Distribution of amyloidosis subtypes based on tissue biopsy site

– Consecutive analysis of 729 patients at a single amyloidosis center in Japan –

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Abbreviations:

AA amyloidosis, (apo) serum amyloid A amyloidosis; AANF amyloidosis, atrial natriuretic factor amyloidosis; AApoAI amyloidosis, apolipoprotein A I amyloidosis; Aβ2M amyloidosis, β2-microglobulin amyloidosis; ACys amyloidosis, cystatin C amyloidosis; AFib amyloidosis, fibrinogen α amyloidosis; AGel, gelsolin amyloidosis; AH amyloidosis, immunoglobulin heavy chain amyloidosis; AHL amyloidosis, immunoglobulin heavy chain and light chain amyloidosis; AL amyloidosis, immunoglobulin light chain amyloidosis; ATTRv amyloidosis, hereditary transthyretin amyloidosis; ATTRwt amyloidosis, wild-type transthyretin amyloidosis; GI, gastrointestinal; GSRA-J, group for surveys and research of amyloidosis in Japan; IHC, immunohistochemistry; LC-MS/MS, liquid-chromatography tandem mass spectrometry; LMD, laser microdissection

Abstract

This study was performed to elucidate the distribution of amyloidosis subtypes based on tissue biopsy site. Samples obtained from 729 consecutive patients with amyloidosis were analyzed by immunohistochemical staining (IHC) and supplemental mass spectrometry (MS). The correlations between the type of organs from which samples were obtained and amyloidosis subtypes were investigated retrospectively. Among the patients, 95.1% were diagnosed by IHC and 4.9 % were diagnosed by MS. The distribution of amyloidosis subtypes was as follows: AL, 59.1%; ATTR, 32.9%; AA, 4.0%; AH, 1.4%; Aβ2M, 0.8%; and others, 0.9%. AL was the most common subtype in most organs, including the liver, lung, kidney, lower urinary tract, bone marrow, gastrointestinal tract, and skin/subcutaneous tissue. ATTR was the most common subtype in the heart, carpal tunnel, and peripheral nerves. AH was the second most common subtype in renal biopsy. Three or more amyloidosis subtypes were detected in each organ. In conclusion, AL was the most common subtype in most biopsy sites except the heart, carpal tunnel, and peripheral nerve, in which ATTR was more common. Because several types of amyloidogenic protein were detected in each organ, amyloid typing must be pursued, no matter the site from where biopsy was obtained.

Key words: amyloidosis subtypes, biopsy, diagnosis, immunoglobulin heavy chain amyloidosis, immunoglobulin light chain amyloidosis, immunohistochemistry, mass spectrometry, transthyretin amyloidosis

Introduction

Amyloidoses are characterized by the presence of extracellular amyloid deposits, consisting of fibrillary aggregates of misfolded β-sheet proteins [1]. To date, 36 amyloid precursor proteins that can cause amyloidosis have been reported in humans [2], including immunoglobulin light chain, transthyretin (TTR), β2-microglobulin (β2M), and serum amyloid A (SAA). Although amyloidoses had been considered as incurable fatal diseases, several types have become treatable or controllable due to recent advances in therapeutic methodologies; for example, high-dose melphalan with stem cell transplantation [3] as well as newly emerging chemotherapeutic agents, including proteasome inhibitors [4, 5] and immunomodulatory drugs [6, 7, 8] for immunoglobulin light chain amyloidosis (AL amyloidosis), orthotopic liver transplantation [9, 10], TTR tetramer stabilizers [11, 12, 13], gene-silencing therapies for transthyretin amyloidosis (ATTR amyloidosis) [14, 15], and biologics, such as anti-TNF and anti-IL-6 drugs for rheumatic disease-induced serum amyloid A amyloidosis (AA amyloidosis) [16, 17, 18].

