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ARTICLE

SCA17, a novel autosomal dominant cerebellar ataxia caused by an expanded polyglutamine in TATA-binding protein

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Genetic etiologies of at least 20% of autosomal dominant cerebellar ataxias (ADCAs) have yet to be clarified. We identified a novel spinocerebellar ataxia (SCA) form in four Japanese pedigrees which is caused by an abnormal CAG expansion in the TATA-binding protein (TBP) gene, a general transcription initiation factor. Consequently, it has been added to the group of polyglutamine diseases. This abnormal expansion of glutamine tracts in TBP bears 47–55 repeats, whereas the normal repeat number ranges from 29 to 42. Immunocytochemical examination of a postmortem brain which carried 48 CAG repeats detected neuronal intranuclear inclusion bodies that stained with anti-ubiquitin antibody, anti-TBP antibody and with the 1C2 antibody that recognizes specifically expanded pathological polyglutamine tracts. We therefore propose that this new disease be called SCA17 (TBP disease).

INTRODUCTION

Autosomal dominant cerebellar ataxias (ADCAs) make up a complex group of neurodegenerative disorders characterized by progressive degeneration of the cerebellum, brain stem and spinal cord (1,2). Presently they are classified according to molecular criteria, but the genetic etiologies of at least 20% of the ADCAs have yet to be determined (3). Interestingly, six of the eight gene-proven spinocerebellar ataxias (SCAs): dentatorubral pallidoluysian atrophy (DRPLA), SCA1, SCA2, SCA3/Machado–Joseph disease (MJD), SCA6 and SCA7 have been shown to be caused by an unstable CAG trinucleotide expansion mutation coding for polyglutamine tracts in the responsible genes (4). These findings strongly suggest that expansion of the polyglutamine tract is a major pathogenic mechanism for dominant ataxias. By screening for expanded polyglutamine tracts by western blotting analysis with a monoclonal 1C2 antibody, we recently identified a new dominant ataxia caused by the expansion of polyglutamine tracts in the TATA-binding protein (TBP), a general transcription initiation factor. This locus was registered as *SCA17* with the approval of the Genome Nomenclature Committee in HUGO (http://www.gene.ucl.ac.uk/nomenclature/). Despite intensive investigation over several years, the genetic pathways and molecular mechanisms that underlie the neuronal degeneration in this group of diseases remain unknown. Recent findings show that in the mouse and fly models transcriptional dysregulation may have importance in cell dysfunction and eventual cell death (5–10). Our findings provide necessary information for clarifying the pathogenesis of polyglutamine diseases.

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RESULTS

1C2 antibody immunoscreening and gene identification

We first conducted 1C2 antibody immunoscreening to identify the expanded polyglutamine tract in lymphoblastoid cell lines from 22 probands, for whom expansions of the eight known CAG repeats associated with neurodegenerative diseases [Huntington's disease (HD), spinobulbar muscular atrophy (SBMA), DRPLA, SCA1, SCA2, SCA3, SCA6, SCA7] had already been excluded by PCR analysis. Mouse monoclonal 1C2 antibody specifically recognizes proteins of largely expanded polyglutamine tracts, particularly those in pathological ranges (greater than 40 glutamines) (11). Using the method described by Stevanin *et al.* (12) but with modifications, we identified a new polyglutamine protein of ~49 kDa in one proband (Fig. 1). Its molecular weight differs from that of any of the known polyglutamine proteins, strongly indicative that this ~49 kDa band is a product of a new gene encoding a polyglutamine tract. Given the similarity of its apparent molecular weight to that of the TBP, we speculated that this 49 kDa protein might be mutant TBP, which is supported by the fact that the monoclonal 1C2 antibody was originally raised against TBP (11). Using the primer pairs flanking the CAG/CAA repeat of the *TBP* gene, we found that both the proband and his affected sibling had the same repeat expansions (Fig. 2A). We therefore determined the CAG/CAA repeat numbers of the *TBP* genes of the two affected siblings and their mother in pedigree A (Fig. 3A). The CAG/CAA repeat number for *TBP* in the healthy mother was 37/39, whereas the numbers in the affected siblings were heterozygous, 37/55 and 39/55 (Fig. 3D and Table 1). The identity of the 49 kDa band was confirmed by western blot analysis of protein extracts from the proband's lymphoblasts using the monoclonal anti-TBP antibody which recognizes an epitope within N-terminal amino acid residues 1–20 of TBP. It clearly shows that his lymphoblasts (Fig. 2B, lane 2; pedigree A, III-1) expressed an equal amount of abnormally large TBP (49 kDa, upper band) as compared with the wild-type TBP (lower band). The intensities of the wild-type

