A novel splicing variant of ANXA11 in a patient with amyotrophic lateral sclerosis: histologic and biochemical features

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To the editor
Mutations in the annexin A11 gene (ANXA11) have been shown to cause amyotrophic lateral sclerosis (ALS) [6]. Annexin A11 is a Ca2+-dependent phospholipid-binding protein that possesses an N-terminal low-complexity domain and C-terminal repeated annexin domains, being involved in Ca2+ signaling, cell division, apoptosis, and vesicle trafficking [3, 5, 8]. Recent studies have indicated that ALS-related ANXA11 mutations enhance aggregation propensity, leading to dysregulation of intracellular Ca2+ homeostasis and RNA granule transport [3, 5, 6].

The clinical phenotypes of ANXA11-mutated ALS vary, and differ even in patients harboring the same mutation [6, 7, 9]. Autopsy findings of two patients harboring N-terminal ANXA11 mutation have been reported. One patient with p.D40G ANXA11 mutation showed the features of classical ALS [6], while the other with p.G38R ANXA11 mutation showed those of ALS-TDP with frontotemporal lobar degeneration (FTLD)-TDP type A [4, 7]. Both patients showed aggregation of 43 kDa TAR-DNA-binding protein (TDP-43) and annexin A11 in neurons (Table 2).

However, the variety of neuropathologic features, including the distribution and morphology of TDP-43- or annexin A11-immunoreactive (ir) inclusions, in patients with ANXA11 mutations remain to be further clarified. Here, we investigated the clinicopathologic features of a Japanese patient with sporadic ALS harboring a novel splicing mutation in the annexin domain of ANXA11, and the functional significance of the mutation.

A 57-year-old man, who had no family history of neurologic disorders, presented with progressive limb weakness and stiffness, followed by muscle fasciculations, pyramidal signs, dysphagia and dysarthria. No dementia was noted. He died suddenly 19 months after disease onset. His detailed clinical features are described in the Additional File.

The neuropathologic features were consistent with ALS. Neuronal loss and gliosis were restricted to the motor cortex, brainstem motor nuclei, and spinal anterior horns (Fig. 1a–c, e–h; Table 1). The corticospinal tract in the spinal cord showed severe degeneration (Fig. 1d). Bunina bodies were occasionally found in the lower motor neurons (Fig. 1i, j). Phosphorylated TDP-43 (pTDP-43)-ir neuronal and oligodendroglial cytoplasmic inclusions (NCIs and GCIs, respectively) were evident in the above-mentioned areas (Fig. 1k, o, p, motor cortex; q, white matter adjacent to the motor cortex; l, r, hypoglossal nucleus; s, anterior horn of the lumbar cord) and several others (Fig. 1m, subthalamic nucleus; n, pontine nucleus) but

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not in the hippocampus or temporal cortex (Table 1). Morphologically, the pTDP-43-ir NCIs were granular and/or filamentous (Fig. 1o, p) in all areas where NCIs were observed, and characteristically those in the lower motor neurons were thick skein-like, or tube-shaped (Fig. 1r, s).

Genetic screening of ALS-related mutations through whole-exome sequencing revealed a heterogeneous splice site mutation, c. 1086+1G>A, in the ANXA11 gene (Fig. 2a), which has an allele frequency of 0.08% in the Human Genetic Variation Database (HGVD), and has not been documented in the Exome Aggregation Consortium (ExAC). On the other hand, no variants which are less than 0.1% frequency were found in any of the other ALS-related genes (See Supplementary methods). A splice site prediction program (NetGene2, http://www.cbs.dtu.dk/services/NetGene2/) correctly recognized the splice donor site of intron 11 in the wild type, but not in the mutant sequence, indicating that the mutation disrupts the splice donor site. Sequencing of the reverse transcription PCR products of ANXA11 mRNA obtained from the autopsied brain showed an aberrant transcript containing a 72-bp insertion between exons 11 and 12 that resulted in the insertion of 24 amino acids (Fig. 2b). Using the structure predictive tool, PSIPRED 4.0 (http://bioinf.cs.ucl.ac.uk/psipred/), the inserted amino acid sequence was predicted to create new alpha helices. However, no new motifs were identified in the aberrant protein using a motif finder (Pfam 34.0, http://pfam.xfam.org/ and HMMER v3.3.2, http://hmmer.org/). Hydrophobicity prediction [2] using ExPASy (https://web.expasy.org/protscale/) and protein disorder analysis using PrDOS (http://prdos.hgc.jp/cgi-bin/top.cgi) [1] revealed that the aberrant annexin A11 had increased hydrophobicity (Fig. 2c, upper panel) and disorder probability (Fig. 2c, lower panel), respectively, around the inserted 24-amino-acid site compared with wild type. We then undertook cellular experiments using HEK293T cells transfected with the 72-bp inserted mutant, and wild type GFP-tagged ANXA11 (GFP-ANXA11MT and GFP-ANXA11WT, respectively) constructs. Solubility fractionation of the cells transfected with GFP-ANXA11 constructs, followed by Western blotting using antibodies for annexin A11 (Fig. 2d, left) and GFP (Fig. 2d, right) revealed that GFP-annexin A11MT formed detergent-resistant insoluble species. Furthermore, cells expressing GFP-annexin A11WT showed mainly a diffuse nuclear and cytoplasmic distribution of GFP, whereas cells expressing GFP-annexin A11MT showed cytoplasmic aggregation significantly frequently (Fig. 2e, f).

Indeed, the present patient had many annexin A11-ir skein-like NCIs in the lower motor neurons (Fig. 3a, b). In contrast, sparse dot-like cytoplasmic staining (Fig. 3c) and rare filamentous inclusions were observed in the lower motor neurons of ALS patients with no ANXA11 mutation. Such sparse dot-like cytoplasmic staining was also observed in controls, and not only in motor neurons (Additional file 1: Fig. 1). These findings indicated that annexin A11-ir NCIs are not completely specific, but characteristic to ANXA11-mutated patients. In the motor cortex, only a few, irregularly shaped or round, small NCIs were observed (Fig. 3d, e). Filamentous NCIs were also evident in several regions where pTDP-43-ir NCIs were also present (Fig. 3f, h, i, Table 1). Annexin A11 aggregated preferentially in neurons, although a few GCIs were observed (Fig. 3g). Double-labeling immunofluorescence for pTDP-43 and annexin A11 revealed that annexin A11 and pTDP-43 were partially co-localized in skein-like NCIs, and a few GCIs in the anterior horns (Fig. 3j, k), but not in small annexin A11-ir NCIs in the motor cortex (Fig. 3l). Annexin A11-ir skein-like NCIs in the anterior horns were frequently well labeled for p62 (Fig. 3m). Details of methods are in Supplementary methods, and the primary antibodies used are listed in Supplementary table 1 in Additional file 1.
### Table 1 Distribution of neuronal loss and pTDP-43- and annexin A11-immunoreactive NCIs and GCIs

|                      | Neuronal loss  | pTDP-43  | Annexin A11 |
|----------------------|----------------|----------|-------------|
|                      | NCI            | GCI      | NCI         | GCI    |
| **Cerebrum**         |                |          |             |        |
| Frontal cortex       | —              | —        | —           | —      |
| Motor cortex         | +              | +        | +           | +      |
| White matter*        | +**            | NA       | +           | NA     |
| Temporal cortex      | —              | —        | —           | —      |
| Parietal cortex      | —              | +        | —           | —      |
| Occipital cortex     | —              | —        | —           | —      |
| **Subcortical area** |                |          |             |        |
| Ammon/dentate gyrus  | —              | —        | —           | —      |
| Amygdaloid nucleus   | —              | —        | —           | —      |
| Caudate nucleus/putamen | —/—         | +/+      | —/—         | —/—    |
| Globus pallidus      | —              | —        | —           | —      |
| Thalamus             | —              | —        | —           | —      |
| Subthalamic nucleus  | —              | +        | +           | —      |
| **Brainstem**        |                |          |             |        |
| Oculomotor nucleus   | —              | —        | +           | —      |
| Substantia nigra     | —              | —        | —           | —      |
| Locus ceruleus       | —              | +        | —           | —      |
| Trigeminal motor nuclei | +          | +        | —           | +      |
| Facial nuclei        | ++             | ++       | +           | ++     |
| Pontine nucleus      | —              | +        | +           | +      |
| Ambiguous nucleus    | +              | +        | —           | —      |
| Hypoglossal nucleus  | ++             | ++       | +           | ++     |
| Inferior olivary nucleus | +          | +        | +           | +      |
| Reticular formation  | —              | +        | +           | +      |
| **Cerebellum**       |                |          |             |        |
| Cortex/white matter  | —/—**          | —/NA     | —/—         | —/NA   |
| Dentate nucleus      | —              | —        | —           | —      |
| **Spinal cord**      |                |          |             |        |
| Anterior horn (C/Th/L) | +++++/++   | +/+      | ++++/++/+   | +/+     |
| Lateral funiculus (C/Th/L) | ++/++/++/** | NA       | —/++/+     | NA     |
| Anterior funiculus (C/Th/L) | ++/++/++/** | NA       | —/++/+     | NA     |

* White matter adjacent to the motor cortex; Neuronal loss (*, degeneration with macrophage infiltration); —, none; +, mild to moderate; ++, severe; NCIs and GCIs: —, none; +, rare (1–4/10 high-power fields); ++, occasional (≥ 5/10 high-power fields); pTDP-43, phosphorylated 43 kDa TAR DNA-binding protein; NCIs, neuronal cytoplasmic inclusions; GCIs, glial cytoplasmic inclusions; NA, not applicable; C, cervical cord; Th, thoracic cord; L, lumbar cord
The neuropathologic features of this patient were indistinguishable from those of ALS without known mutations, except for the presence of annexin A11-ir NCIs, being similar to the previously reported patient with ANXA11 mutation (Table 2) [6].

On the other hand, genetic screening revealed the novel splicing mutation in the C-terminal of ANXA11, and the in silico analysis and cellular experimental findings indicated that the aberrantly spliced transcript induced cytoplasmic accumulation and enhanced the aggregation propensity of annexin A11, suggesting that the mutation had pathogenicity. Functional studies of ALS-related ANXA11 mutations have shown that both N-terminal and C-terminal mutations induced abnormal phase separation to form aggregates, leading to the functional defects of annexin A11 [3, 5]. Similar toxic-gain-of-function mechanisms might have contributed to ALS pathogenesis in this patient.

Morphologically, the annexin A11-ir NCIs in this patient differed slightly from those in the previous patients with N-terminal ANXA11 mutation [6, 7]. We observed skein-like, filamentous, and small round inclusions but not the conglomerated, round, or basket-like large inclusions that had been present in the previous patients. Such differences might depend on the specific location of each mutation, since the disorder probability is lower in the annexin domain than in the N-terminal low complexity domain (Fig. 2c), which is responsible for protein aggregation [5]. Furthermore, the co-localization of annexin A11 and p62 in NCIs was clear in this patient, but not in the previous patient with N-terminal ANXA11 mutation [7], implying that clear and frequent superposition of annexin A11 and p62 might be specific for the patients with C-terminal ANXA11 mutation.

Interestingly, annexin A11 was aggregated predominantly in neurons and only very sparsely in glial cells, and topographically, in the brainstem motor nuclei and spinal anterior horns rather than the motor cortex, whereas TDP-43 was aggregated in both neurons and glial cells, and frequently in both the upper and lower motor systems. Thus, TDP-43 aggregated even in annexin A11-negative cells, especially glial cells. Similarly, TDP-43 aggregation in annexin A11-negative cells had also been noted in the two previously reported patients with N-terminal ANXA11 mutation [6, 7]. Together with these previous studies, our present findings indicate that annexin A11 aggregation propensity is probably dependent on cell type, and that annexin A11 aggregation is not indispensable for triggering TDP-43 aggregation and neurodegeneration.

Overall, we have confirmed the pathogenicity of this novel mutation in the C-terminal region of ANXA11. Neuropathologic findings suggested a cell type-dependent annexin A11 aggregation propensity. Further studies are needed to confirm this possibility.
Fig. 3  Expression of annexin A11 in autopsied CNS tissue. a–i Annexin A11-immunohistochemistry. a Annexin A11-immunoreactive (ir) skein-like neuronal cytoplasmic inclusions (NCIs) in the anterior horn and b hypoglossal nucleus. c Small dot-like staining pattern in an anterior horn cell from a patient with amyotrophic lateral sclerosis without ANXA11 mutation. d Small irregular-shaped and e round NCIs in the motor cortex. f Filamentous NCI and g glial cytoplasmic inclusion (GCI) in the putamen. h Filamentous NCI in the pontine nucleus and i inferior olivary nucleus. j–l Double-label immunofluorescence with annexin A11 and pTDP-43. j Annexin A11 and pTDP-43 are partially co-localized in the skein-like NCI. k Weak immunoreactivity of annexin A11 in pTDP-43-ir GCI. l, k, lumbar anterior horn. l Annexin A11-ir and pTDP-43-negative small, irregularly shaped and short linear NCIs, and pTDP-43-ir and annexin A11-negative dystrophic neurite in the motor cortex. m Annexin A11-ir and p62-ir NCI in the lumbar anterior horn. j–l green, annexin A11; red, pTDP-43. m green, annexin A11; red, p62. Bar = 60 µm for a, b; 30 µm for c, d; 10 µm for e–i, l; 15 µm for j, m; 6 µm for k.
Table 2  Clinical and neuropathologic features of patients harboring ANXA11 mutation

| Clinical features | Present patient | Patient with p.D40G [6] | Patient with p.G38R [7] |
|-------------------|-----------------|------------------------|------------------------|
| Age at onset (years) | 57 | 72 | 60's^a |
| Disease duration (months) | 19 | 36 | na^a |
| Initial symptom | Limb | Bulbar | Limb |
| Cognitive impairment or FTD | − | − | + |
| Neuronal loss | + | na | + |
| Motor cortex | + | + | na |
| Lower motor neurons | − | − | + |
| Frontotemporal cortex | + | + | + |
| Degeneration of CST | − | − | + |
| Bunina body | + | na | + |
| pTDP-43-ir NCIs/GCIs | +/+ | +/- | +/+
| Annexin A11-ir NCIs/GCIs | +/- | +/- | +/- |
| Morphology of annexin A11-ir NCIs | Skein-like, filamentous, small round | Skein-like, filamentous, large-caliber and tube-shaped, large basket-like | Tube-shaped, large conglomerate, large round |
| Co-localization of pTDP-43 and annexin A11 | − or + | − | − or + |

+, present; −, absent; a, among 3 patients harboring p.G38R ANXA11 mutation, the autopsied patient was not identified in the reference [7]; FTD, frontotemporal dementia; ALS, amyotrophic lateral sclerosis; FTLD, frontotemporal lobar degeneration; na, not available; CST, corticospinal tract; pTDP-43, phosphorylated 43 kDa TAR DNA-binding protein; ir, immunoreactive

Abbreviations
ALS: Amyotrophic lateral sclerosis; pTDP-43: Phosphorylated 43 kDa TAR-DNA-binding protein.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s40478-021-01202-w.

Additional file 1. Details of methods and case presentation, a list of the primary antibodies used (Supplementary Table 1), and the representative images of annexin A11 immunohistochemistry in CNS tissue of controls (Supplementary Figure 1).

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Authors’ contributions
MT, TI, OO and AK designed research project. MS, MT, HM, YT and AK performed pathological analysis. YH, TI, SA, TK, AY and OO designed the molecular experiments and performed those. JT, GI, TO and OO collected clinical data. KA managed statistical analyses. MS, YH, MT, TI, OO and AK discussed the results and drafted the manuscript for intellectual content.

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Availability of data and materials
The datasets used and analyzed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of Niigata University (G2018-0033 and G2015-0781). Written informed consent for autopsy including the use of tissues for research purposes was obtained from the patients’ family.

Consent for publication
Family members have consented to publication.

Competing interests
The authors declare that they have no competing interests.

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References
1. Ishida T, Kinoshita K (2007) PrDOS: prediction of disordered protein regions from amino acid sequence. Nucl Acids Res 35:W460-464
2. Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105–132
3. Liao Y-C, Fernandopulle MS, Wang G, Choi H, Hao L, Drenup CM et al (2019) RNA granules hitchhike on lysosomes for long-distance transport, using Annexin A11 as a molecular tether. Cell 179:147-164.e20

4. Mackenzie IR, Neumann M, Babione A, Sampathu DM, Du Plessis D, Jaros E et al (2011) A harmonized classification system for FTLD-TDP pathology. Acta Neuropathol 122:111–113

5. Nahm M, Lim SM, Kim YE, Park J, Noh MY, Lee S et al (2020) ANXA11 mutations in ALS cause dysregulation of calcium homeostasis and stress granule dynamics. Sci Transl Med 12:eaax3993

6. Smith BN, Topp SD, Fallini C, Shibata H, Chen H-J, Troakes C et al (2017) Mutations in the vesicular trafficking protein annexin A11 are associated with amyotrophic lateral sclerosis. Sci Transl Med 9:eaad9157

7. Teyssou E, Muratet F, Amador MD, Ferrien M, Lautrette G, Machat S et al (2021) Genetic screening of ANXA11 revealed novel mutations linked to amyotrophic lateral sclerosis. Neurobiol Aging 99:102.e11-102.e20

8. Wang J, Guo C, Liu S, Qi H, Yin Y, Liang R et al (2014) Annexin A11 in disease. Clin Chim Acta 431:164–168

9. Zhang K, Liu Q, Liu K, Shen D, Tai H, Shu S et al (2018) ANXA11 mutations prevail in Chinese ALS patients with and without cognitive dementia. Neurol Genet 4:e237

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