As disease modifying therapies for amyloidoses differ among subtypes, it is essential to determine the amyloidosis subtype to decide on an appropriate treatment regimen. Currently, two major modalities (i.e., immunohistochemical (IHC) analysis and mass spectrometric (MS) analysis) are utilized for amyloid typing. MS analysis can theoretically detect all kinds of amyloid proteins including rare and even novel proteins, although it requires special equipments, trained technicians, and considerable efforts. On the other hand, IHC analysis does not require special equipment or

skills, but detectable amyloid proteins are limited by a set of antibodies. In addition, commercially available immunostaining antibodies for immunoglobulin light chains and TTR do not have sufficient sensitivity or specificity [19], and therefore it is difficult to make an accurate diagnosis for two major subtypes of amyloidosis (i.e., AL and ATTR) immunohistologically in non-amyloidosis centers. In Japan, reliable antibodies for immunoglobulin light chains and TTR were developed by the group for surveys and research of amyloidosis in Japan (GSRA-J) founded by the Ministry of Health, Labour and Welfare, and GSRA-J started the nation-wide pathology consultation of amyloidosis in 2018 [20]. Our institute is a member of GSRA-J and has provided amyloidosis diagnostic support, including Congo red staining for detection of amyloid deposits and immunostaining for amyloid fibril subtype determination with or without supplemental mass spectrometry for many years. In this study, we retrospectively analyzed 729 consecutive amyloidosis cases diagnosed in our institute and elucidated the distribution of amyloidosis subtypes based on tissue biopsy site.

Materials and methods

Samples and clinical information collection

We examined samples sent to the Department of Medicine (Neurology and Rheumatology), Shinshu University, Nagano, using our diagnostic methods for amyloidosis [21]. Samples and associated clinical information were obtained from the attending physicians of patients diagnosed or

suspected to have amyloidosis. Clinical information included age, sex, medical history, and clinically affected organs of the patients. Clinically affected organs were determined by attending physicians according to their own clinical estimations but not to histological confirmation (Table 1). Samples included unstained paraffin-embedded slides, paraffin-embedded block tissue, and/or frozen tissue, which were obtained at biopsy or autopsy. Data on samples from 810 consecutive patients examined during the period from July 2008 to December 2018 were collected and analyzed retrospectively.

Congo red staining and immunostaining

Unstained paraffin-embedded slide samples were subjected to Congo red staining and immunostaining, as described previously [21]. Congo red staining-positive structures, which showed typical apple-green birefringence under polarized light, were histologically confirmed as amyloid deposits and then evaluated immunohistologically using anti-κ (116-133), anti-λ (118-134) [22], anti-AA (Dako M0759), and anti-TTR (115-124) [23] antibodies. Anti-β2M (5511; Nordic-MUbio, Susteren, The Netherlands) immunostaining was added for those with a history of dialysis and anti-amyloid β (Aβ) (1-40, 18580; IBL, Minneapolis, MN) immunostaining was added for brain samples. “Immunohistological” final diagnosis of amyloidosis-subtype was concluded if Congo red staining positive amyloid deposit showed specific positive reaction for only one type of our routine antibody (Figure 1).

Proteomics analysis with mass spectrometry

We utilized liquid-chromatography tandem mass spectrometry (LC-MS/MS) analysis for cases in which amyloid deposits were not significantly reactive with any of our antibodies or amyloid deposits showed positive reaction for two or more antibodies on routine IHC analysis. Amyloid proteins were extracted from frozen tissue samples and purified as described previously [24] before March 2015. After March 2015, a laser microdissection (LMD) system (LMD7000; Leica Microsystems Inc., Tokyo, Japan) became available to collect amyloid deposits directly from paraffin-embedded slides. Collected tissues were solubilized into the buffer and analyzed by LC-MS/MS (Nano LC DiNa; KYA Technologies Co., Tokyo, Japan and VelosPro; Thermo Fisher Scientific Inc., Waltham, MA) as reported previously [25, 26]. If an antibody against the candidate protein was available, IHC analysis was performed for confirmation.

Statistical analysis

We compared demographic and clinical data among patients with each amyloidosis subtype (i.e., AL, ATTR, AA, immunoglobulin heavy chain (AH), and Aβ2M amyloidosis). For statistical analysis, Fisher's exact test with Bonferroni correction was applied for binary outcomes and the Mann–Whitney U test with Bonferroni correction was applied for continuous variables.

Protocol approval and ethical concerns

All procedures in studies involving human participants were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. This study protocol was approved by the Ethics Committee of Shinshu University School of Medicine (No. 4225). The design of this study (an anonymized retrospective observational study without any medical intervention) did not necessarily require personal informed consent from the participants, but we provided the opportunity to decline enrollment in the study in an opt-out manner through the website of this study.

