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【新評価方法を用いたフォールディング病の分子シャペロン療法の検討班】
（分担）研究報告書

凝集阻害化合物のポリグルタミン病モデルマウスに対する治療効果

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研究要旨

ポリグルタミン（PolyQ）病を含む多くの神経変性疾患では、異常蛋白質のミスフォールディングが共通して神経変性を引き起こすと考えられている。我々はこれまでに変性蛋白質のミスフォールディング・凝集を標的とした共通の治療薬開発を目指して、*in vitro* のアッセイ系を用いた PolyQ 凝集阻害化合物スクリーニングにより、血液脳関門透過性・安全性の高い化学シャペロン QAI1 を同定した。本研究では、QAI1 の臨床応用に向けて、様々な PolyQ 病モデルに対する QAI1 の効果を検討した。その結果、QAI1 が異常伸長 PolyQ 蛋白質のβシート構造変移、オリゴマー形成を抑制することを明らかにした。さらに QAI1 が PolyQ 病モデルショウジョウバエ、マウスにおいても PolyQ 凝集体形成および神経症状を抑制することを見出した。

A．研究目的

アルツハイマー病、パーキンソン病、ポリグルタミン（PolyQ）病などの多くの神経変性疾患では、異常蛋白質のミスフォールディング・凝集が共通して神経変性を引き起こすと考えられていることから、「フォールディング病」と総称されている。PolyQ 病は、種々の脊髄小脳失調症やハンチントン病などを含む９疾患の総称で、PolyQ 鎖の異常伸長により原因蛋白質がミスフォールディング・凝集を生じ、その結果神経変性を引き起こすと考えられている。

我々はこれまでに変性蛋白質のミスフォ

ールディング・凝集を標的とした共通の治療薬開発を目指して、*in vitro* のアッセイ系を用いて化合物ライブラリーのスクリーニングを行い、いくつかの PolyQ 凝集阻害化合物を同定した。本研究では、その中で血液脳関門透過性・ヒトへの安全性が高く、蛋白質のフォールディングに作用する化学シャペロン QAI1 に注目し、様々な神経変性疾患に広く共通の分子標的治療薬を創薬することを目的として、以下の研究を行った。

B&C&D．研究方法、結果および考察

①QAI1 の *in vitro* での PolyQ 蛋白質凝集体形成に対する効果の検討：まず異常伸長 PolyQ 蛋白質の凝集体形成に対する QAI1 の効果を、*in vitro* の アッセイ系を用いて詳細に検討した。その結果、QAI1 が濃度依存的に異常伸長 PolyQ 蛋白質の凝集体形成を抑制し、そして既に凝集した PolyQ 蛋白質に対してもさらなる凝集の進行を抑制することを明らかにした。

②QAI1 の PolyQ オリゴマー形成に対する効果の検討：次に、異常伸長 PolyQ 蛋白質の凝集体形成の前に生じる、より毒性が高いβシート構造変移やオリゴマー形成に対する QAI1 の効果を検討した。Native PAGE の結果から、QAI1 が *in vitro* での異常伸長 PolyQ 蛋白質のβシート変移を抑制することを確認した。さらに FCS 解析の結果から、QAI1 が細胞内での異常伸長 PolyQ 蛋白質のオリゴマー形成を阻害することが明らかとなった。

③QAI1 の PolyQ 病モデルショウジョウバエに対する治療効果の検討：次に、QAI1 の PolyQ 病モデルショウジョウバエに対する *in vivo* での治療効果を検討した。その結果、QAI1 が PolyQ 病モデルショウジョウバエにおける PolyQ 凝集体形成および神経変性を抑制することを明らかにした。

④QAI1 の PolyQ 病モデルマウスに対する治療効果の検討：次に、QAI1 の PolyQ 病モデルマウスに対する治療効果を検討した。その結果、QAI1 が PolyQ 病モデルマウスにおける PolyQ 凝集体形成および運動障害を有意に抑制することを明らかにした。さらに QAI1 は発症後からの投与でも運動障害を抑制することを示した。

(倫理面への配慮)

本研究では直接ヒトを対象とした研究は行っていない。実験動物の取り扱いにあたっては国の法律・指針および国立精神・神経医療研究センター動物実験倫理指針を遵守した。

E．結論

QAI1 は異常伸長 PolyQ 蛋白質のβシート構造変移、オリゴマー・凝集体し、さらに PolyQ 病モデルショウジョウバエ、マウスにおいて治療効果を発揮することを見出した。

F．健康危険情報

なし

G. 研究発表

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なし

H. 知的財産権の出願・登録状況（予定を含む）

III　研究成果の刊行に関する一覧表

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Original Article

Abnormal RNA structures (RNA foci) containing a penta-nucleotide repeat $(UGGAA)_n$ in the Purkinje cell nucleus is associated with spinocerebellar ataxia type 31 pathogenesis

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Spinocerebellar ataxia type 31 (SCA31) is an autosomal-dominant cerebellar ataxia showing a Purkinje cell (PC)-predominant neurodegeneration in humans. The mutation is a complex penta-nucleotide repeat containing $(TGGAA)_n$, $(TAGAA)_n$, $(TAAAA)_n$ and $(TAGAATAAAA)_n$ inserted in an intron shared by two different genes *BEAN1* and *TK2* located in the long arm of the human chromosome 16. Previous studies have shown that $(TGGAA)_n$ is the critical component of SCA31 pathogenesis while the three other repeats, also present in normal Japanese, are not essential. Importantly, it has been shown that *BEAN1* and *TK2* are transcribed in mutually opposite directions in the human brain. Furthermore, abnormal RNA structures called "RNA foci" are observed by a probe against $(UAGAAUAAAA)_n$ in SCA31 patients' PC nuclei, indicating that the *BEAN1*-direction mutant transcript appears instrumental for the pathogenesis. However, it is not known whether the critical repeat $(TGGAA)_n$ contributes to the formation of RNA foci, neither do we understand how the RNA foci formation is relevant to the pathogenesis. To address these issues, we investigated two SCA31 cerebella by fluorescence *in situ* hybridization using a probe against $(UGGAA)_n$. We also asked whether the mutant *BEAN1*-transcript containing $(UGGAA)_n$ exerts toxicity compared to the other three repeats in cultured cells. Histopathologically, we confirm that the PC is the main target of SCA31 pathogenesis. We find that the RNA foci containing $(UGGAA)_n$ are indeed observed in PC nuclei of both SCA31 patients, whereas similar foci were not observed in control individuals. In both transiently and stably expressed cultured cell models, we also find that the mutation transcribed in the *BEAN1*-direction yields more toxicity than control transcripts and forms RNA foci detected with probes against $(UGGAA)_n$ and $(UAGAAUAAAA)_n$. Taking these findings together, we conclude that the RNA foci containing *BEAN1*-direction transcript $(UGGAA)_n$ are associated with PC degeneration in SCA31.

Key words: cerebellar ataxia, neurodegeneration, Purkinje cell, repeat expansion, RNA foci.

INTRODUCTION

Spinocerebellar ataxia (SCA) is defined as a group of autosomal-dominant neurodegenerative disorders affecting the cerebellum and its related nervous system.[1] SCA31 is caused by a complex penta-nucleotide repeat insertion containing a complex of penta-nucleotides, $(TGGAA)_n$, $(TAGAA)_n$, $(TAAAA)_n$, and the combination of the latter two $(TAGAATAAAA)_n$, in an intron shared by two different genes called *BEAN* (brain expressed, associated with Nedd4; recently re-named as *BEAN1*) and *TK2* (thymidine kinase 2) in human chromosome 16q22.1.[2] Clinically, this disease shows purely cerebellar ataxia with average age of onset at 60 years.[3,4] This is one of the most common SCAs in Japan.[5,6] The neuropathology of SCA31 shows predominant degeneration of the Purkinje cell (PC),[3] whereas neurons in the granule cell and molecular layers are

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preserved compared to PC. The neurodegeneration in SCA31 is characterized by a peculiar shrinkage of PC bodies surrounded by synaptophysin-immunoreactive structures called "halo-like amorphous materials".[3,7] In addition to presynaptic endings, calbindin D_{28k}-positive and ubiquitin-positive granules are seen in these structures, indicating that degeneration of PC dendrites are taking place.

