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Accumulation of α-synuclein in hepatocytes in nonalcoholic steatohepatitis and its usefulness in pathological diagnosis

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ABSTRACT

Backgrounds: Nonalcoholic steatohepatitis (NASH) is characterized by fat deposition, inflammation, and hepatocellular damage. The diagnosis of NASH is confirmed pathologically, and hepatocyte ballooning is an important finding for definite diagnosis. Recently, α-synuclein deposition in multiple organs was reported in Parkinson's disease. Since it was reported that α-synuclein is taken up by hepatocytes via connexin 32, the expression of α-synuclein in the liver in NASH is of interest. The accumulation of α-synuclein in the liver in NASH was investigated. Immunostaining for p62, ubiquitin, and α-synuclein was performed, and the usefulness of immunostaining in pathological diagnosis was examined.

Methods: Liver biopsy tissue specimens from 20 patients were evaluated. Several antibodies against α-synuclein, as well as antibodies against connexin 32, p62, and ubiquitin were used for immunohistochemical analyses. Staining results were evaluated by several pathologists with varying experience, and the diagnostic accuracy of ballooning was compared.

Results: Polyclonal α-synuclein antibody, not the monoclonal antibody, reacted with eosinophilic aggregates in ballooning cells. Expression of connexin 32 in degenerating cells was also demonstrated. Antibodies against p62 and ubiquitin also reacted with some of the ballooning cells. In the pathologists' evaluations, the highest interobserver agreement was obtained with hematoxylin and eosin (H&E)-stained slides, followed by slides immunostained for p62 and α-synuclein, and there were cases with different results between H&E staining and immunostaining

Conclusion: These results indicate the incorporation of degenerated α-synuclein into ballooning cells, suggesting the involvement of α-synuclein in the pathogenesis of NASH. The combination of immunostaining including polyclonal α-synuclein may contribute to improving the diagnosis of NASH.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases [1]. Nonalcoholic steatohepatitis (NASH) is the inflammatory subtype of NAFLD, and in addition to fatty deposits, hepatocellular injury, balloon-like degeneration (ballooning), and inflammation are seen, which may be accompanied by fibrosis. Although often clinically asymptomatic, over time NASH is known to progress to cirrhosis, end-stage liver failure, and the need for liver transplantation [2]. The diagnosis of NAFLD requires the absence of excessive alcohol consumption and imaging or histological evidence of $\geq$ 5 % fat deposition in liver tissue [1]. On the other hand, the diagnosis of NASH requires biopsy and histological evidence of $\geq$ 5 % hepatic fat deposition, ballooning of hepatocytes, and inflammation of the hepatic lobules.

The histological grade of NASH is also assessed using a scoring system (NAFLD activity score) that includes steatosis, lobular inflammation, and ballooning [3]. Liver biopsy is currently the only accepted method to reliably distinguish NASH from simple fatty liver or uncomplicated NAFLD [1,4,5].

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Hepatocyte ballooning is an important finding in the pathological diagnosis of NASH. It is defined as enlarged hepatocytes with eosinophilic aggregates in the cell chamber [6–9].

Immunostaining shows decreased CK8 and CK18, and Mallory-Denk bodies found in some ballooning cells are known to be positive for anti-p62 and anti-ubiquitin antibodies, which are thought to be associated with cytoskeletal damage [10–15]. However, the definition of ballooning is descriptive, and discrepancies in diagnosis among pathologists are often problematic [16]. Immunostaining is available as an adjunct to pathological diagnosis, but its usefulness is uncertain, and many cases are currently diagnosed using hematoxylin and eosin (H&E) staining.

Alpha-synuclein (α-syn) is a protein present in the presynaptic terminals of neurons in the central nervous system. Accumulation of α-syn is a key sign of Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA), which are neuropathological disorders called synucleinopathies [17]. Pathologically, PD and DLB are characterized mainly by accumulation of α-syn in neurons, whereas in MSA, accumulation of α-syn is observed mainly in oligodendrocytes [18]. Although α-syn accumulation in the brain is a pathological feature of PD, several studies have identified α-syn accumulation in gastrointestinal areas including the liver, retina, skin, and heart, suggesting that PD is a multiorgan disorder [19,20]. In addition, a recent study reported that, in human liver tissue, cases with neuropathologically confirmed α-syn showed α-syn within hepatocyte structures to a higher degree than cases without α-syn accumulation in the brain, suggesting that the liver is involved in pathological α-syn metabolism [21,22]. It has been reported that α-syn oligomers are incorporated into neurons and oligodendrocytes via Connexin 32 (Cx32), a gap junction protein, in PD and MSA [22,26,27]. Hepatocytes express Cx32 and Cx26, with Cx32 accounting for the majority. Previous studies have also shown that fatty hepatitis and fibrosis progression in NAFLD patients correlate with low expression of Cx32 in hepatocytes and are associated with NAFLD progression. These facts indicate that the physiological uptake and excretion of α-syn via Cx32 may be impaired in the hepatocytes of NASH patients, but the dynamics of α-syn in the NASH liver have not yet been investigated. We hypothesized that ballooning, a hepatocellular degenerative phenomenon in NASH, may be related to the accumulation of α-syn.

In this study, the accumulation of α-syn in the NASH liver was investigated using monoclonal and polyclonal antibodies against phosphorylated α-syn. The expressions of Cx32 and α-syn oligomers were also examined in representative NASH cases containing marked ballooning cells. In addition, to evaluate the usefulness of immunostaining in determining ballooning, immunostaining for p62 and ubiquitin, as well as α-syn, was performed, and interobserver agreement (IOA) among multiple pathologists was evaluated.

2. Materials and methods

2.1. Design

This retrospective, cohort study using formalin-fixed, paraffin-embedded tissue blocks. Liver biopsy tissue specimens from 20 patients at Tokushima University Hospital (Tokushima, Japan) who were histologically diagnosed with steatohepatitis between 2010 and 2020 were used.

2.2. Patients' background characteristics

Patients in this cohort ranged in age from 33 to 75 years, and 11 (55 %) were female.

NAFLD was clinically suspected as a differential diagnosis at biopsy in 17 patients (85 %), with other differential diagnoses including alcoholic hepatitis (ASH), autoimmune hepatitis (AIH), and drug-induced hepatitis (Table 1).

Table 1
Patients' clinical characteristics.

Variable	
Female, n (%)	11 (55 %)
Age at biopsy, (mean)	33–75(58)
Number of NAFLD, n (%)	17 (85 %)

2.3. Immunohistochemistry

Immunostaining was performed using anti-phosphorylated α-syn monoclonal, anti-phosphorylated α-syn polyclonal, anti-oligomer-specific α-syn, anti-Cx32 antibodies and anti-cytokeratin 8/18 in patients diagnosed with NASH.

The immunostaining protocol after deparaffinization with xylene and rehydration in a graded ethanol bath (100 %, 95 %, 70 %) is described below. Immunostaining for anti-phosphorylated α-syn monoclonal antibody (1:1000 dilution; WAKO, Richmond, VA; pSyn#64), anti-phosphorylated α-syn polyclonal antibody [23–25] (1:4000 dilution), anti-p62 antibody (1:5000 dilution; Enzo Life Sciences, Farmingdale, NY; PW9860), anti-ubiquitin antibody (1:1000 dilution; Sigma-Aldrich, St. Louis, MO; AB1690) and anti-cytokeratin 8/18 antibody (1:1000 dilution; Abcam, Cambridge, MA; ab17139) was performed using a Leica BOND-MAX™ system (Leica Biosystems, Wetzlar, Germany) with Bond polymer refined detection reagents (Leica Biosystems). For anti-oligomer-specific α-syn antibody (1:200 dilution; Agrisera, Vännäs, Sweden; AS13-2718) and anti-Cx32 antibody (1:200 dilution; Invitrogen, Carlsbad, CA; 71-0600), antigen activation was performed at room temperature (RT) for 7 min with proteinase K solution (Nichirei Biosciences, Tokyo, Japan). After 2 h of incubation with these primary antibodies at RT, the secondary antibody, labeled Polymer-HRP Mouse/Rabbit (DakoCytomation, Glostrup, Denmark) was applied and incubated for 40 min at RT. HRP was colored by diaminobenzidine (DAB) using DAB Substrate Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

2.4. Histological evaluation

Staining results were evaluated by several pathologists with varying experience using hematoxylin and eosin (H&E) staining and immunostaining. Liver pathologist evaluated Steatosis, lobular inflammation and hepatocyte ballooning using NAS score and fibrosis stage using Brunt's system. H&E-stained slides were evaluated for the presence of ballooning with NAS score 1 or 2. Polyclonal phosphorylated α-syn, p62, and ubiquitin-immunostained slides were evaluated as positive or negative.