Results

Diagnosis of amyloidosis and its subtypes

Among 810 referred patients, 81 patients were histologically confirmed not to have amyloid deposition in the samples provided at our center. Among the 729 amyloid-positive patients, 689 patients were successfully diagnosed by IHC only. Among the 40 remaining patients, LC-MS/MS analysis was available in 36 patients and 34 patients were successfully diagnosed by LC-MS/MS (Table 2), so that final diagnosis remained to be undetermined in six patients (Table 1). All ATTR amyloidosis and all AA amyloidosis patients were diagnosed immunohistochemically, with the exception of one case of hereditary ATTR (ATTRv) amyloidosis. On the other hand, 12 of 431

(2.9%) AL amyloidosis patients were negative on IHC and LC-MS/MS analysis was required to obtain the final diagnosis. All patients with AH, gelsolin (AGel), cystatin C (ACys), apolipoprotein AI (AApoAI), fibrinogen α (AFib), and atrial natriuretic factor (AANF) amyloidosis were diagnosed by LC-MS/MS analysis. Five of six Aβ2M amyloidosis patients required LC-MS/MS analysis because their dialysis history was not clear in the information provided and they were negative on our routine immunostaining protocol, which does not include anti-Aβ2M staining. The remaining one patient had evident history of dialysis and was diagnosed by anti-Aβ2M immunostaining. Figure 1 shows panels of representative results of Congo red staining and immunohistochemical staining using anti-κ light chain, anti-λ light chain, anti-TTR, and anti-AA antibodies.

Demographic characteristics in each amyloidosis subtype

Summary of demographical and clinical information on each amyloidosis subtypes are shown in Table 1. The number of patients in each amyloidosis subtype group was as follows; 431 (59.1%) patients in AL amyloidosis, 240 (32.9%) patients in ATTR amyloidosis, 29 (4.0%) patients in AA amyloidosis, 10 (1.4%) patients in AH amyloidosis, 6 (0.8%) patients in Aβ2M amyloidosis, 2 (0.3%) patients in AGel amyloidosis, and each 1 (0.1%) patient in ACys, AApoAI, AFib, AANF, and Aβ amyloidosis. The average age of entire cohort was 69 ± 12 (mean ± SD). AL and ATTR amyloidoses were the two most dominant subtypes of amyloidosis and they constituted most of the

patients (671 of 729 patients, 92.0%) in this study.

The average age of five major amyloidoses, AL, ATTR, AA, AH and Aβ2M amyloidosis were 66, 75, 69, 70, and 64 years old, respectively. The highest mean age observed in ATTR amyloidosis was notable (Mann-Whitney *U* test; ATTR vs AL ($P < 0.0001$), AA ($P = 0.0009$), AH ($P = 0.0169$), or Aβ2M ($P = 0.0087$)). Moreover, mean age in wild-type ATTR (ATTRwt) amyloidosis (76) was significantly older as compared with ATTRv amyloidosis (66, $P < 0.0001$). With regard to the sex ratio, male dominant tendency was observed in the two most major subtypes (AL and ATTR) as well as in the entire cohort. Particularly, the tendency of male involvement in ATTR amyloidosis (male ratio, 81.9%) was remarkable (Fisher's exact test; ATTR vs AL ($P < 0.0001$), AA ($P < 0.0001$), AH ($P = 0.0091$), or Aβ2M ($P = 0.3060$)). On the other hand, characteristic female dominant tendency (female ratio, 82.1%) in AA amyloidosis was notable (Fisher's exact test; AA vs AL ($P < 0.0001$), ATTR ($P < 0.0001$), AH ($P = 0.1087$), or Aβ2M ($P = 0.0305$)).

Demographic characteristics were also compared between the patients in our prefecture (patients in our institution and those who were consulted from affiliated institutions in Nagano prefecture) and the patients consulted from outside our prefecture (Table 1). No significant difference was detected in the ratio of amyloidosis subtypes between the two groups except for AH amyloidosis (i.e., ratio of AH amyloidosis in our prefecture (3.5%) was significantly higher than that in the other prefectures (0.9%) ($P = 0.0285$)).

Amyloidosis subtypes according to organ sample site

Figure 2 shows the distributions of amyloidosis subtypes according to organ sample site. The number of autopsy cases (35 patients) was very small and most of the cases (694 of 729 patients, 95%) were diagnosed by biopsy. The gastrointestinal (GI) tract, heart, kidney, and skin/subcutaneous tissue were the most common sites for obtaining tissue samples to detect amyloid deposition. AL amyloidosis was the most common subtype in most organs, including the liver (92.8%), lung (86.9%), kidney (80.5%), lower urinary tract (78.5%), bone marrow (76.4%), GI tract (69.8%), and skin/subcutaneous tissue (54.3%). Among AL amyloidosis, κ-type was dominant in the liver and carpal tunnel, unlike other organs where λ-type was dominant. ATTR amyloidosis was the most common subtype in the heart (65.2%), carpal tunnel (88.2%), and peripheral nerves (54.6%). On the other hand, ATTR amyloidosis was very rare in renal (0.8%) and liver (0%) biopsy specimens. In renal biopsy, AH amyloidosis was the second most common subtype after AL amyloidosis, accounting for 7.8% of cases. The ratio of AH to AL was 1:10.2 in renal biopsy specimens, which was much higher than previously thought.

Three or more amyloidosis subtypes were detected in each organ as follows (Figure 2): 8 in the GI tract (i.e., ALκ, ALλ, ATTRwt, ATTRv, AA, AH, Aβ2M and ACys); 7 in the heart (i.e., ALκ, ALλ, ATTRwt, ATTRv, AA, Aβ2M and AANF), 7 in the kidney (i.e., ALκ, ALλ, ATTR, AA, AH, AGel, and AFib), 7 in the subcutaneous tissue (i.e., ALκ, ALλ, ATTRwt, ATTRv, AA, AH, and Aβ2M), 3 in the lung (i.e., ALκ, ALλ, and ATTRwt), 4 in the peripheral nerves (i.e., ALκ, ALλ,

ATTRv, and AH), 6 in the bone marrow (i.e., ALκ, ALλ, ATTRwt, AA, AH, and Aβ2M), 3 in the carpal tunnel (i.e., ALκ, ATTRwt, and ATTRv), 3 in the liver (i.e., ALκ, ALλ, and ApoAI), 3 in lower urinary tract (i.e., ALκ, ALλ, and ATTR) and 3 in the brain (i.e., ALκ, ATTRv, and Aβ).

Discussion

This is the first report describing the practical experience of providing amyloidosis diagnosis support in a large cohort at a single amyloidosis center in Japan. Firstly, it was noted that 10% of referred patients (81 out of 810 patients) were revealed to have no amyloid deposition. The reasons why these patients were considered to have amyloid deposition in primary institutions were assessable in 41 patients. Among them, Congo red staining evaluation with polarized view inspection was performed in only 6 patients. Eleven patients were evaluated by Congo red staining "without" polarized view inspection. In other 9 patients, Congo red staining was not performed and only DFS staining was applied. Therefore it is strongly recommended to use Congo red staining but not DFS staining to detect amyloid deposition and also to apply polarized view inspection to confirm Congo red staining positive materials to be "real" amyloid.

Among the amyloid positive referred cases, most of them were confirmed to consist of two types of amyloidosis, AL and ATTR (671 of 729 patients, 92.0%). This is attributed to the fact that these two subtypes are the most prevalent amyloidosis [27] and therefore primary institutes may encounter many patients with these subtypes. Another possible reason is the high demand for

accurate diagnosis of AL and ATTR amyloidosis, as both have subtype-dependent disease modifying therapies and accurate diagnosis by IHC analysis is difficult outside of the tertiary-referral center setting. On the other hand, the numbers of patients with AA, Aβ2M, and Aβ amyloidosis were relatively small in this study, although much larger number of patients is epidemiologically estimated. This finding may be attributed to the fact that other types of amyloidosis are not as commonly recognized or considered in the patients who developed amyloidosis during the long history of rheumatic disease (AA) or dialysis (Aβ2M). In addition, clinical diagnosis of Alzheimer's disease (Aβ amyloidosis) does not include histopathological confirmation of amyloid deposition due to considerably invasive aspect in brain biopsy. Lack of effective disease modifying therapies for Aβ2M and Aβ amyloidosis might be another reason of weak demand of diagnosis in primary institutes. Furthermore, reliable commercial antibodies are available in AA, Aβ2M, and Aβ amyloid, which makes possible proper diagnosis in the primary institutes. In addition to aforementioned patient selection bias, our study was limited by biopsy site selection bias. For example, the heart is commonly involved in AL amyloidosis, however, most AL patients are diagnosed by non-cardiac biopsies such as renal, GI tract, or abdominal fat biopsy. On the other hand, endomyocardial biopsy is necessary to demonstrate amyloid deposition in many ATTRwt patients, as positive rates of other organs' biopsy are relatively low [27, 28]. As described above, proportion of amyloidosis subtypes in this study was influenced by biases and does not represent the accurate epidemiological population of each amyloidosis subtypes in the real world.