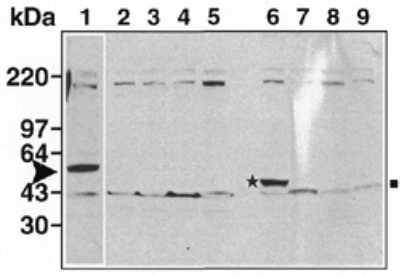

Figure 1. Identification of a novel ~49 kDa polyglutamine protein in lymphoblasts from the proband in pedigree A. The immunoblot of solubilized protein extracts from lymphoblasts, made with monoclonal 1C2 antibody, has a TBP (41 kDa) band in all the lanes (closed square). Two proteins, at ~200 and ~230 kDa, which have the same intensity are visible in the lymphoblastoid extracts from the control MJD patient (lane 1) and all the probands (lanes 2–9). MJD protein with an expanded polyglutamine tract, molecular weight ~60 kDa, (arrowhead) is present in lane 1. The novel ~49 kDa polyglutamine protein (indicated by an asterisk) is present only in the lymphoblastoid extract of the proband in pedigree A (lane 6).

TBP bands in the western blots differ in Figure 1, lane 6 and Figure 2B, lane 2. This is probably due to differences in the reactivities of the 1C2 and anti-TBP antibodies.

PCR screening of expanded CAG/CAA repeat in the *TBP* gene

In pedigree A, the proband's father showed dysarthria at the age of 26 and ataxic gait at the age of 34. He became bedridden at the age of 35 and died of pneumonia at the age of 37. The disease phenotype in pedigree A, including the two affected siblings, is an autosomal dominantly inherited young-onset cerebellar ataxia and progressing dementia with the later addition of a pyramidal and an extrapyramidal sign, e.g. parkinsonism or trunkal dystonia. This suggests that expansion of the CAG repeats of the *TBP* gene is associated with a new form of dominant SCA. To investigate this possibility, we screened 316 disease probands whose clinical phenotypes included

Table 1. SCA17 (TBP disease) clinical features

Patient	NA	AA	Onset	Course	Dementia	Ataxia	Hyper-reflexia	Parkinsonism	Other signs	Epilepsy
A III-1	37	55	19	20[a]	+	+	+	+	Dystonia	–
A III-2	39	55	25	12[a]	+	+	+	+	Dystonia	–
C III-3	39	48	ND	ND[a]	+	+	ND	ND	ND	+
C III-5	36	48	39	13[a]	+	+	+	+	Dystonia	+
C III-6	36	48	48	10[a]	+	+	+	+	Chorea	+
B II-2	36	47	40	15	+	+	+	+	Chorea	+
B III-1	38	47	–	–	–	–	–	–	–	–
B III-2	38	47	–	–	–	–	–	–	–	+
D III-8	32	47	28	9	+	–	+	+	–	+

Dementia is the fundamental symptom common to all the probands. The severity of cerebellar ataxia varies with the case, from minimum trunkal ataxia to obvious dysmetria in the four limbs. Hyper-reflexia tends to appear first in the lower limbs, gradually spreading to all four, but there are no definite pyramidal tract signs, such as Babinski and Chaddock signs. Parkinsonism here indicates bradykinesia and postural reflex disturbance (antero- and retropulsion). Tremor and rigidity are less common symptoms. Epilepsy indicates either an abnormality seen in the EEG or actual episodes of general convulsion.
NA, CAG repeats in normal alleles; AA, CAG repeats in affected alleles; ND, no data.
[a]Patient death.

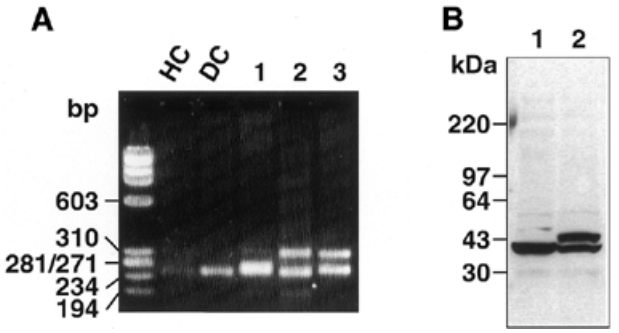

Figure 2. PCR and immunoblot analysis of TBP of pedigree A. (**A**) Genomic DNAs from a control individual (lane HC), a patient with MJD (lane DC), the mother of the proband (lane 1), the proband (lane 2) and the younger sister of the proband (lane 3) were amplified by the TBP-F/TBP-R primer. The father's genomic DNA was not available because he had died. The expected molecular size of the PCR product is 245 bp when the *TBP* gene has 38 CAG/CAA polymorphic repeats. Abnormally expanded PCR products of ~300 bp are present only in the lanes for the proband and his sister (lanes 2 and 3). Numbers on the left side of the gel give the molecular marker size ($\phi \times 174$/HaeIII). HC, healthy control; DC, disease control. (**B**) Total protein lysates of lymphoblasts from the proband's mother (lane 1) and the proband (lane 2) of pedigree A were immunoblotted with anti-TBP antibody (1TBP18; QED Bioscience). Only the proband has an abnormally large TBP. Because no lymphoblast cell line had been established, the proband's sister's (pedigree A, III-2) lymphoblasts were not available.