A previous study has shown that transcripts of *BEAN1*-direction form RNA foci in the SCA31 PC nucleus conforming to the general rule in the RNA-mediated non-coding repeat disorders,[8,9] which include SCA8, 10, 12, myotonic dystrophy type 1 (DM1), DM2, Huntington's disease-like disease type 2 (HDL2), fragile X ataxia/tremor syndrome (FXTAS), SCA36[10] and *C9orf72*-linked amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD).[11] However, the implications of these foci for the SCA31 pathogenesis is totally unknown. A previous study demonstrated that the RNA foci contain non-pathogenic $(UAGAAUAAAA)_n$ repeat.[2] However, such foci may also be present in healthy individuals harboring a rare insertion $(TAGAATAAAA)_n$. Therefore, it is particularly important to address whether the RNA foci in the SCA31 PC nucleus contain the critical $(UGGAA)_n$ repeat sequence, the *BEAN1*-direction transcript of $(TGGAA)_n$. In addition, it is not known how its formation is related to PC degeneration.

To clarify whether the SCA31 RNA foci contains the presumably toxic $(UGGAA)_n$ repeat, we studied two new patients with SCA31 by fluorescence *in situ* hybridization (FISH) technique using a new probe against this repeat. We also asked whether the $(UGGAA)_n$-containing repeat is toxic and forms RNA foci in cultured cells.

PATIENTS, MATERIALS AND METHODS

Patients' clinical information

The first patient, aged 80 years at death, is a relative of the first pathologically reported SCA31 patient.[3] The second individual, aged 74 years at death, is unrelated with the first individual. The following are brief clinical summaries of the two individuals. Of note is that these two SCA31 patients were both in a mild stage of the disease. The length of the mutation was 2.81 kilo-base-pairs (kb) in Patient 1 and 2.86 kb in Patient 2. This roughly corresponds to 562 (Patient 1) and 572 (Patient 2) penta-nucleotide repeats, respectively.[2]

Patient 1

This patient first noticed hearing impairment at 61 years, and it gradually worsened. At the age of 70, he noticed a very mild truncal ataxia. Neurological examination disclosed very mild truncal ataxia that was pronounced when he tried to stand in tandem. MRI disclosed isolated cerebellar atrophy most prominent in the midline region. At the age of 80, his cerebellar ataxia was still mild so that he could walk by himself with a cane. The Scale for the Assessment and Rating of Ataxia (SARA) score during his final days was 10. He suddenly died of aspiration pneumonia.

Patient 2

This male individual first developed imbalance in walking at the age of 70. He also noticed difficulties in articulation. At the age of 73, he was found to have an advanced gastric cancer which had already extended to his retroperitoneal space. He was referred to us (TI and KI) for neurological investigation which revealed very mild ataxia also scoring 10 on SARA. He subsequently developed emaciation and complications of limb edema. He was confined to his bed for his final 3 months and died at 74 years, 4 years after the onset of ataxia.

Autopsy was performed on each patient under the family's written consent. The investigation was approved by the Institutional Ethics Committee of the Tokyo Medical and Dental University.

Brain tissue samples

The left halves of the brains and spinal cords were subjected for neuropathological studies. The right halves of the brain tissues were frozen at –80°C for various investigations. The brains and spinal cords were immersed and fixed in formalin. Then, selected regions, including the entire left half of the cerebellum, were embedded in paraffin. Conventional histological investigations were undertaken by using hematoxylin & eosin (HE), Klüver-Barrera (KB) and Bodian stains.

For FISH analysis, 6 μm-thick sections were created from all the blocks containing the cerebellum. Besides the two SCA31 patients' cerebella, we studied six control brains which included two patients with sporadic amyotrophic lateral sclerosis (age at death: 61 and 73 years), one of each with cerebral infarction (age at death: 83 years), Parkinson's disease (age at death: 88 years), SCA2 (age at death: 67 years) and SCA6 (age at death: 75 years) (Table 1).

Studies on cultured cells

Cloning the pentanucleotide repeats

As an initial step, the SCA31 insertion was amplified by PCR from DNA, as previously described.[2] This insertion originated from a patient who harbored a heterozygous 3.0 kb-long insertion. The control insertion of a

Table 1 Profiles of the investigated patients

Diagnosis	Age at death (years)/gender
SCA31	
Patient 1	80/male
Patient 2	74/male
Control	
Sporadic amyotrophic lateral sclerosis	61/male
Sporadic amyotrophic lateral sclerosis	73/male
Cerebral infarction	83/female
Parkinson's disease	88/male
SCA2	67/male
SCA6	75/female

SCA, spinocerebellar ataxia.

2.5 kb length was similarly amplified. The SCA31 constructs contained a complex penta-nucleotide repeat consisting of $(TGGAA)_n$, $(TAGAA)_n$, $(TAAAA)_n$ and $(TAGAATAAAA)_n$. On the other hand, the control insertion lacked $(TGGAA)_n$ (please refer Reference 2 for the detail).

The PCR amplicons were digested with *Hae*III, purified and cloned into a pTracer-CMV Bsd vector at the *EcoR*V/*EcoR*V site in the multi-cloning site (Invitrogen, Carlsbad, CA, USA). This vector has an advantage in validating the expression of the gene-of-interest (in the present study, SCA31 or control insertion) by detecting the expression of green fluorescent protein (GFP) driven by a different promoter (human elongation factor 1α (hEF-1α)) within the pTracer-CMV Bsd vector. As the present study focused on *BEAN1*-direction, two constructs designed to express the *BEAN1*-transcripts were generated: (i) the SCA31-insertion transcribed in the *BEAN1*-direction, which we abbreviated as "SCA31"; and (ii) the control insertion transcribed in the *BEAN1*-direction, abbreviated as "Control". As these constructs have either TAG- or TAA-translation stop codons just upstream of the repeat sequences, our genes-of-interest were considered to be faithfully transcribed but not translated, unless a recently discovered mechanism translating the non-coding repeat takes place.[12]

Cell culture and transfection

The rat pheochromocytoma-derived PC12 cells purchased from ATCC (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM) (WAKO, Saitama, Japan) containing final concentrations of 10% (v/v) horse serum (HS) (Gibco, Tokyo, Japan), 5% (v/v) fetal bovine serum (FBS) (Gibco) and 1% (v/v) penicillin/streptomycin (PS) (Gibco). The human embryonic kidney (HEK)293T cells purchased from ATCC were grown in DMEM containing 10% (v/v) FBS and 1% (v/v) PS in a humidified atmosphere of 5% CO_2 at 37°C. The lipofection method was used to transfect these vectors by Lipofectamine 2000 following manufacturer's protocol (Invitrogen).

Generation and culture of inducible PC12 stable cell lines

PC12 Tet-off cell lines (Clontech, Foster City, CA, USA) were grown in DMEM containing 10% (v/v) HS, 5% (v/v) Tet-system approved FBS (Clontech), 1% (v/v) PS and 200 μg/mL G418 (SIGMA, Tokyo, Japan) in a humidified condition of 5% CO_2 at 37°C. Tet-off PC12 cells were transfected with either of the SCA31 or Control insertion at 70% confluence. Monoclonal cells were selected by using complete medium containing 200 μg/mL G418 (SIGMA), 200 μg/mL hygromycin (WAKO) and 2 μg/mL doxycycline (Dox) (Clontech). SCA31 and Control cells started to express the transfected genes when Dox was removed from the culture media.

Cellular toxicity and viability analysis

Cellular toxicity was assessed by lactate dehydrogenase (LDH) assay with CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA). Cellular toxicity with LDH assay was calculated using the following formula (experimental LDH release/maximum LDH release).

Cell viability was assessed by (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (MTS) assay with CellTiter96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega).

In transiently expressed cells, LDH and MTS assay were measured 3 days after transfection to HEK293T cells. This long duration was set because the constructs did not show obvious cell death until 3 days after transfection. In stably expressed cells, LDH assay was measured 5 and 7 days after removal of Dox and MTS assay was 6 days.

