Involvement of autophagic protein DEF8 in Lewy bodies

Makoto Timon Tanaka [a, b], Yasuo Miki [a, c], Conceição Bettencourt [c, d], Taku Ozaki [b], Kunikazu Tanji [a], Fumiaki Mori [a], Akiyoshi Kakita [e], Koichi Wakabayashi [a]

[a] Department of Neuropathology, Institute of Brain Science, Hirosaki University Graduate School of Medicine, Hirosaki, Japan

[b] Department of Biological Science, Graduate School of Science and Engineering, Iwate University, Morioka, Japan

[c] Queen Square Brain Bank for Neurological Disorders, UCL Queen Square Institute of Neurology, 1 Wakefield Street, London WC1N 1PJ, UK

[d] Department of Neurodegenerative Disease, UCL Queen Square Institute of Neurology, 1 Wakefield Street, London WC1N 1PJ, UK

[e] Department of Pathology, Brain Research Institute, Niigata University, Niigata, Japan

Correspondence to Yasuo Miki, MD, PhD

Department of Neuropathology, Institute of Brain Science, Hirosaki University Graduate School of Medicine, Hirosaki 036-8562, Japan

Email: yasuomiki@hotmail.com; y.miki@ucl.ac.uk

Key words:

Autophagy, DEF8, Parkinson's disease, Dementia with Lewy bodies, synucleinopathy, α-synuclein

Abstract

Dysregulation of autophagy, one of the major processes through which abnormal proteins are degraded, is a cardinal feature of synucleinopathies, including Lewy body diseases [Parkinson's disease (PD) and dementia with Lewy bodies (DLB)] and multiple system atrophy (MSA), which are characterized by the presence of abnormal α-synuclein in neurons and glial cells. Although several research groups have reported that Rubicon family proteins can regulate autophagosome-lysosome fusion or positioning, little is known about their involvement in synucleinopathies. In the present study, by studying patients with PD (N = 8), DLB (N = 13), and MSA (N = 5) and controls (N = 16), we explored the involvement of Rubicon family proteins [Rubicon, Pacer and differentially expressed in FDCP8 (DEF8)] in synucleinopathies. Immunohistochemical analysis showed that not only brainstem-type Lewy bodies but also cortical Lewy bodies were immunoreactive for DEF8 in Lewy body diseases, whereas Rubicon and Pacer were detectable in only a few brainstem-type Lewy bodies in PD. Glial cytoplasmic inclusions in patients with MSA were not immunoreactive for Rubicon, Pacer or DEF8. Immunoblotting showed significantly increased protein levels of DEF8 in the substantia nigra and putamen of patients with PD and the temporal cortex of patients with DLB. In addition, the smear band of DEF8 appeared in the insoluble fraction where that of phosphorylated α-synuclein was detected. These findings indicate the involvement of DEF8 in the formation of Lewy bodies. Quantitative and qualitative alterations in DEF8 may reflect the dysregulation of autophagy in Lewy body diseases.

1. Introduction

α-Synuclein is a 14-kDa protein present in presynaptic nerve terminals under physiological conditions [1]. Under pathological conditions, however, α-synuclein self-assembles into oligomers and forms highly insoluble fibrils [2]. This toxic and abnormal form of α-synuclein accumulates in the brains of patients with synucleinopathies, including Lewy body diseases [Parkinson's disease (PD) and dementia with Lewy bodies (DLB)] and multiple system atrophy (MSA) [3, 4]. Histopathologically, Lewy body diseases are characterized by the presence of abnormal α-synuclein in neurons (i.e., Lewy bodies), whereas in MSA, abnormal α-synuclein accumulates predominantly in oligodendrocytes [i.e., glial cytoplasmic inclusions (GCIs)] [5]. While the cell type affected by abnormal α-synuclein is different among synucleinopathies, an excessive α-synuclein load can lead to neurodegeneration and ultimately cell death.