hereditary spinocerebellar degeneration, sporadic spinocerebellar degeneration, multiple system atrophy, dementia of unknown etiology, chorea and spastic paraplegia. In all these cases, diseases in which the CAG expansions could be attributed to one of the previously identified genes were excluded. The number of CAG/CAA repeats in the *TBP* gene ranged from 29 to 42 (including one chromosome of 42 repeats) in 116 healthy control chromosomes (Fig. 3B, gray bars). The CAG repeat of the *TBP* gene has been intensively analyzed (13,14). In a large population study, alleles corresponding to a range of 25–42 glutamine residues were detected, the most common alleles encoding stretches of 32–39 glutamines. A gln42 allele was found only once in 2003 chromosomes representing several different ethnic backgrounds (15). We therefore concluded that a *TBP* gene having a CAG/CAA number in excess of 43 is pathological. Our 316 disease cases provided three new probands (Fig. 3A, pedigrees B, C, D) whose CAG/CAA repeat number ranged from 47 to 48 (Fig. 3B, black bars; Table 1). Therefore, 43–46 CAG/CAA repeats might be intermediate alleles.

The disease phenotype associated with CAG/CAA expansion in the *TBP* gene is complex and appears to be transmitted in a manner that defies easy categorization. In pedigrees A and B, the phenotype is transmitted autosomal dominantly, whereas in pedigree C, it seems to occur autosomal recessively. In addition, it seems to occur sporadically in pedigree D. We therefore investigated the nucleotide sequence of the CAG/CAA repeat of the *TBP* loci of the family members in all the pedigrees to clarify the inheritance pattern of the expanded CAG repeat in the *TBP* gene. Both siblings (III-1 and III-2) in pedigree A have a heterozygous pattern of gln55 expansion expression at this site (Fig. 3D and Table 1). Their healthy mother (pedigree A, II-9) has gln37/gln39 alleles. These findings indicate that the normal alleles of the proband and his sister were probably maternal transmissions, and the expanded gln55 alleles were paternal ones. Although it was not possible to study their father's DNA, both the proband's father and grandfather had a history of spinocerebellar degeneration. In pedigree B, the expanded gln47 allele of the proband was transmitted to both her daughters (III-1 and III-2). At the time of our study, the younger daughter of the 29-year-old had a history of general convulsion, and epilepsy was diagnosed based on her abnormal EEGs. In contrast, the elder daughter at the age of 33 does not yet show any symptom of neurological deficit. We speculate that she may be asymptomatic due to her young age, because our clinical observations show that this disease can have its onset as late as age 48. We could not obtain any clinical information on the proband's parents (II-1, II-8) in pedigree C, but all three manifesting siblings (III-3, III-5 and III-6) expressed abnormal gln48 alleles heterozygously, whereas the healthy siblings had only normal alleles. In pedigree D, the proband (III-8) has gln32/gln47 alleles, whereas her healthy father (II-5) and brother (III-7) have only normal alleles. No examination of the proband's mother (II-3) could be made because she had died of breast cancer at the age of 43.

We also found two CAA/CAG expansion patterns by nucleotide sequencing of the *TBP* gene. One pattern is intragenomic partial duplication, as observed in patients III-1 and III-2 in pedigree A. The other, which is most frequently seen in this disease, is the simple $(CAG)_y$ expansion in lesion IV of the *TBP* gene (Fig. 4D).

Clinical features of SCA17 (TBP disease)

Age at onset ranged from 19 to 48 years, mean age 33.2 years (n = 6, Table 1). There was a strong inverse correlation (R = –0.88) between age at onset and the number of CAG repeats in the *TBP* gene (Fig. 3C) inspite of the narrow range of expanded alleles (47–55 repeats) and difficulty in determining the exact age of the first sign of ataxia, bradykinesia or dementia in this disorder. Most individuals presented in the third decade with gait ataxia and dementia, progressing over several decades to include bradykinesia, dysmetria, dysdiadokokinesis, hyper-reflexia and paucity of movement. The first symptom (e.g. ataxia, dementia or parkinsonism) varied with the patient. Parkinsonism in these cases mainly showed bradykinesia, gait disturbance (accelerated gait and *marche á petits pas*) and postural reflex disturbance (retropulsion), tremor and muscle rigidity being less prominent. The appearance of epilepsy varied, showing first as a neurological symptom in patient III-2 in pedigree B, but appearing in the late stage in patients III-6 in pedigree C and III-8 in pedigree D. No abnormal eye movements were present in any patient. MRI or CT findings for all the patients indicated diffuse cortical and cerebellar atrophy (Fig. 4). Initial clinical diagnoses varied: dementia-related atypical Holme's type spinocerebellar degeneration (pedigree A), DRPLA-like spinocerebellar degeneration (pedigree B), atypical HD (pedigree C) and atypical parkinsonism with progressive dementia (pedigree D).