Flow cytometry-assisted cell viability assay

Transiently expressing vectors (pTracer-CMV Bsd), which contained either SCA31 or Control insertion, were transfected to PC12 cells. At desired time points, cells were harvested with trypsin, collected by centrifugation and re-suspended in phosphate buffered saline (PBS) at a concentration of 10^6/mL. Then, the cells were stained with propidium iodide (PI) 1 μg/mL, as previously described.[13,14] For each sample, 10,000 gated events were acquired with FACS (fluorescence-activated cell sorting) Calibur Instrument (Beckman Coulter, Fullerton, CA, USA). Results were expressed as a percentage of GFP/PI double positive cells relative to total GFP positive cells.

RNA isolation and real time quantitative PCR (qPCR) and sequencing analysis from transiently and stably expressed cells

Total cellular RNA was isolated by TRIzol (Invitrogen) according to the manufacture's protocol. Cellular extracts were then treated with DNaseI (Invitrogen) and total RNA was quantified on a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Total RNA was then reverse transcribed using a PrimeScript™ RT Master Mix (Perfect Real Time) (Takara Bio, Shiga, Japan). For real time qPCR, the primers and probe of insertion from stably expressed cells were designed by Applied Biosystems (Carlsbad, CA, USA) and those were tentatively named as pTRE2-F, pTRE2-R and TGGAA probes, respectively. Real time qPCR was carried out with a LightCycler 480 (Roche, Basel, Switzerland). Quantities measured by this analysis were adjusted by pre-designed, gene-specific primers and probe sets of β-actin (Rn00667869-m1, ACTb) (Applied Biosystems).

To confirm the nucleotide sequence of expressed genes-of-interest, the cDNAs were synthesized from transiently and stably expressed cells, and then subjected to PCR amplification using primers T7 (5′-TAATACGAC TCACTATAGGG-3′) and bovine growth hormone (BGH; 5′-TAGAAGGCACAGTCGAGG-3′). For the stably expressed cells, primers F2 (5′-TTCAGGTTCAGG GGGAGGTG-3′) and R (5′-GACCCCAAGGACTTT CCTTCAGA-3′) were used instead of T7 and BGH primers. For both PCR reactions, each reaction tube contained a final volume of 10 μL, with 0.25 μmol/L (each) of deoxynucleotide triphosphate (dNTP), 1.5 mmol/L of $MgCl_2$, 0.25 U of LA Taq HS polymerase (Takara Bio), and 100 ng of cDNA. Thermal cycles were as follows: initial denaturing at 95°C for 5 min followed by 30 cycles of denaturing at 95°C for 30 s and annealing at 68 or 60°C (transiently or stably expressed cells, respectively), for 30 s and extension at 72°C for 30 s.

The PCR-amplified cDNAs were subjected for nucleotide sequencing using T7, BGH, F2 and R primers with BigDye Terminator v3.1 Cycle Sequencing Kit® (Applied Biosystems) in ABI PRISM 3100® (Applied Biosystems).

Manipulations of cultured cells for FISH analysis

For transiently expressed cells, PC12 cells were first grown on BioCoat® Poly-D-Lysine Cellware 4-well CultureSlide (BD Falcon, Bedford, MA, USA) and then vectors that had the SCA31 or the Control insertion were transfected. On the next day of transfection, cells were grown in DMEM containing final concentrations of 1% (v/v) HS, 1% (v/v) PS and neuronal differentiation was promoted by adding the nerve growth factor (NGF) 2.5S (Invitrogen) with a final concentration of 50 ng/mL. Five days after initiating differentiation, culture slides were washed with PBS and distilled water (DW). After fixation with 4% paraformaldehyde (PFA) in PBS for 5 min, culture slides were dehydrated by hot air, and placed in an air incubator at 45°C overnight. Culture slides were preserved at −80°C until use.

Stably expressed cells were seeded on BioCoat® Poly-D-Lysine Cellware 4-well CultureSlide (BD Falcon). To express genes-of-interest and initiate neuronal differentiation, cells were grown in DMEM containing 1% HS, 1% PS, 200 μg/mL G418, 200 μg/mL hygromycin and 50 ng/mL NGF 2.5S. These plates were separated to either, the plates containing 2 μg/mL of Dox to keep the gene-of-interest expression "off", or the ones without the Dox to turn its expression "on", for 7 days. Culture slides were created similarly as described in the transiently expressed cells.

FISH analysis on cultured cells

To detect repeat insertion transcripts in transiently and stably expressed cells and to find any correlation between the number of RNA foci and cell death, FISH analysis was carried out as previously described[2,15] with several modifications.[16]

Glass slides were washed by PBS and DW, and then they were permeabilized for 20 min with 0.2 N HCl, and for 10 min 0.1% TritonX at room temperature. After fixing with 4% PFA in PBS for 5 min, glass slides were immersed in 2 mg/mL glycine in PBS for 30 min and prehybridized at 68°C in the hybridization mixture solution (2 × SSC, 50% formamide, 0.2 mg/mL yeast tRNA, 0.5 mg/mL heparin). The hybridization with a desired probe was carried out at 68°C with 1 ng/mL digoxigenin (DIG)-labeled, locked nucleic acid (LNA) probes (Exiqon, Vedbaek, Denmark) for 16–18 h for transiently expressed cells. Hybridizations with lower temperatures resulted in high background signals, which led us to find an optimal temperature with a higher stringency at 68°C.

For stably expressed PC12 cells, all procedures were similarly undertaken except that the hybridization and pre-hybridization temperature was set at 37°C instead of 68°C. After being washed at 37°C with 2 × SSC in 50% formamide and subsequently with 0.1% SSC both for three times, the slides were subjected to the detection step at 4°C overnight using anti-DIG Fab conjugated with alkaline phosphatase (Roche) to detect the LNA probe. Anti-DIG antibody was visualized with HNPP/Fast Red TR (Roche) guided by the manufacture's protocol. After nuclei were stained with 4′, 6-diaminino-2-phenylindole (DAPI), glass slides were mounted with an aqueous mounting kit.

To detect the $(UAGAAUAAAA)_n$ repeat, (5′- TATTT TATTCTATTTTATTCTATTT -3′) LNA-oligonucleotide

probe was used which was abbreviated as (TTTTAT TCTA)$_{2.5}$ probe. To detect the (UGGAA)$_n$ repeat, (5′- TTCCATTCCATTCCATTCCATTCCA -3′) LNA-oligonucleotide probe was used which was abbreviated as (TTCCA)$_5$. Two probes for detecting the *TK2*-direction repeat sequence were (TGGAA)$_5$ LNA-probe ((5′-TGGAATGGAATGGAATGGAATGGAA -3′)) and (TAGAATAAAA)$_{2.5}$ LNA-probe ((5′- AAAATA GAATAAAATAGAATAAAATA -3′)).

FISH analysis on human cerebellar tissue sections

To detect the SCA31 repeat insertion transcripts in PCs, FISH was carried out as described above in the section for cultured cells with some modifications.[17] From formalin-fixed, paraffin-embedded tissue blocks, 6 μm-thick sections were created. They were de-paraffinized in xylene, and rehydrated by immersing into the gradually reducing ethanols. After successive treatments with 0.2 N HCl, 0.1% TritonX, and proteinase K (10 μg/mL; Merck, Whitehouse Station, NJ, USA), tissue sections were re-fixed with 4% PFA. After a prehybridization step, hybridization was performed with a solution containing 1 ng/mL (TTCCA)$_5$ LNA probe (Exiqon). After being rinsed, the slides were subjected to a solution containing anti-DIG antibody Fab conjugated with alkaline phosphatase (Roche), and finally developed by HNPP/Fast Red TR (Roche). After a DAPI staining, sections were mounted with an aqueous mounting kit. We conducted two different control experiments to address the specificity of the RNA foci. The first was by using RNase A. We applied RNase A (Invitrogen) at a concentration of 50 μg/mL and incubated at 37°C for 30 min after the sections were treated with 0.2N HCl and 0.1% Triton X. Subsequent procedures were similarly undertaken. The second control experiment was done by omitting the LNA-probe.

FISH analysis was undertaken in all cerebellar cortices of the two SCA31 subjects. In every section of the cerebellum, we randomly picked up various cortical regions and observed PCs to test how many of them contain RNA foci. From the two patients, we counted 1300 PCs in total. The frequency of RNA foci was then calculated.

