly suggested that the misfolding and abnormal aggregation of proteins are crucial in the pathogenesis of these neurodegenerative diseases, which are hence collectively called the "protein misfolding diseases" [1–3] (Figure 1).

Our group has been working towards establishing therapies for these protein misfolding diseases, with a particular focus on the polyQ diseases because of the following reasons. Firstly, they are determined almost solely by a monogenic mutation and are minorly influenced by environmental factors unlike the other diseases. Furthermore, there is a tight correlation between the severity of the genetic mutation and the disease phenotypes. These special characteristics highlight the polyQ diseases as the most suitable model for the protein misfolding diseases.

In this review, we will introduce our research towards establishing a therapy for the polyQ diseases by targeting the protein misfolding and aggregation, using polyglutamine binding peptide 1 (QBP1), a small biologically active peptide that we identified from combinatorial screening.

2. The Polyglutamine Diseases

Molecular genetics studies on inherited neurodegenerative diseases in the last few decades have revealed a common genetic mutation shared by a group of diseases, namely, an expansion (>40) of the CAG repeat encoding a polyQ stretch in each unrelated disease-causing gene, and hence these diseases are called the polyQ diseases [4, 5]. Currently nine diseases have been found to belong to this group, including Huntington's disease, spinocerebellar ataxia (SCA) type 1, 2, 3, 6, 7, and 17, dentatorubral pallidoluysian atrophy, and spinobulbar muscular atrophy (SBMA) [6–17].

The polyQ diseases share many common characteristics, although the responsible proteins share no particular functional or sequence similarities except for the polyQ stretch. Most of the diseases are inherited through an autosomal dominant manner except for SBMA. The threshold of the polyQ repeat size for disease manifestation is approximately 35–40, except for SCA6, and the length of the polyQ repeat is tightly correlated with the age of onset and severity of the disease. These facts taken together strongly indicate that the expanded polyQ stretch itself causes these diseases via a gain of toxic function mechanism, which is unrelated with the normal function of the host protein. Indeed, expression of an expanded polyQ stretch alone or even an expanded polyQ stretch introduced into an unrelated protein has been shown to cause neurodegeneration in various experimental animal models [18–21].

As a common molecular pathogenesis of the polyQ diseases, it has been proposed that proteins with an expanded polyQ stretch become misfolded and form oligomers and amyloid fibrillar aggregates, and subsequently accumulate as inclusion bodies within neurons, eventually resulting in neurodegeneration (Figure 2) [22–26]. Various cellular proteins have been shown to associate with the polyQ aggregates/inclusion bodies, including transcription factors [27, 28], molecular chaperones [29, 30], cytoskeletal proteins [31], and proteasomal subunits [29], and such abnormal associations are thought to play a role in the disease pathogenesis, through dysfunction of the cellular processes involving these proteins. Accordingly, there have been therapeutic approaches targeting each specific cellular process that is compromised in the disease pathogenesis [23, 32]. However, these attempts result in only limited therapeutic effects, since numerous cellular processes are affected by expression of the expanded polyQ protein [33–36]. In contrast to these downstream events, misfolding and aggregation of the

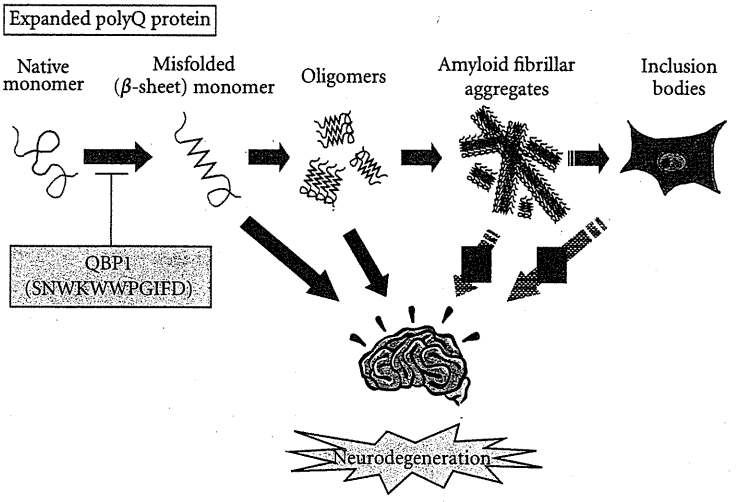

FIGURE 2: Molecular pathogenesis of the polyQ diseases and the therapeutic target of QBP1. Proteins with an expanded polyQ stretch are prone to misfold into a β-sheet dominant structure, leading to their assembly into oligomers and amyloid fibrillar aggregates, followed by their accumulation as inclusion bodies within neurons, eventually resulting in neurodegeneration. The peptide QBP1 inhibits the initial misfolding into a β-sheet dominant structure of the protein by binding to the expanded polyQ stretch, resulting in suppression of polyQ protein aggregation and polyQ-induced neurodegeneration. Question marks indicate structures for which cytotoxicity remains controversial.

expanded polyQ proteins are the most initial events of the pathogenic cascade, and therefore ideal targets since their intervention is expected to lead to the suppression of a broad range of downstream pathogenic events [22, 24, 37, 38]. We therefore aimed towards establishing a therapy targeting misfolding and aggregation of the expanded polyQ protein.

3. Identification of the Aggregation Inhibitor Peptide QBP1

We hypothesized that molecules capable of binding specifically to the expanded polyQ stretch would interfere with its misfolding and aggregation. Identification of the monoclonal antibody 1C2 that selectively binds to the expanded polyQ stretch, probably by recognizing its unique structure [39], prompted us to search for amino acid sequences (domains) or peptides possessing similar properties, which would be more suitable as a drug due to their smaller size and more efficient *in vivo* delivery. We decided to employ phage display screening to identify peptides that bind selectively to the expanded polyQ stretch (Figure 3) [40]. Eleven-amino acid combinatorial peptide libraries expressed on the surface of M13 phage were first screened for their binding to a polyQ62 stretch fused to glutathione *S*-transferase (GST-Q62) by enzyme immunosorbent assay. Phage clones isolated from this first screening were further screened for their selective binding to pathologic length GST-Q62 compared to normal-length GST-Q19. We finally identified six phage clones with greater binding to GST-Q62, and named the encoded peptide sequences polyglutamine binding peptide 1-6 (QBP1-6) (Table 1). Interestingly, most of the peptides were rich in Trp residues, implying that hydrophobic interactions play a role in their binding to the expanded polyQ stretch.

TABLE 1: Polyglutamine binding peptides isolated from phage display screening.

Name	Q62/Q19 binding ratio	Sequence (X_5-fixed-X_5)
QBP1	1.66	Ser-Asn-Trp-Lys-Trp-Trp-Pro-Gly-Ile-Phe-Asp
QBP2	1.31	His-Trp-Trp-Arg-Ser-Trp-Tyr-Ser-Asp-Ser-Val
QBP3	1.30	His-Glu-Trp-His-Trp-Trp-His-Gln-Glu-Ala-Ala
QBP4	1.27	Trp-Gly-Leu-Glu-His-Phe-Ala-Gly-Asn-Lys-Arg
QBP5	1.25	Trp-Trp-Arg-Trp-Asn-Trp-Ala-Thr-Pro-Val-Asp
QBP6	1.23	Trp-His-Asn-Tyr-Phe-His-Trp-Trp-Gln-Asp-Thr
SCR		Trp-Pro-Ile-Trp-Ser-Lys-Gly-Asn-Asp-Trp-Phe

We chose QBP1 (Ser-Asn-Trp-Lys-Trp-Trp-Pro-Gly-Ile-Phe-Asp), which showed the greatest differential binding affinity to pathologic length polyQ compared with normal length polyQ for further analysis.

We first tested our hypothesis that QBP1, a peptide that selectively binds to the expanded polyQ stretch would interfere with polyQ aggregation *in vitro* [40]. We designed thioredoxin-polyQ (thio-polyQ) fusion proteins, and found that thio-polyQ with an expanded polyQ stretch (>40) forms aggregates *in vitro* in a time-, concentration-, and polyQ length-dependent manner, which faithfully mimic the *in vivo* characteristics of disease-causing polyQ proteins. We