Immunocytochemical study of postmortem brain tissues

We conducted an immunocytochemical examination of postmortem brain tissues from a patient (pedigree C, III-5) who had expanded polyglutamine repeats (48) of his TBP. A

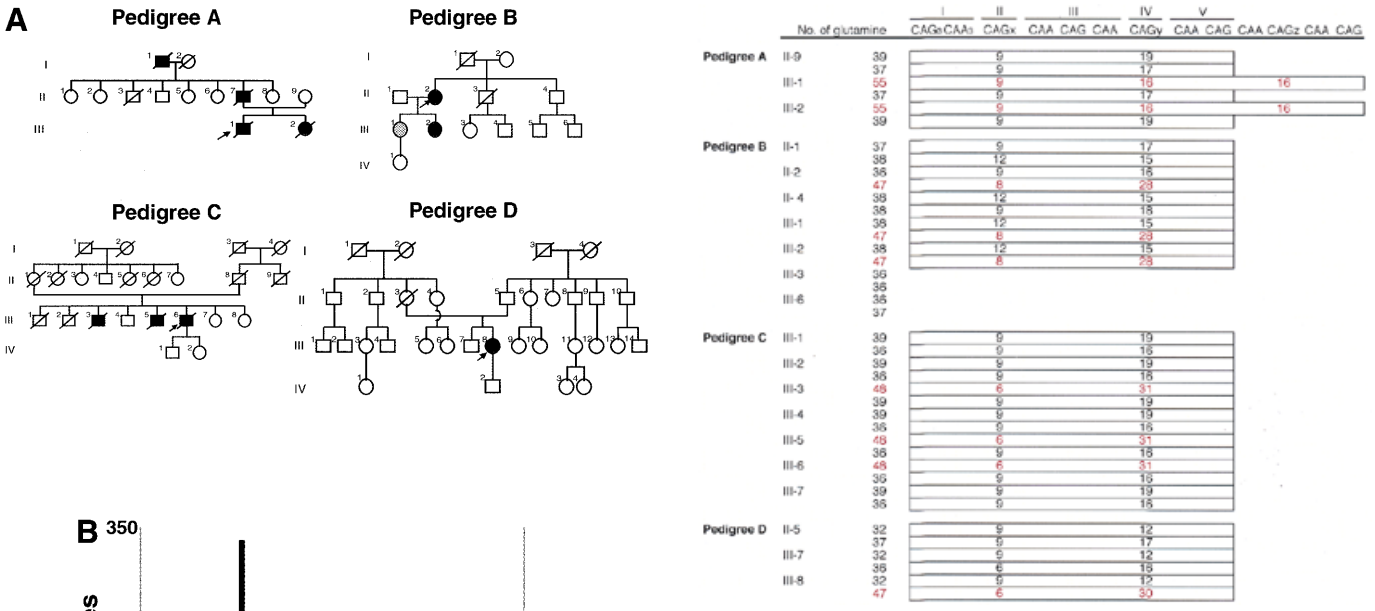

Figure 3. (**A**) Identification of *TBP* mutation in nine individuals from four independent Japanese pedigrees. Pedigree A has an obvious family history of spinocerebellar degeneration. We directly examined all the live members of pedigree B and found no neurological symptoms, except in III-2 who has a history of general convulsion. In pedigree D, except for II-5, III-7 and III-8, there is no clinical information about the family members. (**B**) Distribution of CAG number in the TBP gene. Totally, 748 chromosome analyses of the *TBP* locus were made on 116 control and 632 disease chromosomes. The number of CAG/CAA repeats for the control chromosomes ranged from 29 to 42 (gray bars). Only one chromosome carrying a 42 CAG/CAA repeat was found (0.086%). None of the control chromosomes had more than 43 CAG/CAA repeats, whereas in the disease group, seven chromosomes carried more than 43 CAG/CAA repeats (black bars). (**C**) There is a strong inverse correlation between age at onset and CAG number (R^2 value = 0.783). The closed diamond at the 63 CAG repeat corresponds to the sporadic case of *de novo* mutation reported previously (14). (**D**) Polymorphic CAG/CAA sequences in the *TBP* genes of four probands and family members. Polymorphic regions in the *TBP* gene are divided into five lesions (15). Roman numerals (I–V) above the table denote the lesion domains. The disease phenotype is derived from the heterozygously expanded *TBP* allele and co-segregates with the abnormal expanded *TBP* allele, except in III-1 of pedigree B, who was asymptomatic at the time of our study.

conventional neuropathological examination revealed shrinkage and moderate loss of small neurons with gliosis in the caudate nucleus and putamen. Large neurons were relatively preserved. Similar but moderate changes were detected in the thalamus, frontal cortex and temporal cortex. Moderate Purkinje cell loss and an increase of Bergmann glia were seen in the cerebellum. Torpedoes were occasionally encountered.