Macroautophagy (hereafter referred to as autophagy) is essential for maintaining cellular homeostasis through degradation of aged organelles and misfolded proteins, including abnormal α-synuclein [6, 7]. Autophagy, a complex process, consists of several steps, including the formation of double membrane vesicles that engulf targeted proteins called autophagosomes, maturation of autophagosomes and degradation of engulfed proteins after fusion with lysosomes [8]. Recently, multiple research groups investigated the functions of a group of proteins with a common carboxy-terminal region containing a Rubicon homology (RH) domain [9, 10]. Rubicon, Pacer and differentially expressed in FDCP8 (DEF8) belong to the Rubicon family and interact with multiple autophagic proteins. Rubicon is associated with endosomal trafficking and negatively regulates autophagy by interacting with the class III phosphoinositide 3-kinase (PI3K) complex (PI3KCIII), which consists of Beclin1, vacuolar protein sorting 34 (Vps34) and UV radiation resistance-associated gene protein (UVRAG) [11]. In contrast, Pacer activates

autophagy by recruiting PI3KCIII to autophagosomes and antagonizing Rubicon [11]. In addition, Fujiwara *et al.* revealed the association of DEF8 together with Plekhm1 and Rab7 with lysosomal trafficking in osteoclasts [12].

Similarly to other autophagic proteins, Rubicon family proteins can be associated with the pathogenesis of several neurodegenerative diseases. In an Alzheimer's disease (AD) mouse model, Rubicon protein expression was significantly upregulated in the hippocampus, suggesting that formation of the Rubicon-UVRAG complex inhibits autophagy [13]. Leyton *et al.* reported alterations in DEF8 protein and mRNA levels in an AD mouse model as well as in patients with mild cognitive impairment (MCI) [14]. On the other hand, Pacer protein expression is significantly decreased in the spinal cords of patients with sporadic amyotrophic lateral sclerosis (ALS). Notably, silencing Pacer induces aggregation of superoxide dismutase (SOD1), an ALS-associated protein, in NSC34 cells [15]. In addition, we previously found several CpGs at the *DEF8* promoter showing nominal DNA methylation changes, and the *DEF8* mRNA was upregulated in the cerebellar white matter of patients with MSA [16, 17]. However, it remains unknown whether Rubicon family proteins can contribute to the pathogenesis of synucleinopathies. In the present study, we investigated qualitative and quantitative alterations in Rubicon family proteins in synucleinopathies.

2. Materials and methods

2.1. Human subjects

Tissue samples were obtained from the Department of Neuropathology, Institute of Brain Science, Hirosaki University Graduate School of Medicine, Hirosaki, and the

Department of Pathology, Brain Research Institute, Niigata University, Niigata, Japan. In total, 42 patients that underwent autopsy were included in the present study (Table S1); these included patients with PD (aged 70-83 years, average 74.5 years, N = 8), DLB (aged 66-89 years, average 79.4 years, N = 13), or MSA (aged 49-89 years, average 67.8 years, N = 5) and normal controls (aged 46-85 years, average 72.9 years, N = 16). Written informed consent for autopsy, collection of samples and subsequent analysis was obtained from the next of kin of the deceased subjects involved in this study. This study was approved by the Institutional Ethics Committee of Hirosaki University Graduate School of Medicine, Hirosaki, Japan (No. 2020-063; No. 2020-176; No. 2021-013). The diagnoses were confirmed by experienced neuropathologists (Y.M. and K.W.).

2.2. Immunohistochemistry

The brains were fixed with 10% buffered formalin for 3-4 weeks. Four-micrometer-thick sections were cut from the temporal cortex, cingulate gyrus, hippocampus, midbrain and upper pons of patients with PD (N = 5), DLB (N = 5), or MSA (N = 5) and controls (N = 6). We used the following primary antibodies: rabbit anti-DEF8 (HPA041745; Atlas Antibodies, Bromma, Stockholm, Sweden; 1:50), anti-Rubicon (PA5-38017; Thermo Fisher Scientific, Waltham, MA, USA; 1:200) and anti-Pacer/RUBCNL (HPA026614; Atlas Antibodies; 1:100). The sections were first dehydrated and subjected to antigen retrieval for 10 minutes in 10 mmol/L citrate buffer (pH 6.0) and 98% formic acid for five minutes using an autoclave. The sections were then subjected to immunohistochemical processing using the avidin-biotin-peroxidase complex method with diaminobenzidine. We also studied the proportion of DEF8-

positive Lewy bodies relative to the total number of phosphorylated α-synuclein-positive Lewy bodies using contiguous slides of the midbrain of patients with PD (N = 5) and the cingulate gyrus of patients with DLB (N = 5). We counted total and DEF8-positive Lewy bodies in all areas of continuous sections and estimated the proportion of DEF8-positive Lewy bodies.