2.5. Statistical analysis

Interobserver agreement (IOA) was evaluated using Fleiss' kappa (κ) correlation coefficient, with $\kappa \leq 0$ indicating no agreement, 0.01–0.20 no to poor, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 substantial, and 0.81–1.00 almost perfect agreement. The κ values were calculated with the "irr" package of R version 4.2.1 (R Foundation for Statistical Computing).

3. Results

3.1. Expression of phosphorylated α-syn in ballooning cells

Immunostaining showed positive results for anti-phosphorylated α-syn polyclonal antibodies and negative results for anti-phosphorylated α-syn monoclonal antibodies in the ballooning cells of the NASH liver (Fig. 1A-C). Oligomer-specific α-syn antibodies were positive in granular form in a small number of degenerated hepatocytes (Fig. 1D).

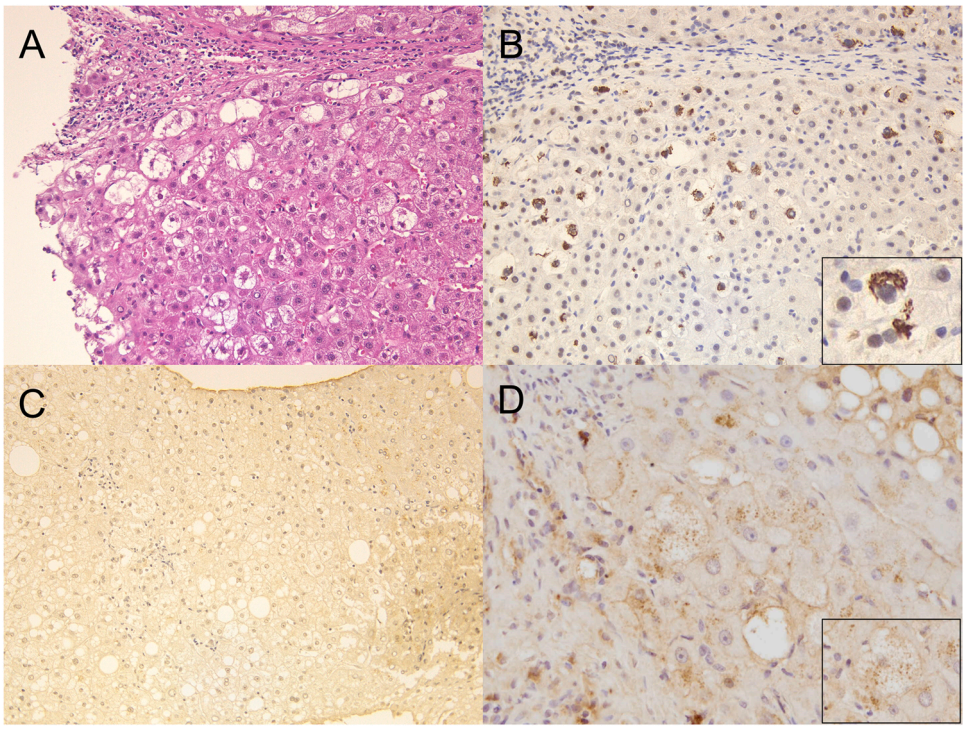

Fig. 1. Phosphorylated α-syn immunostaining in NASH liver. A, H&E-stained slides show degeneration and ballooning of hepatocytes. B, aggregates in ballooning cells are positive for phosphorylated α-syn polyclonal antibody. C, no positive results with phosphorylated α-syn monoclonal antibody are seen. D, granular aggregates in some hepatocytes and ballooning cells are positive for oligomer-specific α-syn antibodies.

3.2. Expression of Cx32 in NASH liver

The Cx32 antibody was positive in the form of dots on the hepatocyte membranes with weak degeneration. In areas of strong degeneration, the antibody was positive in enlarged hepatocytes, and its expression was attenuated in surrounding hepatocytes (Fig. 2A,B).

3.3. Expression of p62, ubiquitin and cytokeratin 8/18 in ballooning cells

Aggregates in ballooning cells were positive for anti-p62 and anti-ubiquitin antibodies, with similar localization to that of anti-α-syn polyclonal antibodies (Fig. 2C,D). Cytokeratin 8/18 showed attenuated expression in ballooning hepatocytes (Fig. 2E).

3.4. Histological evaluation

The evaluation by liver pathologist were 0–3 (mean 1.0) for Steatosis, 0–1 (mean 0.8) for lobular inflammation, 0–2(mean 0.9) for hepatocyte ballooning, and 0–4 (mean 2.2) for fibrosis stage (Supplementary Table 1). Evaluation of ballooning on H&E-stained slides showed that the pathologist with the most experience in liver diagnosis judged 14 of 20 cases to be positive, whereas pathologists with limited diagnostic experience judged 11–14 cases to be positive (Supplementary Table 2).

In the evaluation of polyclonal phosphorylated α-syn-immunostained slides, the pathologist with the most experience in liver diagnosis considered 16 of 20 cases positive, whereas pathologists with limited diagnostic experience considered 12–15 cases positive (Figs. 3 and 4) (Supplementary Table 3).

In the evaluation of p62-immunostained slides, the pathologist with the most experience in liver diagnosis judged 16 of 20 cases to be positive, whereas pathologists with limited diagnostic experience judged 15–16 cases to be positive (Supplementary Table 4).

In the evaluation of ubiquitin-immunostained slides, the pathologist with the most experience in liver diagnosis considered 9 of 20 cases positive, whereas pathologists with limited diagnostic experience considered 8–11 cases positive (Supplementary Table 5).

The IOA among pathologists was 0.73 for H&E-stained slides, 0.62 for polyclonal phosphorylated α-syn-immunostained slides, 0.62 for p62-immunostained slides, and 0.30 for ubiquitin-immunostained slides (Table 2).

4. Discussion

In the pathological diagnosis of NASH, hepatocyte ballooning is an important histological finding. However, the definition of ballooning formation on H&E staining is descriptive, and the mechanism by which ballooning occurs is controversial. In the present study, it was shown that α-syn is positive in ballooning cells, and the association between degenerating cells and Cx32 expression was evaluated. In addition, the usefulness of immunostaining for determining ballooning in the pathological diagnosis of NASH was examined.

It is known that α-syn accumulates in neurons and oligodendrocytes in PD and other neuropathological diseases. Several studies have also confirmed accumulation of α-syn in multiple organs in PD [18–20]. It has been reported that α-syn oligomers are incorporated into neurons and oligodendrocytes via Cx32, a gap junction protein, in PD and MSA [22,26,27]. Hepatocytes express Cx32 and Cx26, with Cx32 accounting for the majority. In contrast, most nonparenchymal cells, such as stellate cells and Kupffer cells, have Cx43 [28–34]. Connexin expression in the liver is known to be altered by differentiation and dedifferentiation. Liver progenitor cells predominantly possess Cx43, which is known to be converted to Cx26 and Cx32 during differentiation into hepatocytes [35–37] In addition, the reverse process is observed in progressive liver injury, such as hepatic fibrosis and hepatitis [38].

Gap junction proteins, including Cx32, are involved in innate immune responses, and several studies have shown that Cx32 plays an important role in injury formation in liver disease models [39–43]. Previous studies have also shown that steatohepatitis and fibrosis progression in NAFLD patients correlate with low expression of Cx32 in

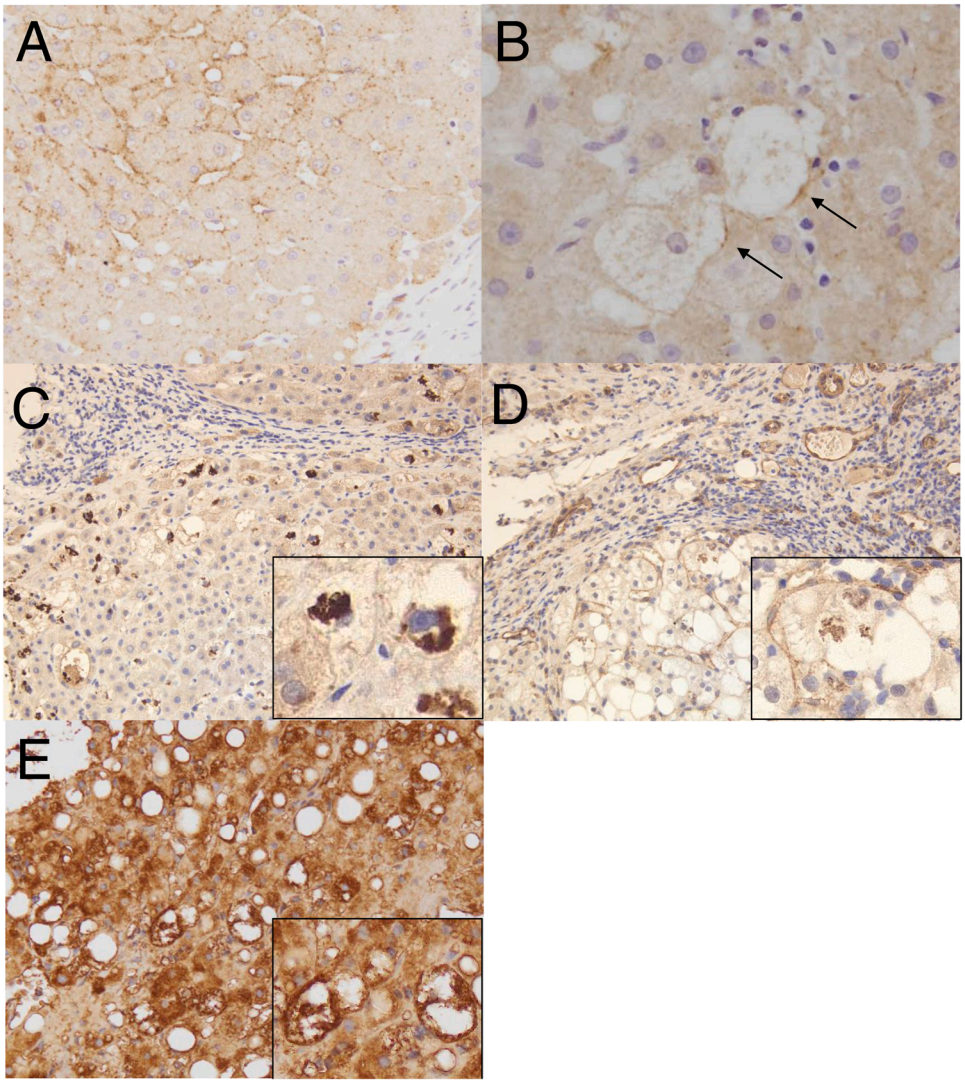

Fig. 2. Cx32, p62, ubiquitin and Cytokeratin CAM5.2 immunostaining in NASH liver. A, mildly degenerated hepatocytes are dot-like positive for Cx32 at the plasma membrane (arrow). B, enlarged hepatocytes are positive for Cx32 at the plasma membrane, whereas the expression of Cx32 is attenuated in the surrounding hepatocytes. C, aggregates in ballooning hepatocytes are positive for p62. D, Ubiquitin shows a similar localization to p62. E, cytokeratin 8/18 showed attenuated expression in ballooning hepatocytes.

hepatocytes and are associated with NAFLD progression [44]. In the present study, Cx32, which is normally expressed in the hepatocyte membrane in granular form, was attenuated in NASH, and there was a mixture of strongly positive and mostly negative cells. In addition, hepatocytes showing ballooning were found to have strong Cx32 expression in a portion of the plasma membrane. These results are consistent with previous reports and suggest that abnormal Cx32 expression may disrupt the normal α-syn traffic in hepatocytes. In addition, polyclonal antibodies against α-syn were positive for intracytoplasmic aggregates in degenerated hepatocytes, and oligomer-specific α-syn antibodies were also positive in a small number of degenerated hepatocytes. One of the reasons why no positive images were observed with α-syn monoclonal antibodies in the present study is that the α-syn accumulated in ballooning cells may be denatured α-syn, in which the recognition epitope of the monoclonal antibody is missing or modified. The present results indicate that some of the denatured α-syn taken up into the hepatocyte by the heterogeneously distributed Cx32 may remain in the hepatocyte in an oligomerized state, and that some of these denatured proteins may be involved in autophagy mediated by p62 or proteolysis mediated by ubiquitin. This is the first time that a protein has been shown to be degraded in the liver.

To test this hypothesis, in addition to observing the uptake of foreign α-syn over time in cultured hepatocytes overexpressing Cx32, it is necessary to observe the dynamics of α-syn in vivo using NASH model mice. We have previously reported several mouse models of NASH [45–47].

However, it is very difficult to recognize ballooning cells in any of these models, and the histological picture is different from that of human NASH. We are currently planning an experimental design in which ballooning cells are induced by intravenous administration of α-syn to increase the influx of foreign α-syn into the liver.

Evaluation by several pathologists with different levels of experience interpreting H&E staining and immunostaining showed that the H&E-stained slides had the highest IOA, followed by α-syn and p62-stained slides, with similar values. However, in some cases, the pathologist evaluation on the H&E-stained slides did not agree with that on the immunostained slides. Fig. 3 showed a case in which the H&E-stained slide was evaluated as negative for ballooning, but the immunostained slide was evaluated as positive. The clinical diagnosis of this case was NAFLD, suggesting the possibility of early NASH. Histology showed only fatty deposits and mild hepatocellular swelling (Fig. 3A), but immunostained slides were positive for aggregates within the enlarged hepatocellular chambers (Fig. 3B-D). These differences in evaluations suggest that, even in the early stage of NASH with little histological changes, the diagnosis may be possible in some cases by adding immunostaining. Fig. 4 shows a case that tested positive for ballooning on the H&E-stained slide but was negative on the immunostained slide. The clinical diagnosis of this case was eating disorder and drug-induced

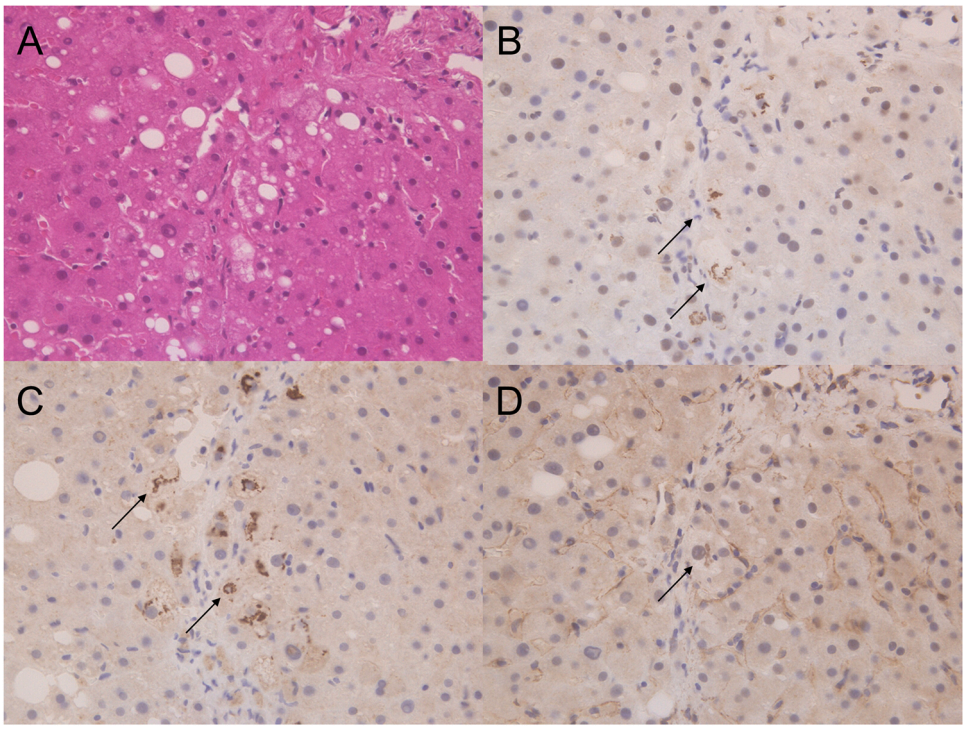

Fig. 3. A, H&E staining shows mild degeneration of hepatocytes, but ballooning is indistinct. B-D, the cytoplasm of enlarged hepatocytes is positive (arrow) for phosphorylated α-syn (B), p62 (C), and ubiquitin (D).

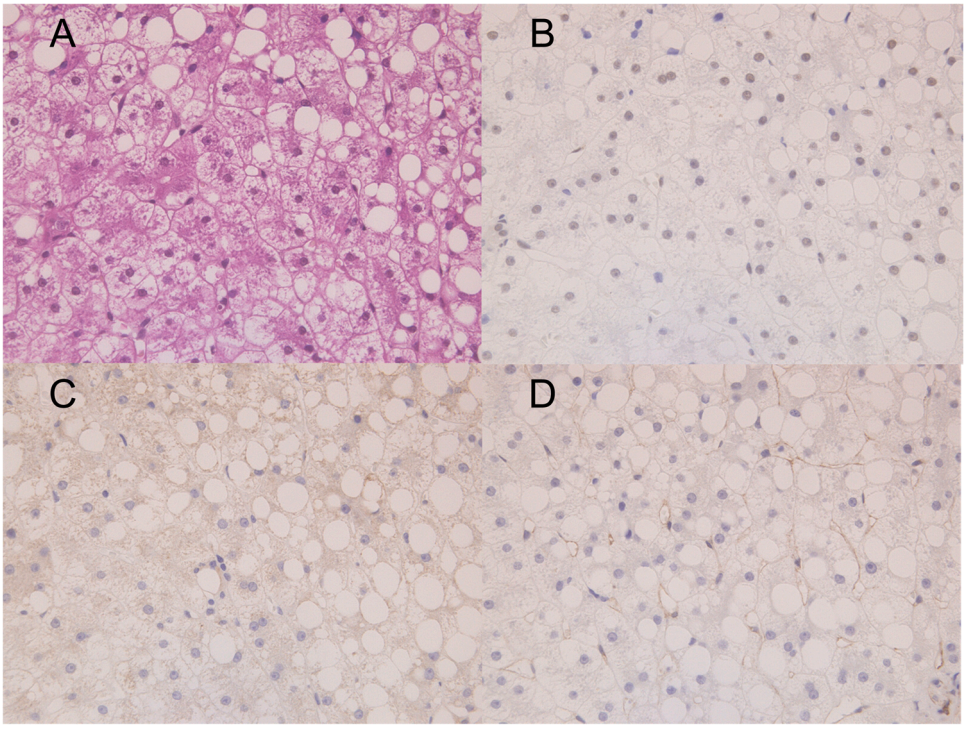

Fig. 4. A, H&E staining shows fatty deposits, hepatocyte degeneration, and ballooning-like changes. B-D, phosphorylated α-syn (B), p62 (C), and ubiquitin (D) are negative.

steatohepatitis, and NAFLD and ASH were negative. Histologically, the patient showed severe steatohepatitis, with numerous enlarged hepatocytes and swelling degeneration, similar to ballooning (Fig. 4A). However, immunostained slides showed that intracytoplasmic aggregates were not positive (Fig. 4B-D). Therefore, the use of immunostaining in the diagnosis of drug-induced steatohepatitis cases with a histology very similar to NASH and ASH may contribute to improve diagnostic accuracy.

This study has several limitations. First, it was conducted using cases from a single institution in Japan, and evaluations were performed by a

Table 2
Assessments of HE and IHC slides and interobserver agreement among pathologists.

	Liver pathologist	General pathologists	IOA (κ)
HE	14	11–14(12.3)	0.73
a-syn	16	12–15(14)	0.62
p62	16	15–16(15.7)	0.62
ubiquitin	9	8–11(8.7)	0.30

limited number of pathologists at the same institution; therefore, the results may not be generalizable, and further studies with larger sample sizes are needed.

Second, this study used only biopsy material and, therefore, only evaluated a portion of the liver; NASH is a diffuse liver injury, and the degree of liver injury may vary depending on the biopsy site. Thus, it is necessary to evaluate specimens including surgical materials for a more accurate study.

In summary, intrahepatic aggregates with ballooning are positive for α-syn and may be involved in the pathogenesis of NAFLD. Immunostaining including α-syn may be useful in the pathological diagnosis of NASH.

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Human and animal rights statement

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. This study was approved by the Ethics Committee of Tokushima University Hospital (No. 4137).

CRediT authorship contribution statement

Takumi Kakimoto : Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Masato Hosokawa** : Investigation, Visualization, Resources. **Mayuko Ichimura-Shimizu** : Formal analysis, Funding acquisition, Supervision. **Hirohisa Ogawa:** Formal analysis, Funding acquisition, Supervision. **Yuko Miyakami** : Data curation, Resources. **Satoshi Sumida** : Data curation, Resources. **Koichi Tsuneyama:** Conceptualization, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Koichi Tsuneyama reports financial support was provided by Japan Society for the Promotion of Science. Hirohisa Ogawa reports financial support was provided by Japan Society for the Promotion of Science. Mayuko Ichimura-Shimizu reports financial support was provided by Japan Society for the Promotion of Science.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prp.2023.154525.

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