Immunocytochemical analysis performed with anti-ubiquitin (Fig. 5B and H) and anti-TBP (Fig. 5D and K) antibodies showed neuronal intranuclear inclusion bodies (NIIs). In addition, most, if not all, neuronal nuclei were diffusely stained with 1C2-Ab (Fig. 5C), whereas none were stained in the healthy control brains (Fig. 5E and F). Except for SCA2 (16,17) and SCA6 (18), in which abnormal inclusions are present in the cytoplasm, NIIs may provide a marker of disease process in many polyglutamine diseases (19,20). The presence of NIIs in the brain of the patient who had expanded polyglutamine repeats (48) further confirmed that this disorder should be categorized as a polyglutamine disease.

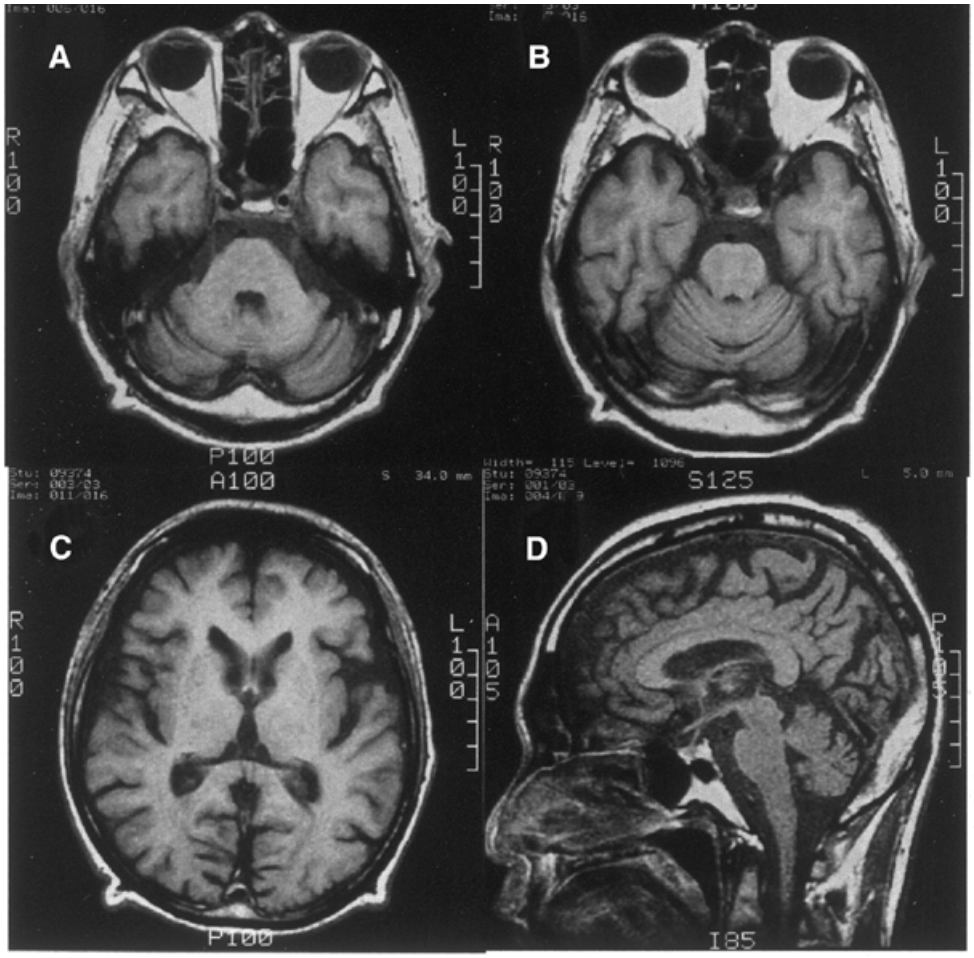

Figure 4. Brain MRI images of a representative patient (pedigree A, III-1) showing diffuse cortical and cerebellar atrophy. Atrophy between the cerebellar hemisphere, brain stem and cortex is not disproportionate. T1-weighted axial images (**A**, **B**, **C**) and a T1-weighted sagital image (**D**).

DISCUSSION

TBP is an important general transcription initiation factor (15,21) and the DNA-binding subunit of RNA polymerase II transcription factor D (TFIID), the multi-subunit complex crucial for the expression of most genes (22,23). The long polyglutamine domain is located in the N-terminus of TBP which regulates the DNA-binding activity of the C-terminus of the protein (24,25). Imbert *et al.* (26) first suggested the possibility of a role for this N-terminal expanded CAG repeat of *TBP* in certain late-onset neurologic disorders. Since then, this gene has been investigated intensively as a candidate for psychiatric disorders (13,26,27).

We show here for the first time that CAG expansion in the *TBP* gene does cause a neurological disorder inherited in an autosomal dominant manner that is classified as a novel polyglutamine disease. Several lines of evidence support our conclusion that polyglutamine expansion in the TBP is pathogenic and the causative agent of a novel type of disease phenotype. (i) The disease phenotype completely co-segregates with the genotype of the abnormally expanded CAG/CAA repeat and arises from a heterozygously expanded *TBP* allele in all the patients in the four pedigrees we have examined. (ii) The clinical features of young-onset ataxia, dementia and parkinsonism are very common. (iii) The disease phenotype detected in this study shares a feature (very common) with a case of *de novo TBP* mutation (14), whereas the clinical feature appears to be more complex and severer in a sporadic case (63 CAG repeats). (iv) Except for one previously reported case (14), there has been no report of a CAG expansion of more than 43 repeats. (v) We found a strong negative correlation between age at onset and the number of polyglutamine repeats (R^2 value = 0.78), a correlation frequently found in other polyglutamine disorders. (vi) NII bodies were present in a diseased brain which carried 48 CAG repeats. We found no evidence, however, indicative of the instability of the CAG repeat in germline transmission, a characteristic feature of polyglutamine diseases.

The precise molecular mechanism that operates in the present disease is not clear. Nuclear localization of the protein with the expanded polyglutamine tract, however, has been indicated as crucial to polyglutamine disease pathogenesis (8,28–30). Of the eight known causative genes of polyglutamine diseases, *TBP* is the third gene, along with the androgen receptor and *CACNL1A4* (the genes responsible for SBMA and SCA6, respectively), whose function is well understood. Taking into account the fact that TBP is abundant in the nucleus and is essential for gene expression, its participation in

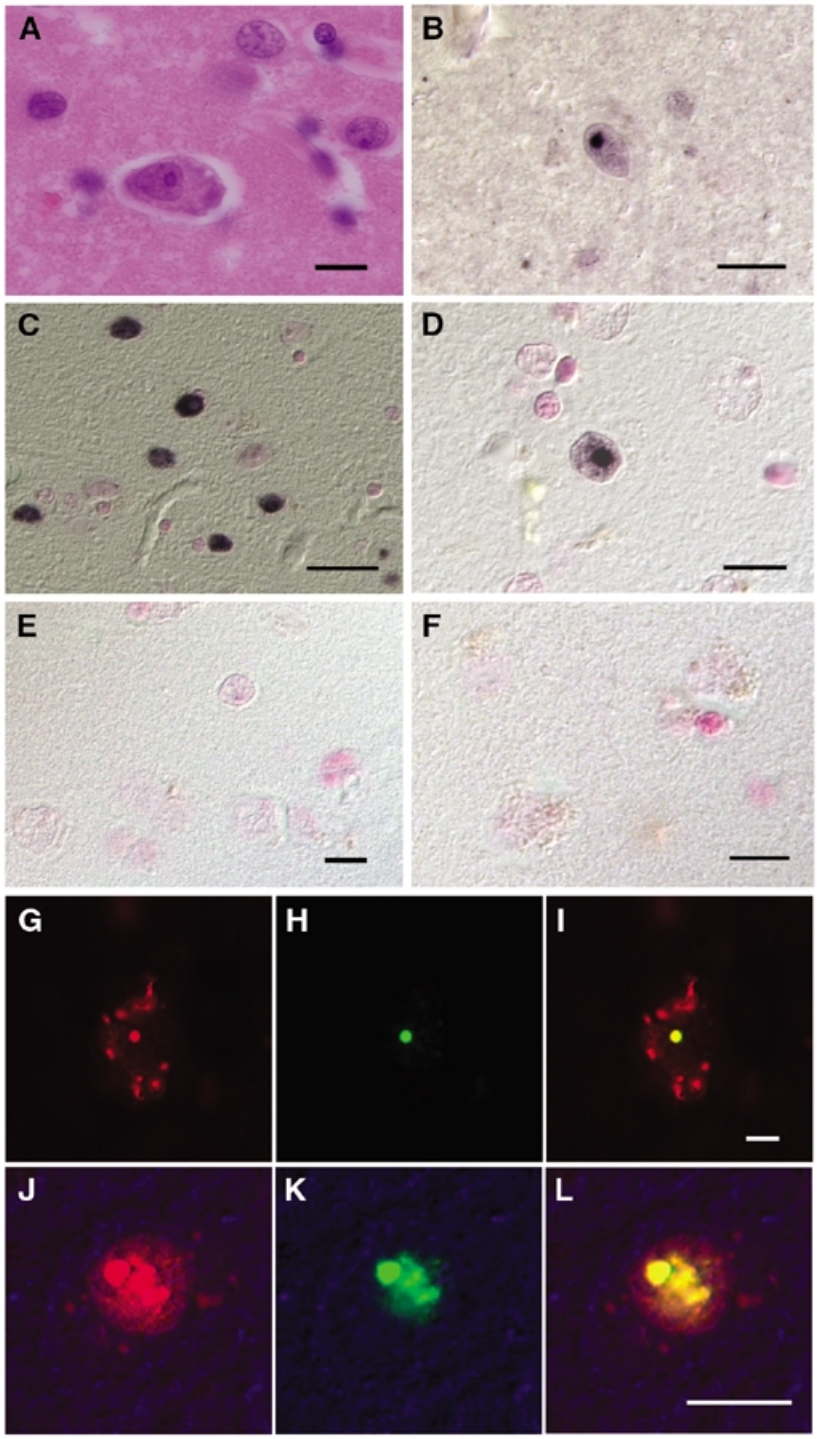

Figure 5. Immunohistochemical study of postmortem brain tissues from a patient (pedigree C, III-5). (**A**) A small neuron in the putamen has an intranuclear inclusion body (HE stain). (**B**) Anti-ubiquitin antibody (Dakopatts; 1:1000): a positive intranuclear inclusion body identified in the putamen. (**C**) Diffuse 1C2-Ab (Chemicon, 1:1000 dilution): immunoreactive neuronal nuclei scattered throughout the frontal cortex. In contrast, no 1C2-Ab immunoreactive neurons are present in the frontal cortex (**E**) or putamen (**F**) of a healthy control brain. (**D**) The intranuclear inclusion body in a neuron of the putamen is also positive for anti-TBP antibody (QED Bioscience; 1TBP18, 1:20000 dilution). An immunofluorescent double-labeling study of the neuronal intranuclear inclusion body (**G–I**). NII in a neuron of the caudate nucleus (G–I) and in a neuron of the putamen (**J–L**). Neurons are stained with monoclonal 1C2-Ab (red, G and J), anti-ubiquitin antibody (green, H) or anti-TBP polyclonal antibody (green, K; Santa Cruz, sc204). This NII consists of an abnormal polyglutamine epitope and ubiquitin (merge, I). It also has TBP (K). A Normarski view (blue channel) is superimposed on (J–L). Scale bar: 10 µm for (A, B, D, E, F, I, J) and 25 µm for (C).

polyglutamine diseases favors a molecular pathogenesis for these diseases. When bound to the TATA box, TBP has a saddle-like shape with its concave face contacting the DNA and its convex one interacting with the other TFIID subunits, the TBP-associated factors (TAFs) (23). The tertiary structural change caused by polyglutamine expansion in TBP may lead

to an aberrant affinity for TAFs, culminating in altered gene expression and cell death. Indeed, human TAFp130 that binds to the polyglutamine domain of the DRPLA protein is length-dependent (9). Lin *et al.* (8) reported that in transgenic mice the expression of several neuronal genes involved in calcium homeostasis and signal transduction are down-regulated very soon after the expression of mutant ataxin-1 protein (8). Polyglutamine-expanded huntingtin, but not normal huntingtin, interacts both *in vivo* and *in vitro* with the transcriptional repressor N-CoR (nuclear receptor corepressor), and N-CoR and at least one of its corepressors, Sin3, are localized ectopically in the cytoplasm of brain sections from HD patients (31). In contrast, co-localization of transcription factors such as CREB-binding protein (CBP) (10,32), TBP itself (33,34), TAFII130 (9) and Sin3a (31), is reported to be recruited into the NIIs. All these findings provide strong evidence that in polyglutamine diseases major transcriptional changes occur in the nucleus.

Further study of what takes place in the dying neuron's nucleus in this disease should provide important information for unraveling the molecular pathogenesis of neuronal cell degeneration as well as for the development of future therapeutic interventions.

MATERIALS AND METHODS

Patients

We interviewed families and collected blood samples after obtaining the fully informed consent of the patients and their families.