2.3. Double immunofluorescence staining

Paraffin sections from the midbrains of PD patients were also processed for double immunofluorescence. The sections were blocked with donkey serum (ab16643; Abcam, Cambridge, UK) and then incubated overnight at 4°C with a mixture of mouse anti-phosphorylated α-synuclein (015-25191; Wako, Osaka, Japan; 1:500) and rabbit anti-DEF8 (HPA041745; Atlas Antibodies; 1:20) antibodies. The sections were then rinsed and incubated with anti-mouse IgG tagged with Alexa Fluor 488 (A-21202; Thermo Fisher Scientific; 1:200) or anti-rabbit IgG tagged with Alexa Fluor 555 (A-31572; Thermo Fisher Scientific; 1:200) for 1 h at 37°C. The sections were then mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) and examined with a confocal fluorescence microscope (EZ-Ci; Nikon, Tokyo, Japan). Adobe Photoshop 2021 (Adobe Systems, San Jose, CA, USA) was used for image processing.

2.4. Immunoblotting

For biochemical analysis, the brains were dissected out at autopsy and rapidly frozen at -70°C. Immunoblotting of the substantia nigra and putamen of patients with PD (N = 3) and age-matched normal controls (N = 3) was performed. We also utilized middle

temporal cortex tissues from patients with DLB (N = 7) and normal controls (N = 7). Each tissue was weighed and homogenized with a 20-fold volume of loading buffer [75 mmol/L Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 25% glycerol, and 5% β-mercaptoethanol]. Immunoblotting was performed as described previously [18]. Anti-DEF8 (TA502669; OriGene, Rockville, MD, USA; 1:500) and anti-β actin (A5044; Sigma–Aldrich, St. Louis, MO, USA; 1:5,000) were used as primary antibodies. We validated the specificity of the DEF8 antibody using a lysate containing DEF8 (NBL1-09817; Novus Biologicals, Centennial, CO, USA) (Fig. S1).

2.5. Fractionation and immunoblotting of brain lysates

Frozen middle temporal cortex tissues from patients with DLB (N = 3) and control subjects (N = 3) were weighed and sequentially extracted with buffers with increasing concentrations of detergent using a previously described protocol [19]. Briefly, the samples were homogenized with 10 volumes of buffer A (10 nmol/L Tris-HCl (pH 7.5), 1 mmol/L EGTA, 10% sucrose, and 0.8 mol/L NaCl) and centrifuged [fraction 1 (f1)]. Subsequently, an equal volume of buffer A containing 2% Triton X-100 was added. The samples were then incubated for 30 minutes at 37°C and spun at 100,000 x g for 30 minutes at 4°C [fraction 2 (f2)]. The resultant pellet was homogenized in 5 volumes of buffer A with 1% sarkosyl and incubated for 30 minutes at 37°C. The homogenate was then spun at 100,000 x g for 30 minutes at room temperature [fraction 3 (f3)]. The sarkosyl-insoluble pellet was homogenized in 4 volumes of buffer A containing 1% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate [(CHAPS) Sigma] and spun at 100,000 x g for 20 minutes at room temperature [fraction 4 (f4)]. The pellet was sonicated in 0.2 volumes of 8 mol/L urea buffer [fraction 5 (f5)]. Fraction 4 tends to

contain less protein than the other fractions when using this method. Immunoblotting was performed as described previously [18]. Anti-DEF8 (TA502669) and anti-β actin (A5044; Sigma–Aldrich; 1:5,000) antibodies were used for this analysis.

2.6. Statistical analysis

In the presence study, all statistical analyses were performed using SPSS (IBM, Chicago, IL, USA). After the Shapiro–Wilk test, Student's t test was performed. Differences with probability values less than 0.05 ($p < 0.05$) were considered significant.