Statistical analysis

Where applicable, data are presented as mean ± standard error (SE). Statistical analysis was performed using Mann-Whitney *U*-test for comparing two groups. The Bonferroni test after one-way analysis of variance (ANOVA) was used for comparing three different groups. Each experiment was repeated four times independently and statistical analysis was performed.

RESULTS

Histopathological features of the present patients with SCA31

The two SCA31 patients both showed cortical cerebellar degeneration. There were a few areas with normal-looking PCs (Fig. 1A). In some other areas, PCs with the halo-like amorphous materials were frequently seen (Fig. 1B). PCs with amorphous materials which sometimes extended to the dendrite often appeared fuzzy (Fig. 1C). Such fuzzy PCs appeared much degenerated than those with well-demarcated cell bodies (Fig. 1D). In the most severely affected areas, very few PCs remained and even PCs with amorphous materials were not encountered. Even in such areas, neuronal loss of the granule cell was not remarkable. The dentate nucleus showed moderate gliosis (Fig. 1E).

In the brainstem, the number of neurons was slightly fewer in the dorsal part of the inferior olivary nucleus compared to that in the ventral part. However, it did not show an obvious neuronal loss (Fig. 1F and G). The pons and midbrain were well preserved in both patients. In Patient 1, a few senile plaques were seen in the cerebral cortex. There was also a moderate neuronal loss in the hippocampal cortex, suggestive of acute anoxic reaction. In the spinal cord, the anterior horn cell (Fig. 1H) and neurons in the intermedial lateral nucleus and the Clarke's column were well preserved in both patients. The dorsal root ganglia was also well preserved (Fig. 1I). From these observations, we confirmed that SCA31 primarily affects the PC.

Detection of RNA foci containing (UGGAA)$_n$ in SCA31 PC nuclei

As a previous study demonstrated that the RNA foci are detected by the probe against non-pathogenic (UAGAAUAAAA)$_n$ with frequencies of 30–50%,[2] we investigated whether the RNA foci contains the pathogenic (UGGAA)$_n$ in the SCA31 PC nuclei using the (TTCCA)$_5$ LNA probe. We found that the two SCA31 patients consistently contained RNA foci in their PC nuclei (Fig. 2A). These foci in the PC nuclei were not observed when RNase A treatment was added. While a few RNA foci were noticeably large with 1.8–2.2 μm diameters (Fig. 2A), most foci were very small with less than 1 μm in diameter that could be confirmed only with a higher magnification (Fig. 2B). The RNA foci were sometimes seen in the nucleus of PCs with amorphous materials (Fig. 2C). Normal-looking PCs also contained the RNA foci. On the other hand, the RNA foci were only sometimes encountered in the nuclei of atrophic PCs. The frequency of RNA foci containing (UGGAA)$_n$ was 31.4% on average, which

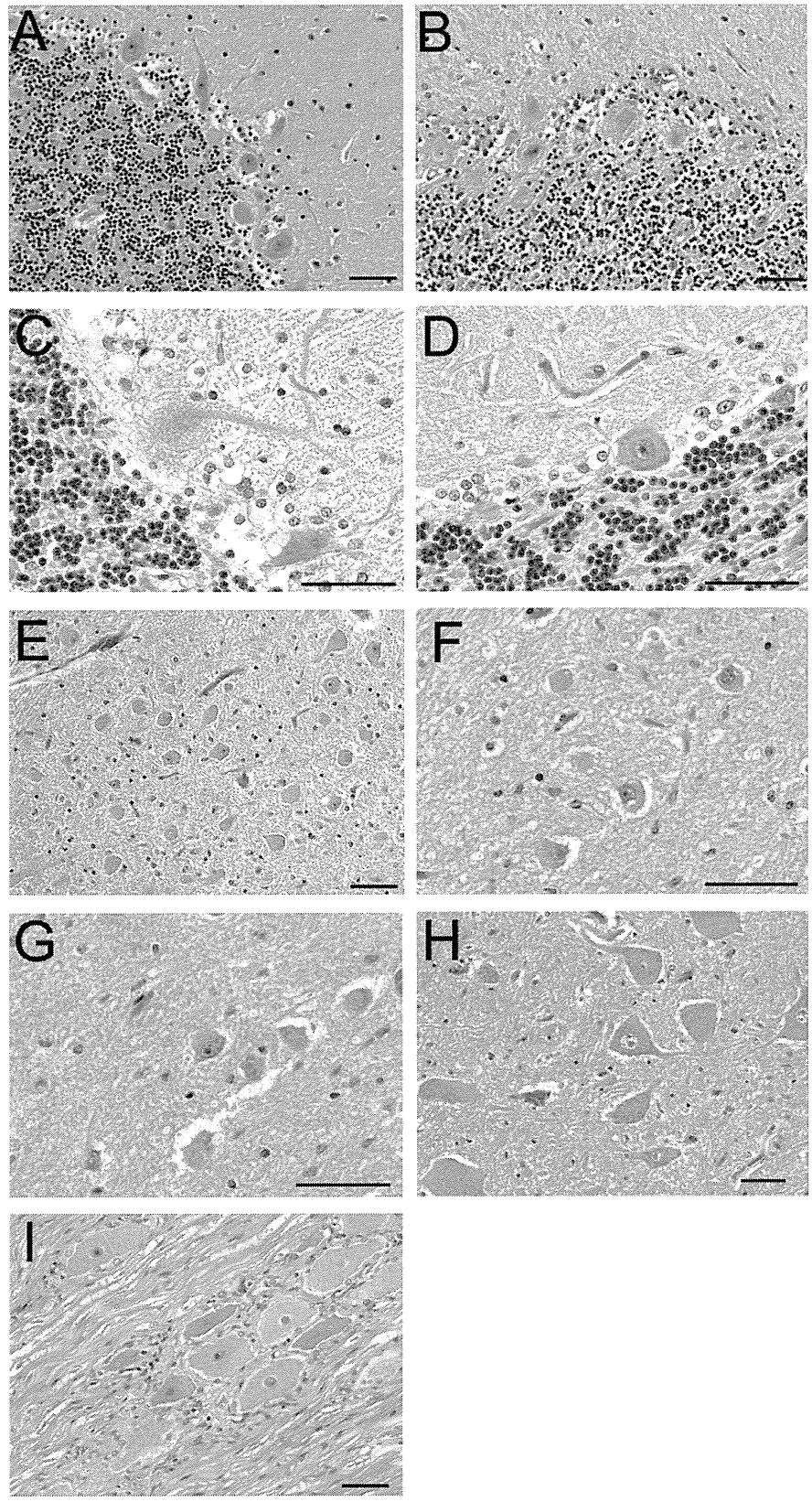

Fig. 1 Histopathological features of two new spinocerebellar ataxia 31 (SCA31) patient brains. (A) An area with relatively well preserved Purkinje cells (PCs) clustered (SCA31, Patient 1). (B) PCs with the halo-like amorphous materials in Patient 1. (C) A fuzzy PC body and a primary dendritic shaft with amorphous materials (Patient 2). (D) A PC with a well-demarcated cell body (Patient 2). Such PCs were very few in number. (E) A mild increase of glial nucleus in the dentate nucleus (Patient 2). (F,G, Patient 1) In the dorso-medial part of the inferior olivary nucleus (F), the neuropil is slightly rarefied with a mild gliosis compared to its vetro-medial part (G). However, the change was very subtle. (H) The anterior horn cells in the lumbar cord are well preserved (Patient 2). (I) The dorsal root ganglia is also well preserved (Patient 2). (Scale bars: 50 μm).

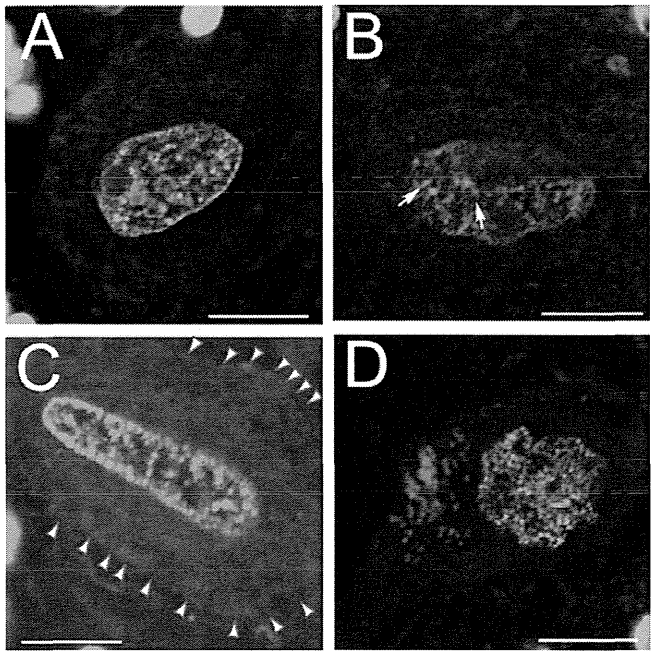

Fig. 2 Fluorescence *in situ* hybridization (FISH) in spinocerebellar ataxia 31 (SCA31) (A–C) and control (D) human cerebella. (A) A relatively large (1.8 μm in diameter) RNA foci granule in an SCA31 Purkinje cell (PC) nucleus. Such noticeably large foci were very few in number. (B) Several smaller RNA foci (arrows), 0.2–0.5 μm in diameter, in another SCA31 PC nucleus. (C) The nuclear RNA foci are sometimes seen in PCs with amorphous materials. White arrowheads indicate outer rim of amorphous materials. Several small RNA foci are seen in the nucleus. (A–C) RNA foci were seen by using $(TTCCA)_5$ locked nucleic acid (LNA)-oligonucleotide probe which detects $(UGGAA)_n$ repeat. (D) Lipofuscin in a PC cell body from a control subject shows auto-fluorescence signal (Control 4). (Scale bars: 10 μm).

is almost comparable with a previous study using a probe against $(UAGAAUAAAA)_n$.[2]

We also noticed structures such as lipofuscin with fluorescence emissions in the cell body (Fig. 2D). However, such structures were also seen in specimens with RNaseA treatment and also in control experiments omitting the LNA probe. From these observations, we concluded that the RNA foci in the nucleus are the pathologic structures. We did not observe obvious RNA foci in neurons other than PCs, nor did we find such structures in glial cells.

The SCA31 insertion transcribed in *BEAN1*-direction induced cell cytotoxicity in transiently expressed HEK293T cells

Plasmid vectors harboring either the SCA31 or the Control insertion were first transfected to cultured HEK293T cells. After confirming that the GFP expression levels were equivalent, RNA was extracted from each transfected plate and reverse-transcribed, yielding cDNA. When amplified using T7 and BGH primers, we confirmed that the two genes-of-interest had equivalent lengths of insertion of approximately 1.5 kb (data shown upon request). The nucleotide sequencing allowed us to confirm that the expressed genes contained $(UGGAA)_n$, $(UAGAA)_n$ and $(UAGAAUAAAA)_n$ from "SCA31" and $(UAGAA)_n$ and $(UAGAAUAAAA)_n$ from "Control".

Simultaneous expression of the GFP, made by a control of hEF-1α promoter, allowed us to check the transfection efficacy. First, the flow cytometry-assisted cell viability assay revealed that the SCA31 insertion showed less viability relative to the Control insertion in PC12 cells (Fig. 3A). Next, we measured cell death by LDH assay to clarify which gene-of-interest (SCA31, Control or an empty vector as a Mock) is the most toxic to cells. We found that the SCA31, corresponding to the SCA31 insertion transcribed in *BEAN1*-direction, showed a significant cytotoxicity. In contrast, the cytotoxicity of the Control was not significant compared to that of Mock (Fig. 3B). The toxicity of the SCA31 insertion was independently confirmed by cell viability, as analyzed by MTS assay (Fig. 3C). Thus, the transiently expressed SCA31 insertion exerts toxicity in cultured PC12 and HEK cells when it is transcribed in *BEAN1*-direction.

The SCA31 insertion transcribed in *BEAN1*-direction also induced toxicity in stably expressed PC12 cells

We next created PC12 cell lines that stably expressed the SCA31 or Control insertions. From each construct (SCA31 and Control), we created 20 cell lines all originating from single cells. Among them, we finally selected four cell lines that were confirmed to express equivalent levels of genes-of-interest by the real time qPCR method using the primer pairs flanking the vector and 5′-insertion site (data shown upon request).

Using these stably expressed SCA31 and Control cells, we investigated cellular toxicity at the designed time-point after the removal of Dox, which turns on the expression of the genes-of-interest. We found that the stable PC12 cells expressing the SCA31 construct $(UGGAA)_n$ $(UAGAAUAAAA)_n$, corresponding to the *BEAN1*-direction transcript, showed a significantly increased cell death measured by LDH assay on Day 5 (Fig. 3D) and Day 7 (Fig. 3E). The cell death was much more prominent on Day 7 than on Day 5, consistent with the toxicity of the SCA31 construct. In Control cells, the cell death was significantly reduced by expressing $(UAGAAUAAAA)_n$ (Fig. 3D,E, right panels). The exact mechanism of this phenomenon is not known. On the MTS assay, there was a tendency for a reduced viability in the SCA31 construct, although it was not statistically significant (Fig. 3F). We did not see significant cell death when Dox was kept in the

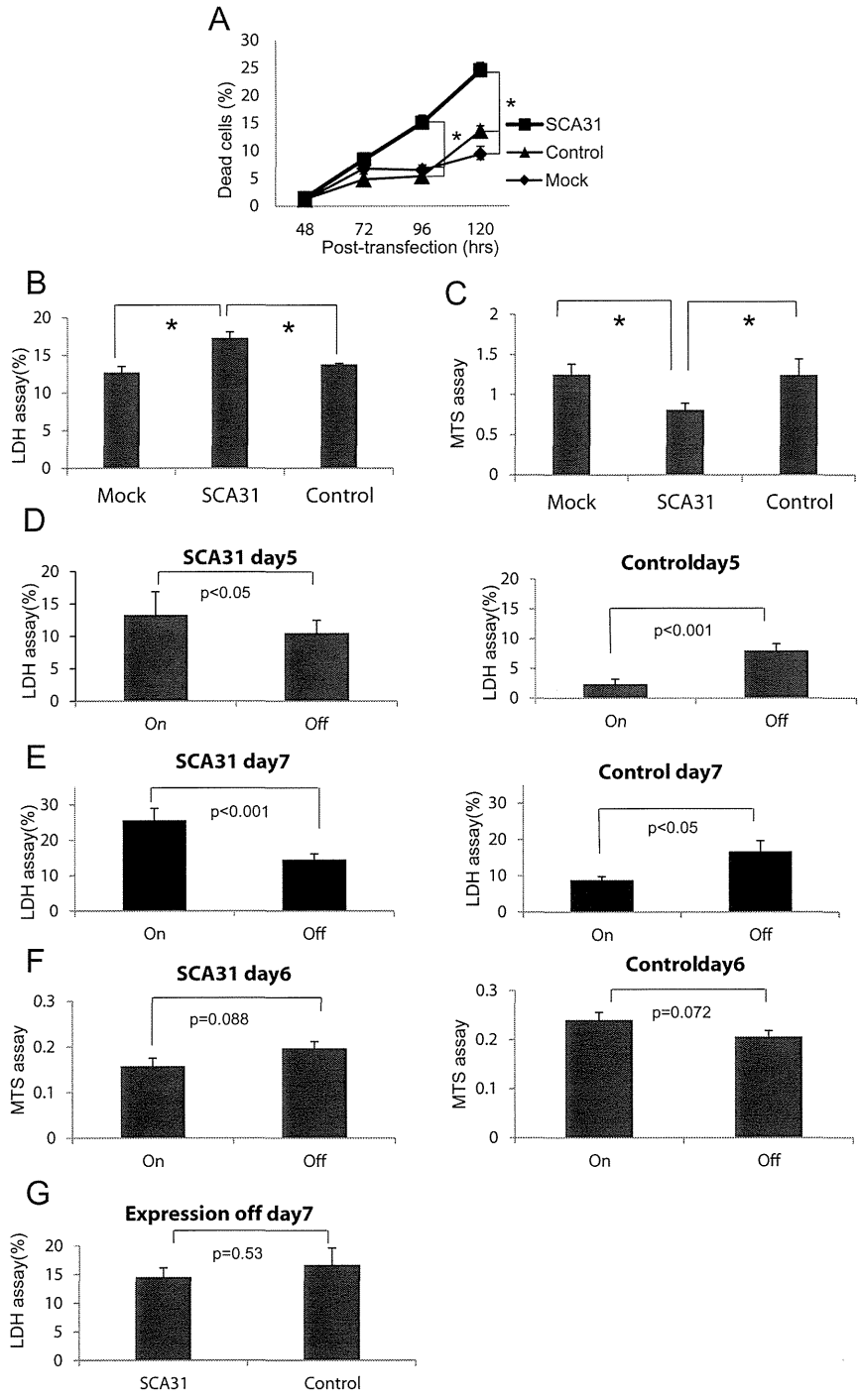

Fig. 3 Toxicities of $(UGGAA)_n$ in cultured cell models. (A) In transiently expressed rat pheochromocytoma-derived PC12 cells, flow cytometry-assisted cell viability assay demonstrating cell death only in the spinocerebellar ataxia 31 (SCA31) compared with Control ($^*P < 0.001$) —■—, SCA31; —▲—, Control; —◆—, Mock. (B) The lactate dehydrogenase (LDH) assay, another indicator of cell death, shows a significant toxicity in SCA31 but not in Mock nor Control ($^*P < 0.01$) when expressed in human embryonic kidney (HEK) cells. (C) The (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (MTS) assay, which indicates cell viability, demonstrates significant reduced cell viability in SCA31 ($^*P < 0.05$). (D) In stably expressed PC12 cells on day 5, the LDH assay demonstrates toxicity in the SCA31 cells expressing the mutant sequence $(UGGAA)_n(UAGAAUAAAA)_n$ compared to cells not expressing the sequence. "On" designates cells expressing the genes-of-interest, while "Off" designates cells without expressing those genes. Control cells expressing the $(UAGAAUAAAA)_n$ sequence even show resistance to cell death by unknown mechanisms. (E) The toxicity of SCA31 construct is more obvious on Day 7 (7 days after the doxycycline (Dox) removal) than on Day 5 (Fig. 3D). (F) The MTS assay, analyzed on Day 6, shows less cell viability in the cells expressing the SCA31 $(UGGAA)_n$ $(UAGAAUAAAA)_n$ construct than the cells without such expression (left panel). However, this was not statistically significant. Likewise, the cell viability was not significant in the Control construct $(UAGAAUAAAA)_n$ (right panel). (G) The LDH assay confirmed that the SCA31 and Control constructs did not exert differences in toxicity when their expressions were shut off.

media (Fig. 3G), supporting that the toxicity was induced by the expression of the SCA31 insertion, and not by any local chromosomal effects of the inserted gene. Overall, the toxicity of the SCA31 construct was confirmed in both transient and stable cell lines, whereas the toxicity of Control constructs was not observed in the two types of cell lines. From these observations, we considered that the $(UGGAA)_n$ repeat, expressed only from the SCA31 construct transcribed in *BEAN1*-direction, is mildly toxic to cells.

FISH demonstrated RNA foci formation when $(UGGAA)_n$ is expressed

We next performed FISH to check whether these cellular models show RNA foci. We designed four LNA probes that

would detect expression of complex repeats *in situ* in either of the two (*BEAN1* and *TK2*) directions (Fig. 4A).

In transiently expressed PC12 cells, we observed RNA foci in the nuclei of cells that expressed the SCA31 insertion transcribed in *BEAN1*-direction. The RNA foci were consistently detected by the probes $(TTCCA)_5$ (Fig. 4B) and $(TTTTATTCTA)_{2.5}$ (Fig. 4C). However, RNA foci were not obvious in the cells expressing the Control insertion (Fig. 4D,E). The PC12 cells stably expressing the SCA31 insertion transcribed in *BEAN1*-direction also formed the RNA foci (Fig. 4F). In contrast, the PC12 cells stably expressing the Control insertion transcribed in *BEAN1*-direction did not form the RNA foci (Fig. 4G). RNA foci were not observed by $(TGGAA)_5$ probe or by the $(TAGAATAAA)_{2.5}$ probe. These experiments allowed us to conclude that the presence of $(UGGAA)_n$ is needed to form the RNA foci. In addition, the pathogenic $(UGGAA)_n$ sequence makes the non-pathogenic $(UAGAA)_n(UAGAAUAAAA)_n$ repeat form abnormal structures as well in cells.

DISCUSSION

We histopathologically investigated two new SCA31 patients. These patients were still mildly ataxic and had been able to walk with a cane. The SARA scores was 10 in both patients. Neuropathologically, PCs were shown to be predominantly affected more than any other neurons. Taking the two previously published SCA31 patients[3,18] together, we confirm that the PC is the main target of this disease.

We here show that the RNA foci in the human SCA31 PC nucleus contain $(UGGAA)_n$ sequence. We did not observe remarkable RNA foci in other cells in the cerebellum, suggesting that RNA foci are restricted in PCs. Such specific distribution of RNA foci may be another characteristic feature of SCA31: other conditions such as FXTAS,[19] SCA8,[20] DM1[21] or HDL-2[22] show widespread distributions of RNA foci distributions. However, we should be cautious about the interpretation of this specificity. It is very difficult to exclude a possibility that RNA foci, particularly when they are small, exist in other neurons such as cerebellar granule cells. To address the specificity of $(UGGAA)_n$-containing RNA foci in SCA31, we need to investigate in a much more sensitive way than the present technique, such as an immunohistochemistry against an RNA-binding protein co-localizing with the foci.

The present FISH analysis demonstrates that "nuclear" RNA foci are specific to SCA31 PCs. However, we do not exclude a possibility that there are some "cytoplasmic" RNA foci that have been abolished by RNaseA in the SCA31 PCs. In fact, we observed some cytoplasmic RNA foci in cultured cell models. Further attempts are necessary to develop an optimal technique to address whether cytoplasmic RNA foci exist in SCA31 PCs.

As we previously described,[2] the mutation causing SCA31 is a complex repeat structure containing $(TGGAA)_n$, $(TAGAA)_n$, $(TAAAA)_n$ and (TAGAAT AAAA)$_n$. Among these various repeat components, normal control Japanese are rarely found to harbor $(TAGAA)_n$, $(TAAAA)_n$ and $(TAGAATAAAA)_n$. As far as we have investigated, none of these controls had family histories of ataxia, supporting a notion that $(TAGAA)_n$, $(TAAAA)_n$ and $(TAGAATAAAA)_n$ are not pathogenic, at least not in the ordinary lifespan of the human being. On the other hand, $(TGGAA)_n$ is specific to SCA31 patients. In Europeans, distinct penta-nucleotides such as $(TACAA)_n$, $(TCAAA)_n$ or $(GAAAA)_n$ have been found with a high frequency of 5.5% in the general French and German populations tested.[23] From these observations, we can consider that SCA31 is a disease caused by a specific sequence, $(TGGAA)_n$. Our finding of the inclusion of $(UGGAA)_n$ sequence in nuclear RNA foci suggests that the $(TGGAA)_n$ transcribed in the *BEAN1*-direction can be an essential feature of SCA31 pathogenesis. However, we do not exclude a possibility that the *TK2*-direction transcript containing $(UUCCA)_n$ is also fundamental for the disease process. We would need to investigate the presence of RNA foci with different probes and conditions to address whether $(UUCCA)_n$ also forms RNA foci. Notably, it may be necessary to explain how *TK2* causes a PC-dominant degeneration because *TK2* is expressed almost ubiquitously in human cells. Furthermore, it is possible that both *BEAN1*- and *TK2*-transcriptions are needed for pathogenesis.

In this context, the present data on cultured cell models are important, proclaiming that the SCA31 insertion exerts toxicity when it is transcribed in the *BEAN1*-direction. Using Dox-inducible stably expressed cell lines, we also revealed that the *BEAN1*-direction SCA31 transcripts show toxicity, while shutting off its expression by adding Dox in culture media did not. Considering that the $(TGGAA)_n$ stretch is the only difference between the control and SCA31 insertions, it is conceivable that the $(UGGAA)_n$, the RNA sequence transcribed in the *BEAN1*-direction, is the critical component of the toxicity. However, it should be remembered that the toxicity was not strong. For example, we needed to wait until 3 days after the transfection in the present transient expression system. In stable cell lines, the cell death caused by the SCA31 construct was not significant when assessed by an MTS assay (Fig. 3F). To validate the RNA toxicity of SCA31 mutation, it may be necessary to create a cultured cell system expressing a construct with much longer $(UGGAA)_n$.

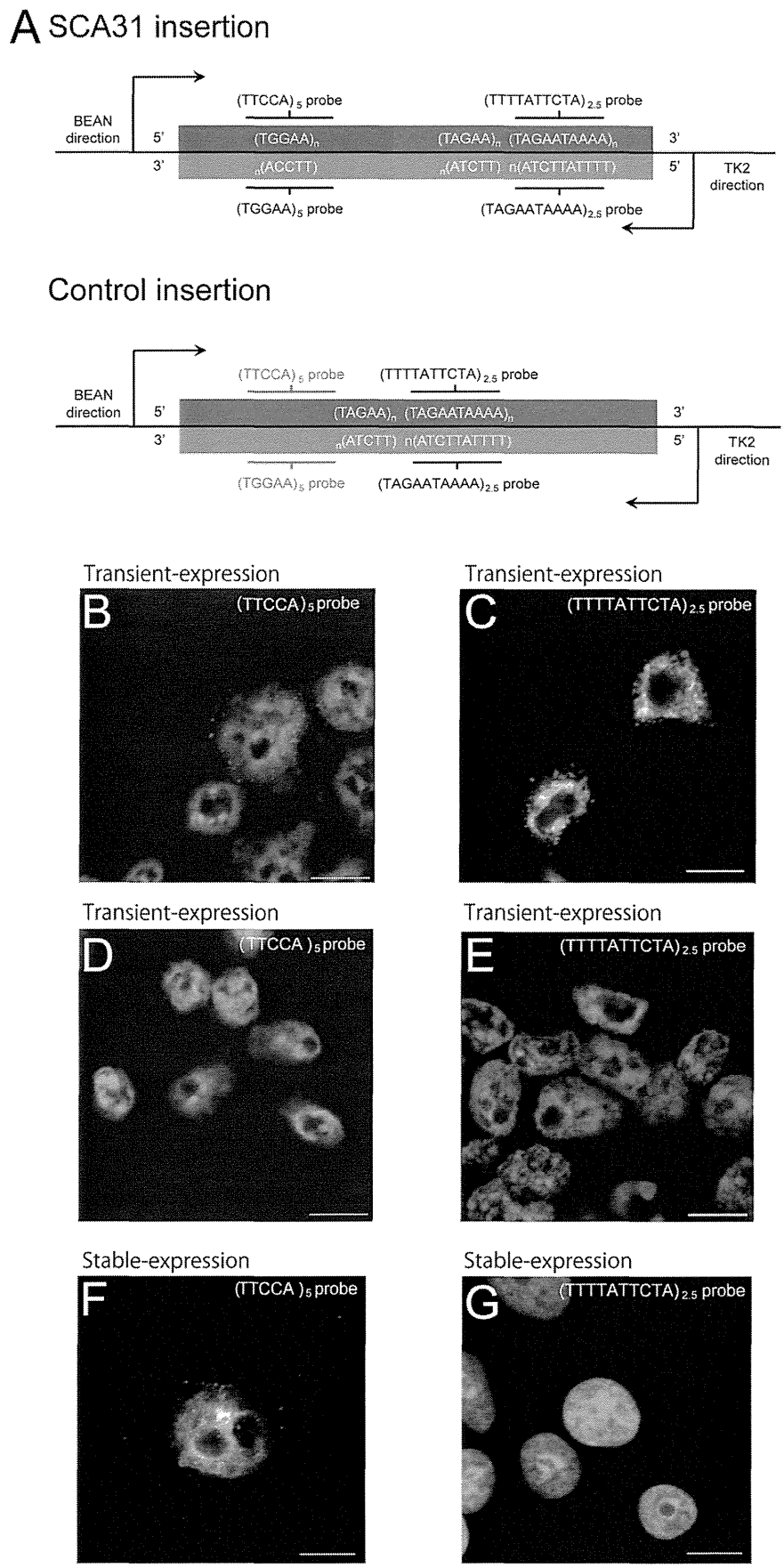

Fig. 4 Detecting the RNA foci in cultured cells using fluorescence *in situ* hybridization (FISH) analysis. (A) A scheme of spinocerebellar ataxia 31 (SCA31) and Control insertion sequences and designed locked nucleic acid (LNA) probes. Note that the $(TGGAA)_n$ repeat is absent in the Control insertion. Therefore, $(TTCCA)_5$ and $(TGGAA)_5$ LNA-probes would not detect any foci in cells expressing the Control insertion. (B–E) FISH analysis on rat pheochromocytoma-derived PC12 cells transiently expressing the SCA31 insertion transcribed in the *BEAN1*-direction ("SCA31") (B, C) and the Control insertion transcribed in the *BEAN1*-direction ("Control") (D, E). Note that only the "SCA31" cells show RNA foci by probes which could detect $(UGGAA)_n$ (B) and $(UAGAAUAAAA)_n$ (C). (F,G) In stably expressing PC12 cells, the RNA foci are also seen in the cells expressing the SCA31 insertion (F), but not in the cells expressing Control insertion (G). (Scale bars in B–G: 10 μm).

Although the present observation suggests that the RNA-mediated gain-of-function is a plausible mechanism underlying SCA31 pathogenesis, it does not exclude other mechanisms such as dysfunction of the BEAN1 or alteration of chromosomal structure due to the insertion. Also, we do not preclude a possibility that the $(UGGAA)_n$ exerts toxicity if translated into an amino-acid repeat by a new mechanism named repeat-associated non-ATG translation (RAN).[12]

In conclusion, we have demonstrated that the $(UGGAA)_n$ at least at the RNA level can be toxic in cells. The RNA foci in the nucleus formed by the presence of $(UGGAA)_n$ appears a pathologic marker of human SCA31 PCs.

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Cytoplasmic Location of α1A Voltage-Gated Calcium Channel C-Terminal Fragment ($Ca_V2.1$-CTF) Aggregate Is Sufficient to Cause Cell Death

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Abstract

The human α_{1A} voltage-dependent calcium channel ($Ca_V2.1$) is a pore-forming essential subunit embedded in the plasma membrane. Its cytoplasmic carboxyl(C)-tail contains a small poly-glutamine (Q) tract, whose length is normally 4~19 Q, but when expanded up to 20~33Q, the tract causes an autosomal-dominant neurodegenerative disorder, spinocerebellar ataxia type 6 (SCA6). A recent study has shown that a 75-kDa C-terminal fragment (CTF) containing the polyQ tract remains soluble in normal brains, but becomes insoluble mainly in the cytoplasm with additional localization to the nuclei of human SCA6 Purkinje cells. However, the mechanism by which the CTF aggregation leads to neurodegeneration is completely elusive, particularly whether the CTF exerts more toxicity in the nucleus or in the cytoplasm. We tagged recombinant (r)CTF with either nuclear-localization or nuclear-export signal, created doxycyclin-inducible rat pheochromocytoma (PC12) cell lines, and found that the CTF is more toxic in the cytoplasm than in the nucleus, the observations being more obvious with Q28 (disease range) than with Q13 (normal-length). Surprisingly, the CTF aggregates co-localized both with cAMP response element-binding protein (CREB) and phosphorylated-CREB (p-CREB) in the cytoplasm, and Western blot analysis showed that the quantity of CREB and p-CREB were both decreased in the nucleus when the rCTF formed aggregates in the cytoplasm. In human brains, polyQ aggregates also co-localized with CREB in the cytoplasm of SCA6 Purkinje cells, but not in other conditions. Collectively, the cytoplasmic $Ca_V2.1$-CTF aggregates are sufficient to cause cell death, and one of the pathogenic mechanisms may be abnormal CREB trafficking in the cytoplasm and reduced CREB and p-CREB levels in the nuclei.

