Additional file 1

Acta Neuropathologica Communications

Spinocerebellar ataxia type 17-digenic *TBP*/*STUB1* disease: autopsy features of an autopsied patient

Rie Saito[1], Yui Tada[2], Daisuke Oikawa[3], Yusuke Sato[4], Makiko Seto[5], Akira Satoh[5], Kodai Kume[2], Nozomi Ueki[6], Masahiro Nakashima[6], Shintaro Hayashi[1,7], Yasuko Toyoshima[1,8], Fuminori Tokunaga[3], Hideshi Kawakami[2], Akiyoshi Kakita[1]

[1]Departments of Pathology, Brain Research Institute, Niigata University, 1-757 Asahimachi, Chuo-ku, Niigata 951-8585, Japan.

[2]Department of Epidemiology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan.

[3]Department of Medical Biochemistry, Graduate School of Medicine, Osaka Metropolitan University, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan

[4]Department of Chemistry and Biotechnology, Graduate School of Engineering, Tottori University, 4-101 Koyama-cho Minami, Tottori 680-8552, Japan.

[5]Section of Neurology, Nagasaki Kita Hospital, 800 Motomurago, Togitsu-cho, Nishisonogi-gun, Nagasaki 851-2103, Japan.

[6]Department of Tumor and Diagnostic Pathology, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.

[7]Department of Neurology, Mishima Hospital, 1713-8 Fujikawa, Nagaoka, Niigata 940-2302, Japan.

[8]Department of Neurology, Brain Disease Center, Agano Hospital, 6317-5 Yasuda, Agano, Niigata 959-2221, Japan.

Correspondence to: Akiyoshi Kakita, MD, PhD
Department of Pathology, Brain Research Institute, Niigata University, 1-757 Asahimachi, Chuo-ku, Niigata 951-8585, Japan.
TEL: +81-25-2270673
FAX: +81-25-2270817
E-mail: kakita@bri.niigata-u.ac.jp

Supplementary methods

Histopathologic analysis

The brain and spinal cord were fixed with 10% buffered formalin, and multiple tissue blocks were embedded in paraffin. Histological examination was performed on 4-μm-thick sections using hematoxylin and eosin and Klüver-Barrera staining. In addition, selected sections were immunostained with antibodies against calbindin D-28k, STUB1 (protein) [2], polyglutamine and p62 (**Supplementary table 1**). Antibodies against amyloid β 11-28, phosphorylated tau and phosphorylated α-synuclein were used to assess senile pathologic changes based on the "ABC" score [7] and the fourth consensus report of the DLB consortium [5]. Bound antibodies were visualized by the peroxidase-polymer-based method using a Histofine Simple Stain MAX-PO kit (Nichirei, Tokyo, Japan) with diaminobenzidine as the chromogen. Immunostained sections were counterstained with hematoxylin.

Genetic analysis

Genomic DNA was extracted from the fresh-frozen frontal cortex of the present patient, and also from the occipital cortex of the patient who was homozygotic for SCA17 [10].

Exome sequencing: The extracted genomic DNA was used to prepare an exome library using a BGI platform sequencer. Details of the method were described in our previous report [4]. Exome capture and library preparation were performed using xGenTM Exome Research Panel v2 (IDT, Coralville, USA). Whole-exome sequencing was performed using a BGI platform sequencer (DNBSEQ-G400). We identified a heterozygous missense mutation in *STUB1* (p.P243L), which was validated by Sanger sequencing (**Fig. 1c**). To evaluate the pathogenicity of the variants, we employed the following criteria: (1) two or more prediction algorithms (SIFT, PP2, Mutation Taster, and CADD) were positive (the CADD score threshold was set to >20) and (2) the allele frequency of the variant was ≤0.005.

Fragment analysis: The *TBP* repeat region was amplified by PCR using an ExTaq (Takara, Japan) based on our previous report [~~5~~8]. The primer sequences were: Forward 5'-(6-FAM) CCTTATGGCACTGGACTGA and Reverse 5'-GTTCCCTGTGTTGCCTGCTG. The amplicon size was measured using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, USA). The size standard was GeneScan™-500 ROX (Applied Biosystems, USA).

Sanger sequencing: *STUB1* exon 6 was amplified by PCR using an Expand High Fidelity PCR System (Roche, Switzerland). The primer sequences were: Forward 5'-GAAGAGGAAGGTGAGTGTGTGTC, Reverse 5'-CAGAGATGAATGCGTCAATAACC. The *TBP* repeat region was amplified using the same method as that for fragment analysis. The amplicons were cloned into pMD20 (TAKARA, Japan) using a TA cloning method. Cycle sequencing of the amplicons was performed using the BigDye Terminator v3.1 Cycle Sequencing

Kit (Applied Biosystems, USA). The sequence of amplicons was analyzed by the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, USA).

Plasmids

The open reading frame of human *STUB1* was amplified by reverse-transcription PCR. P243L mutation was introduced by the QuikChange method, and the nucleotide sequences were verified. The coding sequences were tagged with Myc, amplified by PCR, and cloned into the pcDNA3.1 expression vector (Invitrogen).

Cell culture and transfection

HEK293T cells (ATCC) were cultured in DMEM containing 10% fetal bovine serum, 100 IU/ml penicillin G, and 100 μg/ml streptomycin at 37°C under 5% CO_2. Transfection experiments were performed using polyethyleneimine (PEI MAX; Polysciences).

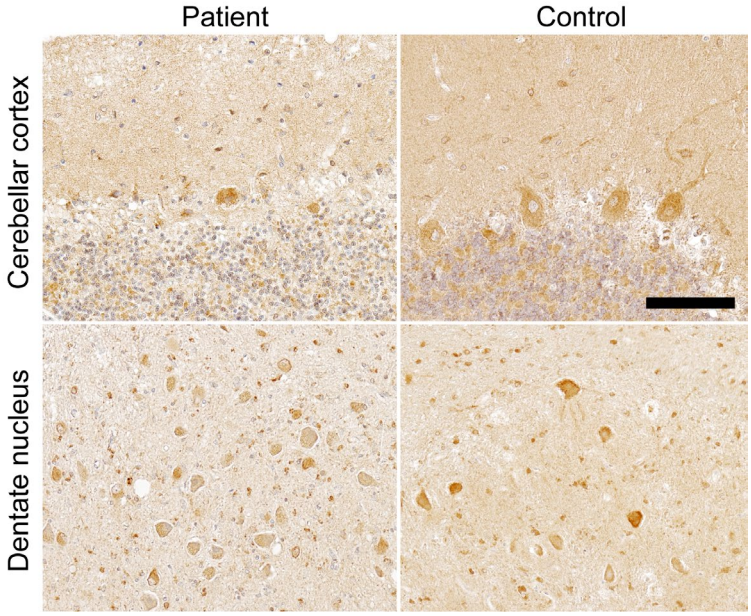
In vitro ubiquitination assay

For in vitro ubiquitination assay, HEK293T cells were transfected with pcDNA3-Myc-*STUB1*-WT or p.P243L mutant. After 24 h, the cells were lysed with lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1 mM DTT, and immunoprecipitated using rabbit anti-Myc antibody and protein A beads (Cytiva). After a wash with lysis buffer, the beads were incubated with 5 μg/ml recombinant mouse His-E1, 20 μg/ml E2 (recombinant human UbcH5a; Boston Biochem), and 250 μg/ml ubiquitin (Sigma-Aldrich), in buffer containing 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, and 2 mM ATP at 30 °C for 4 h. Then, the samples were subjected to SDS–PAGE, followed by immunoblotting.

SDS-PAGE and immunoblotting

Samples were separated by SDS-PAGE using 2/15% gradient gels (Cosmobio) and transferred to PVDF membranes. After blocking the membranes in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) with 5% skim milk for 2 h at room temperature (RT), they were incubated overnight with the appropriate primary antibodies diluted in TBS-T containing 5% skim-milk at 4°C. The following antibodies were used for immunoblotting analyses: Myc (HRP-Conjugate), ubiquitin, and tubulin. For immunoprecipitation, rabbit anti-Myc antibody was used (**Supplementary table 1**). Then, the membranes were incubated with anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (NA931V; 1:10,000; Cytiva) diluted in TBS-T containing 5% skim-milk for 1.5 h at RT. For detection, SuperSignal West Pico PLUS (34577; Thermo Fisher Scientific) or Luminata Forte (WBLUF0100; Millipore) was used. The chemiluminescent images were obtained with a Fusion Solo S imaging system (Vilber).