3. Results

3.1. DEF8 immunoreactivity in Lewy bodies

We performed immunohistochemical analysis of the brains of patients with PD (N = 5), DLB (N = 5), MSA (N = 5) and controls (N = 6) using antibodies against Rubicon, Pacer and DEF8. In controls, very weak DEF8 immunoreactivity was observed granularly in the neuronal cytoplasm (Fig. 1A), whereas no glial cells were stained with the anti-DEF8 antibody (data not shown). We then investigated immunoreactivity for DEF8 in the midbrain, cingulate gyrus and temporal cortex tissues from patients with Lewy body diseases and found that not only brainstem-type Lewy bodies but also cortical Lewy bodies were moderately to strongly immunolabelled with the anti-DEF8 antibody (Fig. 1B, C). However, pale bodies, the precursor of Lewy bodies, were immunonegative for DEF8 in PD (data not shown). On the other hand, only a few brainstem-type Lewy bodies were weakly immunopositive for Rubicon and Pacer in PD (Fig. S2). Moreover, no cortical Lewy bodies were immunopositive for them in DLB (data not shown). No GCIs

were stained with anti-DEF8, anti-Rubicon or anti-Pacer antibodies in MSA (Fig. S3). Next, we counted the number of DEF8-positive Lewy bodies in cases of PD (N = 5) and DLB (N = 5). The proportion of DEF8-positive brainstem-type Lewy bodies relative to the total number of Lewy bodies in the midbrains of PD patients was 69.0% (20/29), while less than 5% of cortical Lewy bodies were immunopositive for DEF8. We further performed double immunofluorescence and confirmed the presence of phosphorylated α-synuclein immunoreactivity in the peripheral portion of Lewy bodies and DEF8 immunoreactivity in the core of Lewy bodies in PD (Fig. 2A-C).

3.2. DEF8 expression levels in the affected regions in patients with Lewy body diseases

Because of the limited Rubicon and Pacer immunoreactivity in Lewy bodies, we focused on the involvement of DEF8 in Lewy body diseases. We performed immunoblotting of substantia nigra and putamen tissues from cases of PD (N = 3) and controls (N = 3) and middle temporal cortex tissues from cases of DLB (N = 7) and normal controls (N = 7). The protein levels of DEF8 were significantly increased in the substantia nigra and putamen in PD patients compared with normal controls (Figure 3A-D) and the middle temporal cortex in DLB patients compared with normal control (Fig. 3E, F).

3.3 DEF8 smeared in the insoluble fraction, similarly to phosphorylated α-synuclein

To further confirm the association of DEF8 with abnormal α-synuclein in Lewy body diseases, we performed immunoblotting of fractionated brain lysates of the temporal cortex of patients with DLB (N = 3) and controls (N = 3). As reported previously [19], phosphorylated α-synuclein was present in the insoluble fraction (f5) of temporal cortex

tissues from patients with DLB (Fig. 4A). The smear band of DEF8 and phosphorylated α-synuclein appeared in the same insoluble fraction (Fig. 4B).

4. Discussion

In the present study, we have demonstrated qualitative and quantitative alterations in DEF8 in Lewy body diseases. DEF8 was involved in the formation of Lewy bodies. The protein levels of DEF8 were significantly increased in the substantia nigra and putamen in cases of PD compared with controls and in the temporal cortex in cases of DLB compared with controls. In addition, we found that similarly to those of other autophagic proteins, including Beclin-1 and Vps34, as reported in our previous study [19], the smear band of DEF8 appeared in the insoluble fraction where that of phosphorylated α-synuclein was observed. Previously, we also reported that the expression of the lipidated form of microtubule-associated protein light chain 3 (LC3-II) is significantly increased in the temporal lobe in cases of DLB compared to controls [20]. LC3-II stays on the inner membrane of autophagosomes and is then degraded together with engulfed proteins in the lysosome [21]. Given that autophagy is dysregulated in synucleinopathies, the increase in the protein levels of autophagic proteins, including LC3-II, in synucleinopathies may indicate the presence of undegraded proteins in lysosomes. DEF8 acts as a regulator of lysosome positioning [12] and may stay with LC3-II until engulfed proteins are degraded. Thus, alterations in DEF8 expression in Lewy body diseases may also reflect the presence of undegraded abnormal α-synuclein in the lysosomes. Further research is necessary to elucidate when and how DEF8 is implicated in the pathogenesis of Lewy body diseases.