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Introduction

Polyglutamine (polyQ) disease is a group of nine neurodegenerative disorders that are associated with protein aggregation caused by an expansion of the polyQ tract. These disorders include Huntington's disease (HD), spinobulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA) and spinocerebellar ataxia (SCA) types 1, 2, 3, 6, 7, and 17 (SCA3 is also known as Machado–Joseph disease (MJD)) [1,2]. In general, the length of the polyQ tract encoded by trinucleotide (CAG) repeat is below 35 in normal individuals. In these diseases, however, the CAG repeat is expanded above 35 to even more than 100, which gives rise to a mutated protein with an expanded polyQ tract that tends to adopt a β-sheet structure, become misfolded, and form oligomers of mutated protein eventually forming microscopic aggregates.

The polyQ expansion causing SCA6 exists in the cytoplasmic carboxyl(C)-tail of the α_{1A} (P/Q-type) voltage-dependent calcium channel protein ($Ca_V2.1$) [3]. The cardinal clinical feature of SCA6

is progressive cerebellar ataxia with an average age-of-onset at 45.5 years and gaze-evoked nystagmus [4,5]. The Purkinje cell of the cerebellar cortex, which expresses $Ca_v2.1$ most abundantly in the brain, undergoes degeneration [5,6]. Previous studies have shown that the polyQ expansion in $Ca_v2.1$ causes functional alterations of $Ca_v2.1$ [7–10]. However, such functional alterations are not considered critical for SCA6 pathogenesis, as $Ca_v2.1$ functions were not obviously altered in two independent studies on knock-in mice [11,12]. Probably more important for the pathogenesis of SCA6 is the formation of microscopic aggregation of $Ca_v2.1$, which has been demonstrated in SCA6 human Purkinje cells by using several antibodies against the $Ca_v2.1$ C-terminus [6,13]. SCA6 has several unique features that make it appear as a different disorder among the rest of other polyQ diseases. First, the length of the polyQ tract in the $Ca_v2.1$ that is responsible for SCA6 falls within the normal range of repeats for other polyQ diseases (4–19 CAG/polyQs in the $Ca_v2.1$ of normal individuals compared with 20–33 CAG/polyQs in SCA6 subjects) [14,15]. Secondly, microscopic $Ca_v2.1$ aggregates can be seen in the cytoplasm (i.e., the cell body or cell processes) of SCA6 Purkinje cells, whereas in other polyQ diseases, aggregates with expanded polyQ are prevalent in the nuclei rather than in the cytoplasm of neurons expressing the responsible proteins [16,17]. These could indicate that SCA6 has a distinct underlying pathophysiology among polyQ diseases. Recently, a study by Western blot analysis showed that a 75-kDa $Ca_v2.1$ C-terminal fragment (CTF), thought to be generated by a proteolytic cleavage of the full-length $Ca_v2.1$, might have a critical role in SCA6 pathogenesis from the following reasons [18]. First, the CTF with a normal-length polyQ tract remains soluble and is localized exclusively in the cytosolic fraction of the normal human cerebellum. Second, the CTF becomes insoluble in the cytosolic fraction of SCA6 cerebellum. Third, a small amount of CTF is additionally detected in the nuclear fraction in the human SCA6 cerebellum, suggesting that the expansion of polyQ causes the CTF to translocate into the nucleus as well as to aggregate in the cytoplasm. These findings raise a fundamental question: where (i.e., the nucleus or the cytoplasm) does the CTF exert serious toxicity? In this context, two previous studies reported that a recombinant (r)CTF, when expressed in cultured cells, tends to localize into the nuclei and exert toxicity in the nucleus rather than in the cytoplasm [19,20]. However, two other studies demonstrated completely opposite data that the rCTF predominantly locates in the cytoplasm where it exerts toxicity [18,21]. We therefore investigated the relationship between the location of CTF and cell death by using newly created cultured cell models. In addition, we also pursued alterations in protein expression and intracellular localization of the cAMP response element-binding protein (CREB) suggested by the pathogenesis of other polyQ diseases. We finally asked whether the findings in cultured cells are relevant to the human SCA6 pathology. Here we show that the CTF with expanded polyQ is sufficient to cause toxicity in the cytoplasm.

Results

Nuclear localization signal (NLS) and nuclear export signal (NES) faithfully targeted the CTF to the desired intracellular locations

We first asked the primary location of the rCTF. We avoided using the enhanced green-fluorescent protein (EGFP), since a large proportion of the rCTF-polyQ (either Q13 or Q28) dramatically shifted into the nucleus with the presence of EGFP (Figure S1A, S1B, S1C). Instead, we utilized an artificial nuclear localization signal (NLS) and export signal (NES), to regulate the location of the rCTFs (Figure 1A). Two types of polyQ (Q13: normal, Q28: expanded) were used (Figure 1A). We fused the rCTFs with either NLS or NES, transfected each of these in PC12 cells, and then examined the intracellular distributions of these constructs. The rCTF-Q13, the normal version of CTF with an approximate size of 75–80 kDa [18], was predominantly, though not exclusively, expressed in the cytoplasm. In the presence of the NLS tag, the rCTF-Q13-NLS dramatically translocated to the nucleus, whereas the NES tag made the rCTF-Q13-NES anchor in the cytoplasm (Figure 1B). This result was confirmed in human embryonic kidney (HEK) 293T cells (Figure S2), and was consistent with the location of native CTF in human brain [18]. The expanded version of the CTF, rCTF-Q28 was also expressed mainly in the cytoplasm with some obvious amounts in the nucleus (Figure 1C). In contrast, the rCTF-Q28 tagged with NLS showed much stronger tendency to locate in the nuclei, while the rCTF-Q28 tagged with NES appeared to remain entirely in the cytoplasm, showing that both NLS and NES are efficient signals to make the rCTF confine in a desired location. When the intracellular locations of various rCTFs were rated into 4 groups (N: exclusively seen in the nucleus; N-c: predominantly located in the nucleus; n-C: predominantly located in the cytoplasm; C: exclusively seen in the cytoplasm)(*See, Materials and Methods for details*), the effects of NLS and NES were confirmed (Figure 1D). This allowed us to examine the roles of CTF in the nucleus and in the cytoplasm separately by using the NLS and NES.

rCTF-NES/-NLS stably expressed in PC12 cell lines with doxycyclin removal

To clarify the chronological sequence of the CTF expression, the formation of CTF aggregates and the point of cell death, we created PC12 cell lines that stably expressed the rCTF by removing the doxycyclin (Dox) (Tet-off PC12 system). In basal conditions when the Dox is kept added in culture medium, Tet-off PC12 cells do *not* express rCTF (termed "Dox(+)" condition). However, when the culture medium was replaced with the one lacking the Dox, cells began to express rCTFs (termed "Dox(−)" condition). Taking an advantage of the fact that the PC12 cells differentiate into cells with neuronal characteristics on exposure to the nerve-growth factor (NGF), we added the NGF and removed the Dox at the same day, which we designate "Day0". We chose six stable PC12 cell clones (rCTF-Q13, rCTF-Q13-NLS, rCTF-Q13-NES, rCTF-Q28, rCTF-Q28-NLS, rCTF-Q28-NES), which had been confirmed to express equivalent rCTF levels by quantitative real-time reverse-transcription PCR (qRT-PCR) (*data shown upon request*). At the mRNA level, the quantitative RT-PCR showed that the rCTF mRNA level starts to be detected from Day3 (Figure 2A). At the protein level, Western analysis using the A6RPT-#5803 antibody against the C-terminus of $Ca_v2.1$ [18] showed that the rCTF expression starts to be detected on the fourth day after Dox removal ("Day4") and reaches abundant levels by Day6 (Figure 2B). The maximum protein expression level at Day6 was approximately 10% of that expressed in transiently over-expressed PC12 cells (*data shown upon request*).

The fluorescent immunocytochemistry revealed that in the stable PC12 cells expressing rCTF-Q28-NES, the recombinant protein was expressed mainly in the cytoplasm (Figure 2C, upper panels), whereas in stable PC12 cells expressing rCTF-Q28-NLS, the protein was confined to the nucleus (Figure 2C, lower panels). The restricted intracellular distribution by NES and NLS was also confirmed by Western blotting (Figure 2D).

We next searched the timeline of the formation of rCTF aggregates. In PC12 cells expressing rCTF-Q28-NES, the cytoplasmic rCTF aggregates visualized by using A6RPT-#5803