Western blot analysis of lymphoblasts

This procedure is described in detail elsewhere (12,35). Briefly, lymphoblast cells (5×10^6) were washed in phosphate-buffered saline (PBS) and resuspended in 2 ml of Tris-buffered saline (TBS, 20 mM Tris–HCl, 150 mM sodium chloride pH 7.5) with 1 mM EDTA and the protease inhibitors (Complete, Boehringer Mannheim, Germany). The cells were then homogenized in an ultrasonicator and centrifuged at 10 000 *g* at 4°C for 30 min. The supernatant was collected, and the protein concentration determined with a BCA protein assay kit (Pierce, Rockford, IL). Samples were stored at –80 C° until they were used. Protein samples (50 µg/lane) were separated by SDS–PAGE in a 0.1% SDS/5–20% gradient polyacrylamide gel then transferred to Immobilon membranes (Millipore, MA). The membranes were blocked with 1% bovine serum albumin and 4% dry milk in TBST buffer (TBS with 0.05% Tween 20) for 1 h at room temperature, after which they were incubated overnight at 4°C in 3% dry milk/TBST with the 1C2 Ab (1:2000; Chemicon, Temecula, CA) and given three 10 min washes in TBST. The filters then were incubated in 3% dry milk/TBST with HRP-conjugated secondary anti-mouse Ig Ab (1:5000). Finally, the reaction was made visible with an enhanced chemiluminescence (ECL) western blotting kit (Amersham Pharmacia Biotech, UK) according to the manufacturer's protocol. Mouse monoclonal anti-TBP antibody (1TBP18; QED Bioscience, CA) at 1:2000 dilution was used for the TBP immunoblotting.

PCR and GeneScan analysis of the *TBP* gene

The PCR of the *TBP* gene was done with the forward primer TBP-F, 5′-CCTTATGGCACTGGACTGAC-3′, and reverse primer TBP-R, 5′-GTTCCCTGTGTTGCCTGCTG-3′. The patient's genomic DNA was amplified in a 25 µl reaction mixture containing DNA template (60–150 ng), dNTP (200 µM each), 10 pmol of each primer, 2.5 µl of 10× PCR buffer and *AmpliTaq* Gold polymerase (5 U; Perkin Elmer, Foster City, CA). Optimal PCR conditions were initial denaturation at 95°C for 12 min, followed by 40 cycles of 20 s at 95°C, 1 min at 60°C, 1 min at 72°C, and final extension at 72°C for 10 min. All the PCR reactions were done in a thermal cycler (iCycler; Bio-Rad, Hercules, CA). PCR products were separated by electrophoresis through 3.5% agarose and made visible by CYBER Green I (Molecular Probes, Eugene, OR) staining. For the GeneScan analysis, forward primer TBP-F was replaced by FAM-labeled primers (TBP-FAM). The GS500 TAMRA-labeled size standard (Perkin Elmer) and PCR products were run simultaneously in the same lanes. The CAG repeat size was determined with an ABI 377XL DNA sequencer and GeneScan version 3.1 software.

Direct sequencing of CAG/CAA polymorphism in the *TBP* gene

Amplified DNA fragments bearing the *TBP* gene (nucleotides 338–563; NM_003194, gi: 4507378) were subcloned into pGEM-T Easy vector (Promega, Madison, WI). DNA sequences of the polymorphic site in the *TBP* gene were verified in 10 individual clones from each genomic DNA template. Homozygotic or heterozygotic CAG alleles of the *TBP* gene were also authenticated on the basis of the CAG/CAA numbers obtained from the GeneScan analysis.

Immunocytochemical analysis of postmortem brain tissue

We obtained postmortem brain tissue (pedigree C, III-5) from the brain bank (NH No. A-157) at the Tokyo Metropolitan Institute for Neuroscience. Formalin-fixed, paraffin-embedded sections, 4 mm thick, were prepared from the caudate nucleus, putamen and frontal cortex of the patient and the control subjects. Two pre-treatment protocols were used. Sections were autoclaved and treated with 99% formic acid for 5 min at room temperature or microwaved in 10 mM citrate buffer (pH 6.0), then treated with 1% periodic acid for 15 min, after which they were immunostained by the ABC method (Vector, Burlingame, CA) with mouse monoclonal 1C2 (1:1000–16 000, Chemicon), rabbit polyclonal (sc204, 1:20 000, Santa Cruz; Santa Cruz, CA), mouse monoclonal (1TBP18, 1:20000, QED Bioscience; San Diego, CA), anti-TBP antibody or rabbit polyclonal antibody against ubiquitin (1:1000; Dakopatts, Carpinteria, CA). Diaminobenzidine was used as the chromogen. Double immunofluorescence analysis was performed by incubating de-paraffinized sections with a mixture of 1C2 monoclonal antibody (1:1000) and anti-ubiquitin (1:500) or anti-TBP (1:250) rabbit polyclonal antibody. Anti-mouse IgG coupled with Rhodamine red (1:200, Jackson ImmunoRes, West Grove, PA) was used to make the 1C2 epitope visible. Anti-rabbit Ig G coupled with horseradish peroxidase (1:1000, Pierce, Rockford, IL) reacted with biotinylated tyramide which enabled the other epitope to be

made visible with FITC conjugated with streptavidin (1:200, Vector). After being mounted with Vectashield (Vector), the sections were observed under a fluorescent microscope equipped with a laser confocal system (TCS-SP, Leica, Heidelberg, Germany).

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