Despite these limitations, our study showed intriguing biopsy site-dependent amyloid subtype distributions. In the clinical practice, biopsy site is selected based on several factors, including clinically affected organs, accessibility, and invasiveness. Therefore, we consider that our study elucidated the amyloid subtype distributions of each biopsy site in the actual clinical practice. In our study, AL amyloidosis was found in all organs (Figure 2) suggesting that the possibility of AL must be considered when inspecting all types of amyloid-containing samples. AL amyloidosis was the most common subtype in most organs, including the liver, lung, kidney, lower urinary tract, bone marrow, GI tract, and skin/subcutaneous tissue. Especially, liver biopsy samples were positive for AL amyloidosis in all except one case with hereditary AApoAI amyloidosis. It is noteworthy that κ-type dominance was observed in the liver [29] and carpal tunnel in contrast to the λ-type dominance in other organs (Figure 2), although the mechanism underlying this finding is unclear. ATTR amyloidosis was the most common subtype in the cardiac, carpal tunnel, and peripheral nerve biopsy specimens (Figure 2). In particular, most carpal tunnel samples were positive for ATTR (88.2%), largely due to the high frequency of carpal tunnel syndrome in ATTRwt amyloidosis [21, 30].

Another important finding of the study was that a considerable number of patients with AH amyloidosis were diagnosed at our amyloidosis center. Because routine IHC analysis and fluorescent antibody analysis cannot detect AH amyloid deposition, AH amyloidosis has been considered very rare subtype of amyloidosis with only 19 AH and 11 immunoglobulin heavy chain

and light chain (AHL) amyloidoses cases reported previously [31 – 44]. However, we found 10 cases of AH amyloidosis in this study (four had been reported previously [37 – 39, 44] and six were unpublished), and they accounted for 2.3% of immunoglobulin-related amyloidosis cases (10 of 441, 431 AL and 10 AH amyloidosis), representing a much higher incidence than previously thought. Intriguingly, ratio of AH/AHL to AL in renal biopsy in our study (10:103, 9.7%) is almost identical to that in the Mayo Clinic (16:222, 7.2%) [42]. In our study, AH amyloidosis represented the fourth largest subtype group in the total cohort after AL, ATTR, and AA amyloidosis (Table 1) and also the second largest number of cases in renal samples (Figure 2). Despite unfavorable renal prognosis, life prognosis of AH amyloidosis is not as bad as AL amyloidosis because life-threatening cardiac and hepatic involvements are rare in AH amyloidosis [31- 44]. Therefore, the accurate diagnosis of AH amyloidosis can help the attending physicians to make a better treatment plan using chemotherapy with optimal intensity. Super dominant renal involvement and in contrast rare visceral organ involvement other than the kidney were demonstrated in this study (Table 1). Furthermore, significantly low ratio of AH amyloidosis patients outside our prefecture suggested the possibility that many AH amyloidosis patients might have been overlooked in primary institutions. Therefore, it is very important to pay attention to the possibility of underdiagnosing AH amyloidosis in patients with a clinical diagnosis of renal AL amyloidosis without proper immunohistopathological or mass spectrometric confirmation.

Lastly, it should be noted that 3 or more amyloidosis subtypes were detected in each biopsy

sites (Figure 2). Organs historically believed to be involved only in AL amyloidosis, such as the liver, bone marrow, and lower urinary tract, were shown to occasionally be involved in other subtypes. Our study indicats that clinicians and pathologists should consider the systemic deposition of various amyloidosis subtypes more broadly, and amyloid typing must be pursued, no matter the site from where biopsy was obtained.