Previously, we studied peripheral blood mononuclear cells (PBMCs) from patients with PD and controls, and found upregulation of the protein expression of ULK1, Beclin-1 and AMBRA1, core regulators of upstream autophagy, and negative feedback of mRNA transcription for these proteins [22]. Interestingly, we found a positive correlation between the expression levels of these proteins and that of α-synuclein oligomers. This level of α-synuclein oligomers in PBMCs correlated with the clinical severity of PD and degeneration of the cardiac sympathetic nerves, as assessed by cardiac uptake of metaiodobenzylguanidine. These findings suggest that autophagic activity in PBMCs from cases of PD can mirror that in the brain. Recently, Leyton *et al.* examined lymphocytes and PBMCs from patients with MCI and Alzheimer's disease (AD). *DEF8* mRNA levels were significantly downregulated in PBMCs from MCI and AD patients compared with healthy controls, and correlated with cognitive tests scores. Additionally, DEF8 protein levels were increased in lymphocytes from MCI compared with healthy controls [14]. It will be interesting to examine whether the protein and mRNA levels of DEF8 in PBMCs from patients with Lewy body diseases correlate with the clinical severity of the diseases.

In summary, we have demonstrated for the first time the involvement of DEF8 in the formation of Lewy bodies. The quantitative and qualitative alteration in DEF8 may further suggest that autophagy is impaired in Lewy body diseases.

Funding

Author's contributions

MT and YM jointly conceived the study, and MT, YM, TK and FM carried out the experiments and analyzed the data. MT, YM, CB, TO, TK, FM, AK and KW prepared the figures and wrote the manuscript. All authors read and approved the final version of the manuscript and figures.

Declaration of Competing Interest

The authors declare no competing interests.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Numbers 21K07452 (Y.M.), 20K06887 (K.T.), 18H02533 (K.W.), The Sakurai Memorial Fund for Medical Research (Y.M.), the Collaborative Research Project of the Brain Research Institute, Niigata University (K.T.) and AMED under Grant Number JP21wm0425019 (A.K.). C.B. is supported by the Multiple System Atrophy Trust and Alzheimer's Research UK (ARUK-RF2019B-005).

The authors wish to express their gratitude to M. Nakata for her technical assistance.

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Figure legends

Fig. 1. DEF8 immunoreactivity in patients with PD and DLB and controls. (A) Very weak granular DEF8 immunoreactivity in the neuronal cytoplasm in the oculomotor nucleus in controls (arrows). (B, C) DEF8 immunoreactivity in the core of brainstem-type Lewy bodies in the substantia nigra in patients with PD (arrowhead) (B) and cortical Lewy bodies in the cingulate gyrus in patients with DLB (arrowhead) (C). Bars = 10 μm.

Fig. 2. Double immunofluorescence staining of substantia nigra tissues from patients with PD. (A-C) The peripheral portion of brainstem-type Lewy bodies is immunopositive for phosphorylated α-synuclein, while the core of Lewy bodies is immunoreactive for DEF8 (white arrowheads). (A) Phosphorylated α-synuclein appears green. (B) DEF8 appears red. Bars = 10 μm.

Fig. 3. Western blot analysis of total brain lysates from patients with PD and DLB and controls. (A-D) DEF8 expression levels normalized to actin protein levels are significantly higher in the putamen and substantia nigra in patients with PD than in controls (PD, N = 3; control, N = 3). (E, F) DEF8 expression levels in the temporal cortex

are also significantly increased in patients with DLB compared with controls (DLB, N = 7; control, N = 7).

Fig. 4. Western blot analysis of fractionated brain lysates of the temporal cortex from patients with DLB and controls (DLB, N = 3; control, N =3). (A) A band with a molecular weight of 16 kDa corresponding to phosphorylated α-synuclein is found in the insoluble fraction (f5) from a representative patient with DLB but not in a representative control subject. In addition, the molecular mass of phosphorylated α-synuclein in the same fraction is higher in a patient with DLB (asterisk). (B) A band with a molecular weight of 58 kDa corresponding to DEF8 is observed in the insoluble fraction (f5) in a patient with DLB and a control subject (arrow). A smear band of DEF8 is evident in the insoluble faction (f5) and corresponded to phosphorylated α-synuclein (arrowhead). These findings were also seen in the other patients with DLB and controls.

Appendix A: Supplementary data

The following is the supplementary data to this article: