—Original—

Immunohistochemical Diagnosis of Amyloid Typing: Utility and Limitations as Determined by Liquid Chromatography-Tandem Mass Spectrometry

Yukako Shintani-Domoto[1], Kousuke Ishino[2], Takenori Fujii[2], Taeko Kitamura[2], Kiyoshi Teduka[2], Hironobu Naiki[3], Takashi Sakatani[1] and Ryuji Ohashi[1,2]

[1]Department of Diagnostic Pathology, Nippon Medical School Hospital, Tokyo, Japan
[2]Department of Integrated Diagnostic Pathology, Nippon Medical School, Tokyo, Japan
[3]Department of Molecular Pathology, University of Fukui, Fukui, Japan

Background: Although immunohistochemical techniques and proteomic analysis are widely used for typing diagnosis of amyloidosis, the diagnostic utility of immunohistochemical evaluation is not well understood.

Methods: We used immunohistochemical techniques to characterize staining patterns of in-house rabbit polyclonal anti-κ, anti-λ, anti-transthyretin antibodies, and commercial anti-amyloid A and anti-β_2-microglobulin antibodies in 40 autopsy cases.

Results: In thirty cases (75%), the subtype was determined by using the criterion that amyloid is strongly and diffusely positive for one antibody while negative for other antibodies. We then performed proteomic analysis of all 40 cases. In 39 cases, we identified only one amyloid protein and confirmed the immunohistochemically determined subtypes of the abovementioned 30 cases. In seven other cases, we could retrospectively determine subtypes with immunohistochemistry by using information from proteomic analysis, which increased the immunohistochemistry diagnosis rate to 92.5% (37/40). In one case, we identified double subtypes, both immunohistochemically and with proteomic analysis. In the remaining three cases, proteomic analysis was essential for typing diagnosis.

Conclusions: The present findings suggest that combined immunohistochemistry and proteomic analysis is more useful than immunohistochemistry alone. Our findings highlight the importance of carefully interpreting immunohistochemistry for anti-TTR and light chain and offer insights that can guide amyloid typing through immunohistochemistry. (J Nippon Med Sch 2024; 91: 261–269)

Key words: amyloidosis, immunohistochemistry, autopsy, mass spectrometry

Introduction

Amyloidosis is a disease in which amyloid proteins deposited in the extracellular matrix cause organ damage. Currently, 42 different amyloid proteins have been reported[1], the most common of which are amyloid light chain κ, λ (ALκ, ALλ), amyloid transthyretin (ATTR), amyloid A (AA), and amyloid β_2-microglobulin (Aβ2M). In recent years, therapies have been developed for some subtypes of amyloidosis, and accurate diagnosis of amyloid subtypes has become important[2]. Of particular interest are transthyretin (TTR) tetramer stabilizers for ATTR amyloidosis, which were shown to be effective for ATTR cardiomyopathy[3,4]. CD38-targeting antibodies for the treatment of plasma cell myeloma have also been used to treat AL amyloidosis[5].

To diagnose amyloidosis, amyloid deposition is detected by Congo red staining, and amyloid proteins are generally identified by immunohistochemistry (IHC)[6]. When it is difficult to determine subtype because of false-positive or false-negative immunoreactivity or suspected deposition of multiple amyloid proteins, laser microdissection (LMD)-liquid chromatography-tandem mass

Correspondence to Yukako Shintani-Domoto, MD, PhD, Department of Diagnostic Pathology, Nippon Medical School Hospital, 1–1–5 Sendagi, Bunkyo-ku, Tokyo 113–8603, Japan
E-mail: yukakoshintani@gmail.com
https://doi.org/10.1272/jnms.JNMS.2024_91-304
Journal Website (https://www.nms.ac.jp/sh/jnms/)

spectrometry (LC-MS/MS) is used to identify the amyloid proteins[6–8], and absolute quantification of specific amyloid proteins using an isotope labeling technique is applied[9]. We raised anti-κ chain$_{116\text{-}133}$, anti-λ chain$_{118\text{-}134}$ (rabbit polyclonal)[10], anti-TTR$_{115\text{-}124}$ (rabbit polyclonal)[11] antibodies. Antibodies for AL target the constant region and do not stain amyloids derived solely from the variable region, which account for approximately 10-15% of amyloids[10]. TTR antibody reacts exclusively with amyloid deposits, not with serum[11]. These antibodies, in combination with commercially available antibodies for AA and β_2-microglobulin (β_2-m), were used by a government-funded group for amyloidosis surveys and research in Japan (GSRA-J) and were useful for IHC subtype diagnosis in about 95% of amyloidosis cases[2,7]. However, several details, such as the accuracy of IHC studies and the frequency of false-positive and false-negative findings, have not been fully clarified.

Using IHC utilizing antibodies against five amyloid proteins, with further validation of subtypes by LC-MS/MS, we investigated amyloid subtypes in 40 autopsied patients with systemic amyloidosis.

Materials and Methods

Ethics Statement

The study protocol was performed in accordance with the principles of the Declaration of Helsinki and was approved by the Human Ethics Committee of Nippon Medical School (No. B-2020-200). Autopsies were performed after receiving informed consent. As this study was conducted using autopsy records, we could not obtain informed consent from the bereaved family for the use of the records. Therefore, in accordance with the "Ethical Guidelines for Medical Research Involving Human Subjects" (enacted by the Ministry of Health, Labor and Welfare in Japan), the opportunity for the family to refuse access to records by opting out was guaranteed.

Patients and Tissue Samples

Among the 5,298 autopsies performed at Nippon Medical School Hospital from 1982 to 2020, 49 cases with "amyloid" noted in the autopsy report were selected. Six cases were later excluded: four with localized amyloidosis and two that had been diagnosed by direct fast scarlet alone but were confirmed as negative by Congo red staining in this study. Of the remaining 43 (0.81%) cases with systemic amyloidosis, data from 40 cases with available histological specimens were ultimately analyzed (**Supplemental Table 1**: https://doi.org/10.1272/jnms.JNMS.2024_91-304).

Supplemental Table 2 (https://doi.org/10.1272/jnms.JNMS.2024_91-304) summarizes the sites of amyloid deposition in relation to the descriptions in the autopsy reports. Formalin-fixed paraffin-embedded (FFPE) heart specimens with amyloid deposits were chosen in all cases except Case 1, in which a heart specimen was not available and thus kidney tissue with amyloid deposits was used. Serial tissue sections of the specimens were cut at 3-μm thickness for hematoxylin and eosin staining, Congo red staining, and IHC, and at 10-μm thickness for LMD-LC-MS/MS.

Congo Red Staining and IHC

Amyloid deposits were histologically confirmed by Congo red staining, showing typical apple-green birefringence under polarized light. To identify amyloid proteins, five antibodies against individual amyloid proteins were used (**Supplemental Table 3**: https://doi.org/10.1272/jnms.JNMS.2024_91-304)[10,11]. IHC intensity and stained area were evaluated on a scale of 0 to 2+, as follows: 0, negative; 1+, weakly and/or focally positive; 2+, strongly and diffusely positive. Amyloid subtype was determined when the Congo red-positive area was positive for only one antibody (2+) and negative for other antibodies (0).

Tissue Preparation for LMD-LC-MS/MS

FFPE specimens (10-μm-thick) from the heart were used for proteomic analysis using LMD-LC-MS/MS according to a previously reported procedure[12] with slight modifications. The sections were deparaffinized with xylene, rehydrated through a graded alcohol series, and stained with Congo red. Congo red-positive regions (approximately 50,000-60,000 μm^2) were dissected using LMD (LMD7000, Leica Microsystems GmbH, Wetzlar, Germany) and lysed in buffer with 10 mM Tris-base/1 mM EDTA/0.002% Zwittergent 3-16. After sonication and boiling, samples were digested overnight using 0.5 μg Trypsin Gold (V528A, Promega, Madison, WI, USA) at 37 °C. After adding dithiothreitol and boiling, samples were evaporated in a vacuum concentrator centrifuge system at 37°C. The residues were resuspended in water containing 0.1% formic acid and then filtered using an Ultrafree-MC unit (UFC30LG00, Merck KGaA, Darmstadt, Germany). A portion of the digested protein was injected into a NanoLC Trap ChromXP C18-CL column (0.35 × 0.5 mm, 3 μm, 120 Å, AB Sciex, Foster City, CA, USA) and further separated through an Eksigent nano LC Ekspert415 system (AB Sciex) using a spraychip column (0.075 × 120 mm, 3 μm C18, Nikkyo Technos, Tokyo, Japan). The analysis was run with an acetonitrile gradient concentration from 2% to 32% in 0.1% formic acid at a

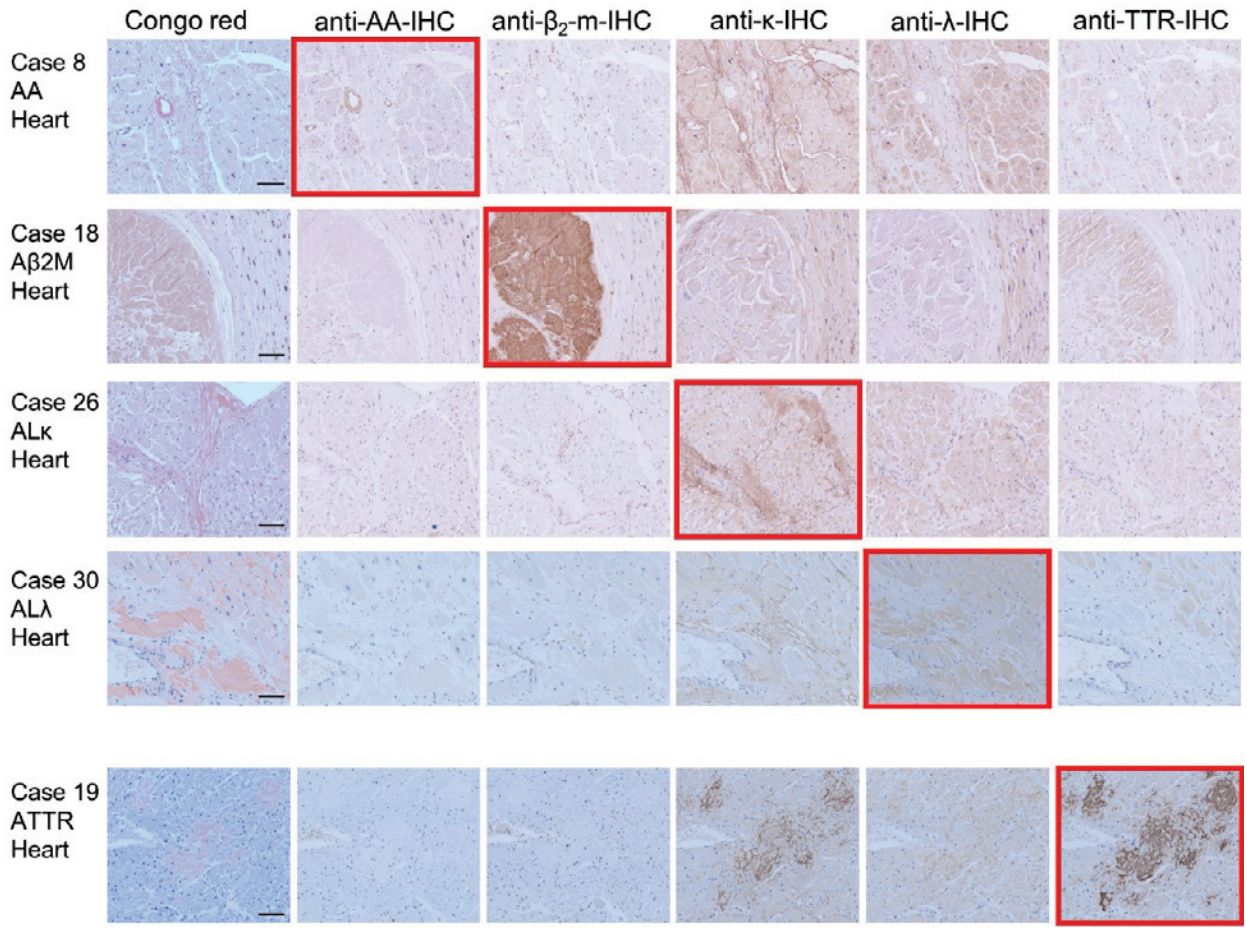

Fig. 1 Representative myocardial images stained with Congo red and anti-AA, anti–β_2-m, anti-κ, anti-λ, and anti-TTR antibodies. Amyloid subtypes were determined when Congo red–positive areas were strongly and diffusely positive (2+) for one antibody only and negative (0) for other antibodies. Note that in Case 19, proteomic analysis revealed that the subtype was ATTR (see Table 2), while the amyloid deposit was focally and weakly positive (1+) for anti-κ antibody, which we interpreted as nonspecific staining. Scale bar, 100 μm.

flow rate of 300 nL/min for 120 min. Eluted peptides were analyzed with a TripleTOF 5600+ mass spectrometer (AB Sciex). The 10 most intense peaks of each full MS scan were acquired in a data-dependent manner. All MS/MS spectral data were analyzed using the Mascot search engine v2.7.0 (Matrix Science, London, UK) against Swiss-Prot (2021_01). The search criteria were set as follows: peptide mass tolerance of ±50 ppm and fragment MS/MS tolerance of 0.05 Da; two missed cleavages were allowed. Search parameters included variable modifications containing methionine oxidation (N-terminus), Gln->pyro-Glu (N-term Q), and methyl (K). The search results were filtered at a false discovery rate of 1% using the decoy search option. The abundance of identified proteins is expressed as the exponentially modified protein abundance index (emPAI)[13,14], which was automatically calculated with the Mascot software. The most abundant amyloid protein detected in each sample was used to determine the amyloidosis subtype.

Results

IHC Subclassification of Systemic Amyloidosis

The patients comprised 20 men and 20 women with a mean age of 65.6 years (**Supplemental Table 1**). Amyloidosis was not the direct cause of death in ten cases. Representative myocardial images stained with Congo red and five different amyloid subtype-specific antibodies are shown in **Figure 1**. Amyloid subtypes were determined when Congo red-positive areas were strongly and diffusely positive (2+) for only one antibody and negative (0) for all other antibodies. In 30 cases (75%), we were able to determine the subtype by using this definition (**Table 1**). Common findings in all cases were that the background plasma components were weakly positive for anti-κ, the cytoplasm of cardiomyocytes was weakly positive for anti-λ, and plasma cells and lymphocytes were positive for anti-β_2-m.

Detection of Amyloid Subtypes by LMD-LC-MS/MS

We performed proteomic analysis of all 40 cases (**Table**

Table 1 Subtypes of 40 cases analyzed by IHC and LC-MS/MS

Case	Amyloid subtype determined by IHC*	IHC scale**					Amyloid subtype determined by LC-MS/MS***	Retrospective evaluation of IHC specimens based on LC-MS/MS data
		AA	β2-m	TTR	Igκ	Igλ		
1	AA	2	0	0	0	0	AA	
2	AA	2	0	0	0	0	AA	
3	AA	2	0	0	0	0	AA	
4	AA	2	0	0	0	0	AA	
5	AA	2	0	0	0	0	AA	
6	AA	2	0	0	0	0	AA	
7	AA	2	0	0	0	0	AA	
8	AA	2	0	0	0	0	AA	
9	AA	2	0	0	0	0	AA	
10	AA	2	0	0	0	0	AA	
11	AA	2	0	0	0	0	AA	
12	AA	2	0	0	0	0	AA	
13	AA	2	0	0	0	0	AA	
14	AA	2	0	0	0	0	AA	
15	AA	2	0	0	0	0	AA	
16	N.D.	1	1	0	0	0	AA	N.D.
17	Aβ2M	0	2	0	0	0	Aβ2M	
18	**N.D.**	**0**	**2**	**1**	**0**	**0**	**Aβ2M**	**Aβ2M**
19	**N.D.**	**0**	**0**	**2**	**1**	**0**	**ATTR**	**ATTR**
20	**N.D.**	**0**	**0**	**2**	**1**	**0**	**ATTR**	**ATTR**
21	**N.D.**	**0**	**0**	**2**	**1**	**1**	**ATTR**	**ATTR**
22	**N.D.**	**0**	**0**	**2**	**1**	**1**	**ATTR**	**ATTR**
23	ALκ	0	0	0	2	0	ALκ	
24	ALκ	0	0	0	2	0	ALκ	
25	ALκ	0	0	0	2	0	ALκ	
26	ALκ	0	0	0	2	0	ALκ	
27	N.D.	0	0	0	1	0	ALκ	N.D.
28	N.D.	0	0	0	1	0	ALκ	N.D.
29	ALλ	0	0	0	0	2	ALλ	
30	ALλ	0	0	0	0	2	ALλ	
31	ALλ	0	0	0	0	2	ALλ	
32	ALλ	0	0	0	0	2	ALλ	
33	ALλ	0	0	0	0	2	ALλ	
34	ALλ	0	0	0	0	2	ALλ	
35	ALλ	0	0	0	0	2	ALλ	
36	ALλ	0	0	0	0	2	ALλ	
37	ALλ	0	0	0	0	2	ALλ	
38	ALλ	0	0	0	0	2	ALλ	
39	**N.D.**	**0**	**0**	**1**	**0**	**2**	**ALλ**	**ALλ**
40	**N.D.**	**0**	**0**	**2**	**0**	**2**	**ATTR** **ALλ**	**ATTR** **ALλ**

*The amyloid subtype was determined when Congo red-positive area was positive only for one antibody (2+) and negative for other antibodies (0).

**0, negative; 1+, weakly and/or focally positive; 2+, strongly and diffusely positive

***The most abundant amyloid protein detected in each sample was determined to be the amyloidosis subtype. As for Case 40, see Table 2.

N.D.; not determined.

Note that in three cases (cases 16, 27, 28), LC-MS/MS data were essential to determine the subtype, while in other seven cases described in bald letters (cases 18-22, 39, 40), we could determine the subtype(s) by the retrospective evaluation of IHC specimens based on LC-MS/MS data (see Fig. 2 and Table 2).

Table 2 List of proteins detected by LMD-LC-MS/MS

Protein/ Cases	16	18	19	20	21	22	27	28	39	40 ATTR	40 ALλ
SAA2*	**1.15****	0	0	0	0	0	0	0	0	0	0
SAA1	**0.47**	0	0	0	0	0	0	0	0	0	0
B2MG	0	**20.69**	0	0	0	0	0	0	0	0	0
TTHY	0	1.42	**0.80**	**196.77**	**80.57**	**24.29**	0.8	0.4	0	**1.48**	0.35
IGKC	0.55	14.9	0	2.26	1.2	0.48	**14.73**	**1.47**	0	0	0.49
KVD13	0	0	0	0	0	0	0	**0.53**	0	0	0
KV401	0	0.42	0	0	0	0	**0.42**	0	0	0	0
KVD20	0	0	0	0.45	0.45	0	0	0	0	0	0
KVD26	0	0.42	0	0.42	0	0	0	0	0	0	0
IGLC2	0	4.16	0	2.41	2.41	0.5	0	0	**0.53**	0	**2.44**
LV223	0	0	0	0	0	0	0	0	**0.50**	0	0
LV301	0	0	0	0	0	0	0	0	0	0	**0.46**
SAMP	0.23	15.18	0.45	12.27	8.15	8.17	6.62	0.24	0.21	2.15	3.49
APOA4	0	15.80	0.52	5.49	6.97	6.99	12.43	0.44	0	0	0.24
APOA1	0.19	3.65	0	0	0	0.36	0	0.20	0	0	0
APOE	0	4.48	0.92	21.74	10.82	4.43	9.41	0	0	0	0.70
CLUS	0.11	0.72	0	0.88	1.05	0.43	0.88	0.37	0.10	0.21	0.31
VTNC	0.10	1.40	0.30	1.39	1.38	0.84	1.19	0.36	0.10	0.20	1.02

Numerical values indicate the exponentially modified protein abundance index (emPAI) assigned to each protein.
*Amyloid proteins are described in bold letters.
**The most abundant amyloid protein detected in each sample was determined to be the amyloidosis subtype, except for Case 40 (see ref. 15).
SAA, Serum Amyloid A; B2MG, Beta-2-microglobulin; TTHY, Transthyretin; IGKC, Immunoglobulin kappa constant; KVD13, Immunoglobulin kappa variable 1D-13; KV401, Immunoglobulin kappa variable 4-1; KVD20, Immunoglobulin kappa variable 3D-20; KVD26, Immunoglobulin kappa variable 2D-26; IGLC2, Immunoglobulin lambda constant 2; LV223, Immunoglobulin lambda variable 2-23; LV301, Immunoglobulin lambda variable 3-1; SAMP, Serum amyloid P-component; APOA4, Apolipoprotein A-IV; APOA1, Apolipoprotein A-I; APOE, Apolipoprotein E; CLUS, Clusterin; VTNC, Vitronectin.

1, 2). In 39 cases, we identified only one amyloid protein and confirmed the immunohistochemically determined subtypes of the abovementioned 30 cases.

In Case 30 (**Fig. 1, 2a**), positive reactivity for anti-λ antibodies was observed in regions surrounding blood vessels and cardiomyocytes showing Congo red staining. However, it is important to note that diffuse positivity for anti-κ antibodies, outlining the tissues, is a result of nonspecific binding, as indicated by proteomic analysis.

Notably, in Case 19 (**Fig. 1, 2b**) proteomic analysis revealed a subtype of ATTR (**Table 2**), while the amyloid deposit was focally and weakly positive (1+) for anti-κ antibody, which we interpreted as nonspecific staining. Other ATTR cases (Cases 20 to 22) were also focally and weakly positive (1+) for anti-κ and/or anti-λ antibodies.

In Case 18, the Congo red-positive region was diffusely positive (2+) for anti-β_2-m, while focally positive (1+) for anti-TTR (**Fig. 3a**). Proteomic analysis revealed a subtype of Aβ2M (**Table 2**). Thus, we interpreted focal positivity for anti-TTR as nonspecific staining.

In Case 39, the Congo red-positive region was diffusely positive (2+) for anti-λ and focally positive (1+) for anti-TTR (**Fig. 3b**). Proteomic analysis revealed a subtype of ALλ (**Table 2**). Thus, we interpreted focal positivity for anti-TTR as nonspecific staining.

Most interestingly, in Case 40, we identified double subtypes with both immunohistochemical and proteomic analysis (**Fig. 3c** and **Table 2**). The deposits in epicardial blood vessels were positive for anti-λ and negative for anti-TTR, while nodular deposits between cardiomyocytes were negative for anti-λ and positive for anti-TTR[15].

Collectively, in the abovementioned seven cases (Case 18 with Aβ2M, Cases 19 to 22 with ATTR, Case 39 with ALλ, and Case 40 with ATTR and ALλ), we could retrospectively determine subtypes with IHC by using information from proteomic analysis, which increased the diagnosis rate of IHC to 92.5% (37/40). In three cases (Cases 16, 27 and 28), proteomic analysis was essential for typing diagnosis (**Table 1, 2**).

Discussion

In the present study, to characterize the staining patterns

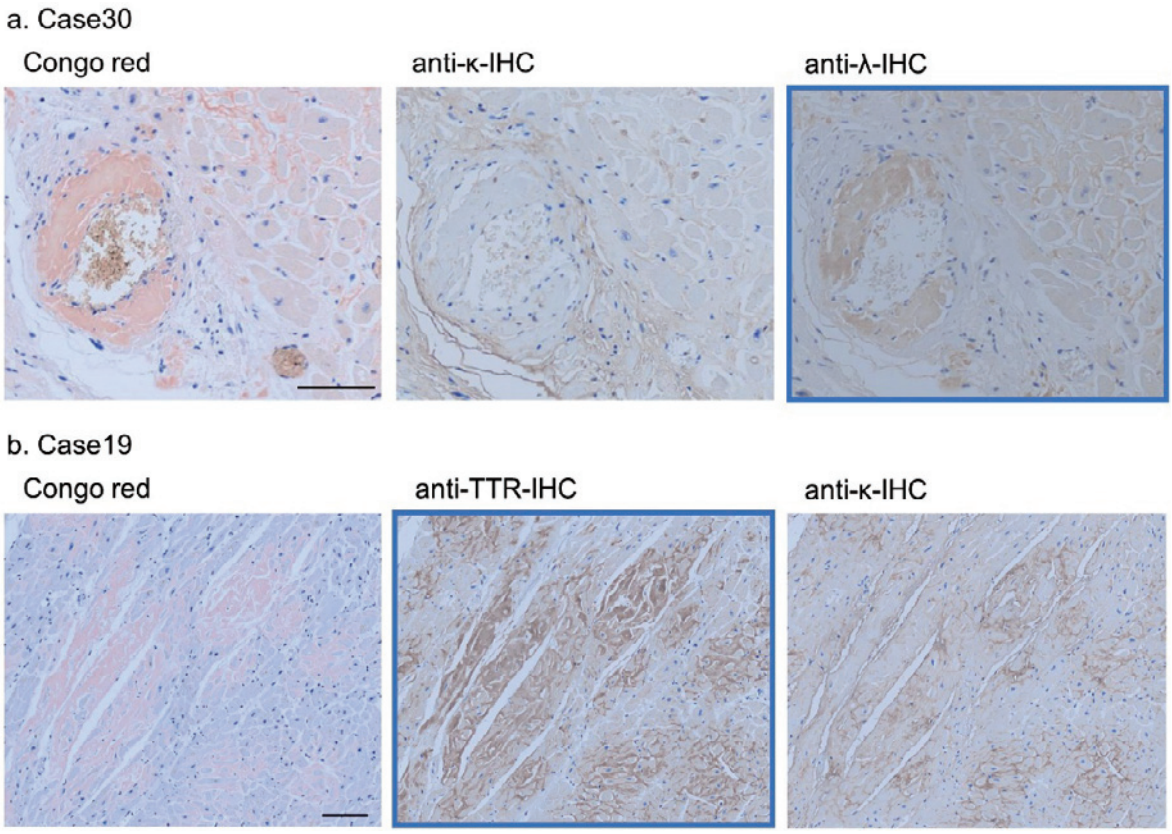

Fig. 2

a. Case 30. From left to right: Congo red staining, anti-κ-IHC, anti–λ-IHC.
Positive reactivity for anti-λ antibodies is observed in the regions surrounding blood vessels and cardiomyocytes that show Congo red staining. However, it is important to note that the diffuse positivity for anti-κ antibodies, outlining the tissues, is a result of nonspecific binding, as indicated by proteomic analysis. Scale bar, 100 μm.

b. Case 19. From left to right: Congo red staining, anti–TTR-IHC, anti–λ-IHC. The solid amyloid deposit appears to be diffusely stained by the anti-TTR antibody and only marginally by the anti-κ antibody, while both antibodies seem to positively stain the pericellular deposit area. Scale bar, 100 μm.

of in-house rabbit polyclonal anti-κ, anti-λ, and anti-transthyretin antibodies, and commercial anti-AA and anti-β_2-m antibodies, we analyzed the subtypes of 40 autopsied cases with IHC and further confirmed them by proteomic analysis. In seven cases, we could retrospectively determine subtypes with IHC by using information from proteomic analysis, which increased the IHC diagnosis rate from 75% (30/40) to 92.5% (37/40).

Jang et al.[16], Gilbertson et al.[8], and Kebbel et al.[17] reported IHC diagnosis rates of 65%, 76%, and 92%, respectively. Furthermore, in two studies using the same antibodies used in the present study the IHC diagnosis rate was approximately 95%[2,7]. These reports mainly used biopsy specimens for diagnosis, while our study utilized FFPE specimens from autopsy cases. Our findings indicate that the confirmed amyloid diagnosis rate was lower when using autopsy samples than in studies that mainly focused on biopsy specimens with the same antibodies. The amyloid diagnosis rate is clearly lower when using autopsy samples. Sato et al. reported that a maximum fixation time of 1 week can be used as a practical standard for IHC[18]. As autopsy specimens tend to have longer fixation times, they are likely to exhibit nonspecific positivity or false negativity for IHC antibodies, which may have influenced our results. Although we confirmed post-mortem intervals, it is uncertain whether these intervals affected our study results (**Supplemental Table 1**).

In two ALκ cases (Cases 27 and 28), the amyloid was weakly positive (1+) for anti-κ and negative for other antibodies. Proteomic analysis revealed a subtype of ALκ (**Table 2**). We recently reported that in some AL consultation cases, IHC could not detect the AL protein[19]. One explanation for this is the absence of most of the constant region of the κ/λ light chains, because our anti-κ/λ antibodies recognize the N-terminal 18/17 amino acids of the respective constant region[10]. Additionally, as shown in **Figure 2**, caution is warranted when considering the

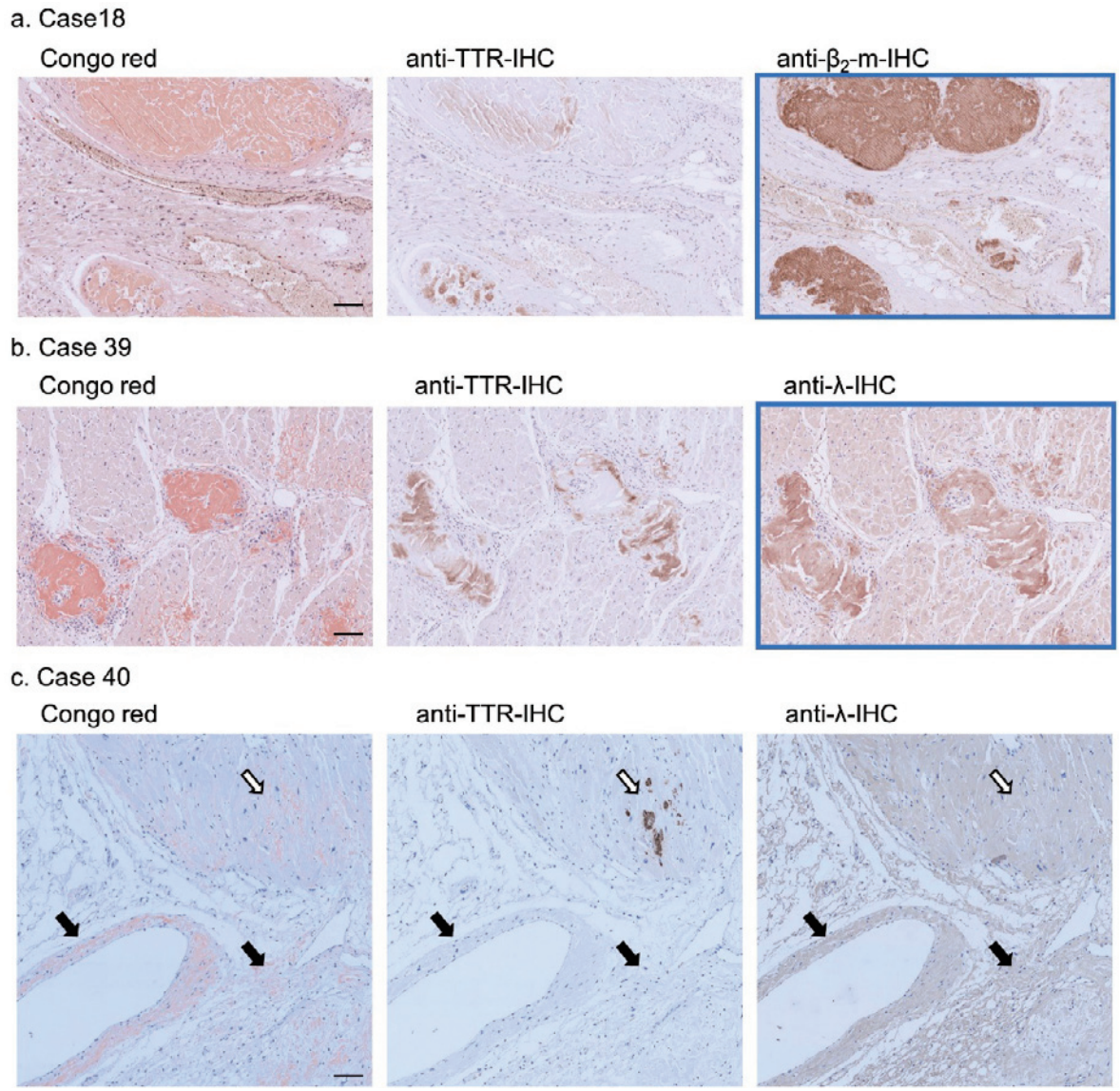

Fig. 3 Exceptional immunohistochemistry cases

a. Case 18. From left to right: Congo red staining, anti–TTR-IHC, anti–β_2-m-IHC. The Congo red-positive region was diffusely positive for anti–β_2-m (2+) and focally positive for anti-TTR (1+). Proteomic analysis revealed that the subtype was Aβ2M (see Table 2). Scale bar, 100 μm.

b. Case 39. From left to right: Congo red staining, anti–TTR-IHC, anti–λ-IHC. The Congo red–positive region was diffusely positive (2+) for anti-λ and focally positive (1+) for anti-TTR. Proteomic analysis revealed that the subtype was ALλ (see Table 2). Scale bar, 100 μm.

c. Case 40. From left to right: Congo red staining, anti–TTR-IHC, anti–λ-IHC. The deposits in epicardial blood vessels were positive for anti-λ and negative for anti-TTR (black arrows), while nodular deposits between cardiomyocytes were negative for anti-λ and positive for anti-TTR (white arrows). Scale bar, 100 μm.

staining characteristics of κ and λ, as κ and λ proteins are abundant in serum. In Case 16 (**Fig. 4a**), although amyloid deposit was weakly positive (1+) for both anti-AA and anti-β_2-m antibodies, proteomic analysis revealed a subtype of AA (**Table 2**). Hoshii et al. reported coexistence of AA and Aβ2M by IHC in two patients with rheumatoid arthritis who were receiving hemodialysis[20]. In Case 16, however, the patient had no history of dialysis for renal disease. Thus, we believe that positivity for β_2-m antibody was a false-positive result.

Limitations of this study include its single-center design and small sample size. However, our study has some diagnostic utility for objective interpretation of IHC staining with the five subtype-specific antibodies used in this study. First, ATTR amyloid deposits are strongly and diffusely positive (2+) for anti-TTR antibody but sometimes weakly and/or focally positive (1+) for anti-κ and/or anti-λ antibodies, which can be interpreted as a false

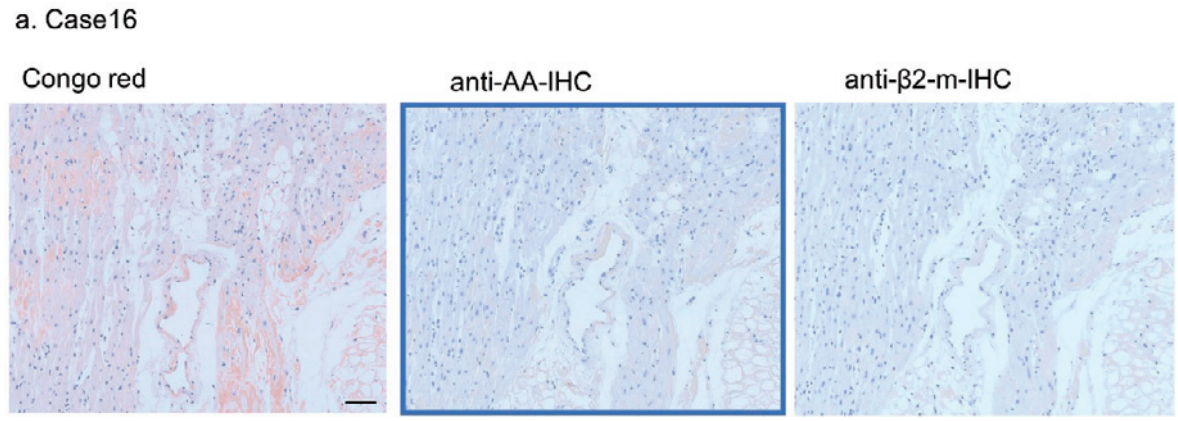

Fig. 4 A case that was indistinguishable by immunohistochemical staining
a. Case 16. Although the amyloid deposit was weakly positive (1+) for both anti-AA and anti–β_2-m antibodies, proteomic analysis revealed that the subtype was AA. We believe that the positivity for β_2-m antibody was a false-positive result. Scale bar, 100 μm.

positive. Second, if Congo red-positive regions are strongly and diffusely positive (2+) for anti-κ, anti-λ, or anti-β_2-m antibodies, weak and/or partial positivity for anti-TTR antibody can be interpreted as a false positive. Finally, if Congo red-positive regions are strongly and diffusely positive (2+) for two different antibodies (e.g., anti-TTR and anti-λ) and the positive regions for these antibodies are mutually exclusive, we can diagnose these cases as double amyloidosis.

In this study, we used the five antibodies employed by GSRA-J and revealed that the combination of IHC and proteomic analysis had greater utility than IHC alone. We have re-evaluated the staining properties using IHC, underpinned by proteomic analysis, and have provided detailed figures, including instances of false positives and false negatives. Especially noteworthy is the need for special attention in interpretating IHC for anti-TTR and light chain. Given these insights, we believe that the present findings are invaluable for researchers examining specimens stained by GSRA-J.

Data availability: The datasets used and analyzed in this study are available from the corresponding author on request.

Author contributions: Y.S-D. designed the study, collected and interpreted data, carried out experiments, and drafted the manuscript. T.F. prepared the slides and carried out IHC and other staining. K.I. carried out experiments and drafted the manuscript. R.O., H.N., and T.S. supplied resources and edited the manuscript.

Acknowledgements: This work was supported by The Japan Society for the Promotion of Science (Grant No. 20K07389) to Y.S-D. and the Grant for Surveys and Research of Amyloidosis from the Ministry of Health, Labour and Welfare, Japan to H.N. (2017-2022).

We are grateful to Fuyuki Kametani and Masayoshi Tasaki for their technical advice regarding LC-MS/MS.

Conflict of Interest: None declared.

References

1. Buxbaum JN, Dispenzieri A, Eisenberg DS, et al. Amyloid nomenclature 2022: update, novel proteins, and recommendations by the International Society of Amyloidosis (ISA) Nomenclature Committee. Amyloid. 2022;29:213–9.
2. Naiki H, Sekijima Y, Ueda M, et al. Human amyloidosis, still intractable but becoming curable: the essential role of pathological diagnosis in the selection of type-specific therapeutics. Pathol Int. 2020;70:191–8.
3. Bulawa CE, Connelly S, Devit M, et al. Tafamidis, a potent and selective transthyretin kinetic stabilizer that inhibits the amyloid cascade. Proc Natl Acad Sci U S A. 2012;109:9629–34.
4. Maurer MS, Schwartz JH, Gundapaneni B, et al. Tafamidis Treatment for patients with transthyretin amyloid cardiomyopathy. N Engl J Med. 2018;379:1007–16.
5. Kastritis E, Palladini G, Minnema MC, et al. Daratumumab-based treatment for immunoglobulin light-chain amyloidosis. N Engl J Med. 2021;385:46–58.
6. Kitaoka H, Izumi C, Izumiya Y, et al. JCS 2020 guideline on diagnosis and treatment of cardiac amyloidosis. Circ J. 2020;84:1610–71.
7. Abe R, Katoh N, Takahashi Y, et al. Distribution of amyloidosis subtypes based on tissue biopsy site - Consecutive analysis of 729 patients at a single amyloidosis center in Japan. Pathol Int. 2021;71:70–9.
8. Gilbertson JA, Theis JD, Vrana JA, et al. A comparison of immunohistochemistry and mass spectrometry for determining the amyloid fibril protein from formalin-fixed biopsy tissue. J Clin Pathol. 2015;68:314–7.
9. Ogawa M, Shintani-Domoto Y, Nagashima Y, et al. Mass spectrometry-based absolute quantification of amyloid proteins in pathology tissue specimens: merits and limita-

tions. PLoS One. 2020;15:e0235143.
10. Hoshii Y, Setoguchi M, Iwata T, et al. Useful polyclonal antibodies against synthetic peptides corresponding to immunoglobulin light chain constant region for immunohistochemical detection of immunoglobulin light chain amyloidosis. Pathol Int. 2001;51:264–70.
11. Gustavsson A, Engstrom U, Westermark P. Mechanisms of transthyretin amyloidogenesis. Antigenic mapping of transthyretin purified from plasma and amyloid fibrils and within in situ tissue localizations. Am J Pathol. 1994; 144:1301–11.
12. Theis JD, Dasari S, Vrana JA, Kurtin PJ, Dogan A. Shotgun-proteomics-based clinical testing for diagnosis and classification of amyloidosis. J Mass Spectrom. 2013; 48:1067–77.
13. Ishihama Y, Oda Y, Tabata T, et al. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics. 2005;4:1265–72.
14. Dowle AA, Wilson J, Thomas JR. Comparing the Diagnostic classification accuracy of iTRAQ, peak-area, spectral-counting, and emPAI methods for relative quantification in expression proteomics. J Proteome Res. 2016; 15:3550–62.
15. Shintani-Domoto Y, Ishino K, Naiki H, Sakatani T, Ohashi R. Autopsy case with concurrent transthyretin and immunoglobulin amyloidosis. Pathol Int. 2022;72:65–71.
16. Jang B, Koh Y, Seo JW. Immunohistochemical classification of amyloid deposits in surgical pathology. Basic Appl Pathol. 2009;2:1–8.
17. Kebbel A, Rocken C. Immunohistochemical classification of amyloid in surgical pathology revisited. Am J Surg Pathol. 2006;30:673–83.
18. Sato M, Kojima M, Nagatsuma AK, Nakamura Y, Saito N, Ochiai A. Optimal fixation for total preanalytic phase evaluation in pathology laboratories: a comprehensive study including immunohistochemistry, DNA, and mRNA assays. Pathol Int. 2014;64:209–16.
19. Naiki H, Yamaguchi A, Sekijima Y, et al. Steep increase in the number of transthyretin-positive cardiac biopsy cases in Japan: evidence obtained by the nation-wide pathology consultation for the typing diagnosis of amyloidosis. Amyloid. 2023;30:321–6.
20. Hoshii Y, Takahashi M, Ishihara T, et al. Immunohistochemical classification of 140 autopsy cases with systemic amyloidosis. Pathol Int. 1994;44(5):352–8.

(Received, August 2, 2023)
(Accepted, December 13, 2023)