Supplementary figure 1. STUB1 immunohistochemistry in the patient.



Images of the Purkinje cell layer (affected lesion) and dentate nucleus (unaffected area). The patient and control display positive immune-reactivity in the cytoplasm and dendrites of the Purkinje cells, and cytoplasm and neurites in the dentate nucleus. Bar = 100 μm in the *upper panels*; 150 μm in the *lower panels*

Supplementary references

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Supplementary table 1. Primary antibodies.

Antigen (clone)	Antibody species	Source	Dilution	Antigen retrieval
Immunohistochemistry				
Calbindin-D28k (CB300)	Mouse	Swant, Fribourg, Switzerland	1:50	Microwave
STUB1	Rabbit	Abcam, Cambridge, UK	1:800	Autoclave (pH 9)
STUB1	Mouse	Proteintech, Rosemont, IL, USA	1:200	Autoclave (pH 9)
p62 (3/P62)	Mouse	BD, San Jose, CA, USA	1:1000	Microwave
Polyglutamine (5TF1-1C2)	Mouse	Chemicon, Temecula, CA, USA	1:16000	Formic acid
Amyloid β (11-28, 12B2)	Mouse	IBL, Gunma, Japan	1:50	Formic acid
Phosphorylated tau (AT8)	Mouse	Fujirebio, Ghent, Belgium	1:200	None
Phosphorylated α-synuclein (pSyn#64)	Mouse	Wako, Saitama, Japan	1:1000	Formic acid
Immunoblotting				
Myc (M192-7)	Mouse	MBL, Tokyo, Japan	1:10000	None
Ubiquitin (P4D1)	Mouse	Santa Cruz Biotechnology, Dallas, TX, USA	1:1000	None
Tubulin (CLT9002)	Mouse	Cedarlane, Ontario, Canada	1:3000	None
Immunoprecipitation				
Myc (562)	Rabbit	MBL, Tokyo, Japan	1:500	None

Supplementary table 2. Summary of the clinical features in the autopsied patients with SCA17-DI, SCA17, and SCA48.

Disorder	Patient	*TBP* allele	*STUB1* mutation	Sex	Age at onset (years)	Disease duration (years)	Cerebellar ataxia	Cognitive impairment	Psychiatric disorder	Rigidity	Involuntary movement	Epilepsy
SCA17-DI	This case	41/38	p.P243L	F	62	14	Yes	Yes	No	No	Chorea	No
SCA17-DI	Fujigasaki et al. [3, 9]	46/37	p.R154C	F	55	10	Yes	Yes	No	Yes	No	No
SCA17	Bruni et al. [1]	52/wild	na	F	17	30	Yes	Yes	Yes	Yes	Dystonia, myoclonus	Yes
SCA17	Toyoshima et al. [10]	48/48	No	M	39	10	Yes	Yes	Yes	No	Chorea, ballism	No
SCA48	Roux et al. [9]	na	p.A46P	F	47	15	Yes	Yes	Yes	Yes	Chorea, dystonia	Yes
SCA48	Mol et al. [6]	na	p.C244Y	na	65	18	Yes	Yes	No	na	No	No
			p.C244Y	na	61	10	Yes	Yes	No	na	Subtle	No
			p.C244Y	na	50	14	Yes	Yes	Yes	na	Subtle	No
SCA48	Chen et al. [2]	na	p.I53T	M	40s	<20	Yes	Yes	No	No	Short jerky movements	No
			p.I53T	M	60s	<19	Yes	Yes	No	na	No	No
			p.I53T	F	NA	10-20?	Yes	Yes	Yes	No	No	No
			p.F37L	F	40	21	Yes	Yes	Yes	na	Tremor	No