Study limitations

Our study is limited by patient selection bias and biopsy site selection bias as described above, and therefore proportion of amyloidosis subtypes in this study does not represent the accurate epidemiological population of each amyloidosis subtype. Another limitation that might have affected the results of the study is methods of amyloid typing. In this study, we mainly employed the IHC analysis, and LC-MS/MS analysis was utilized only for cases in which amyloid deposits were not significantly reactive with any of our antibodies on routine IHC analysis. Although we used reliable antibodies [20, 22, 23] and made considerable effort to exclude misinterpreting, methodological limitation of IHC (i.e., over- or under-staining and false-positive or -negative results) could not be completely avoided.

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Disclosure

No potential conflict of interest was reported by the authors.

Author Contributions

RA and NK analysed the data and wrote the manuscript. RA, NK, YT, KT and TY performed immunohistochemical analysis. TY, MY and FK performed mass spectrometric analysis. YS contributed to study design and critical editing and revising of the manuscript.

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Tables

Table 1. Amyloidosis subtypes and clinical information of enrolled patients

	Number (%)	The number of patients in Nagano (%)	The number of patients consulted from outside Nagano (%)	The number of cases in which LC-MS/MS analysis was performed	Age at diagnosis (mean ± SD, years old)	Sex ratio (male %)	Clinically affected organs: %
Total	729	142	587	36	68.6 ± 12.0	65.7	heart 46.6, kidney 28, GI tract 19.5, bone marrow 14.8, nerve 13, carpal tunnel 10.2, liver 4.9, tongue 3.6, lung 3.4, lymph node 1.8, lower urinary tract 1.6, thyroid gland 1.4
AL total	431 (59.1)	87 (61.3)	344 (58.6)	12	65.6 ± 12.2	60.8	heart 38.1, kidney 36.2, GI tract 24.8, bone marrow 21.8, nerve 11.1, liver 7, tongue 5.1, lung 4.9, carpal tunnel 4.2, lymph node 2.6, lower urinary tract 2.1
ALκ	123 (16.9)	22 (15.5)	101 (17.2)	10	66.2 ± 12.3	61.5	heart 35.8, kidney 35.8, GI tract 25.2, bone marrow 25.2, liver 18.7, nerve 12.2, tongue 8.1, carpal tunnel 6.5, lung 4.9, lymph node 2.4, thyroid gland 2.4
ALλ	306 (42.0)	64 (45.1)	242 (41.2)	2	65.4 ± 12.2	60.9	heart 39.2, kidney 36.6, GI tract 24.8, bone marrow 20.6, nerve 10.8, lung 4.6, tongue 3.9, carpal tunnel 3.3, lymph node 2.6, muscle 2.6, liver 2.3, lower urinary tract 2.3
AL unknown	2 (0.3)	1 (0.7)	1 (0.2)	0	65 ± 15.6	0	lung 50, lacrimal gland 50, salivary gland 50, lip 50, skin 50
ATTR total	240 (32.9)	39 (27.5)	201 (34.2)	1	74.7 ± 9.0	81.9	heart 67.9, carpal tunnel 21.7, nerve 18.3, GI tract 8.8, kidney 7.9, bone marrow 2.5, liver 2.1, eye 1.7, tongue 1.3, lymph node 0.8, lower urinary tract 0.8, thyroid gland 0.8
ATTRwt	106 (14.5)	23 (16.2)	83 (14.1)	0	76.1 ± 6.5	93.3	heart 67.9, carpal tunnel 34.9, nerve 14.2, kidney 6.6, GI tract 3.8, bone marrow 2.8, liver 0.9, eye 0.9, lymph node 0.9
ATTRv	44 (6.0)	8 (5.6)	36 (6.1)	1	66.4 ± 12.4	65.9	heart 61.4, nerve 54.6, carpal tunnel 18.2, GI tract 15.9, eye 6.8, tongue 6.8, kidney 4.5, liver 4.5, thyroid gland 2.3, lymph node 2.3
ATTR unknown	90 (12.3)	8 (5.6)	82 (14.0)	0	76.9 ± 7.4	76.4	heart 71.1, GI tract 11.1, kidney 11.1, carpal tunnel 7.8, nerve 5.5, bone marrow 3.3, liver 2.2, lower urinary tract 2.2, lung 1.1, thyroid gland 1.1
AA	29 (4)	7 (4.9)	22 (3.7)	0	68.6 ± 9.4	17.9	kidney 41.4, joint 34.5, GI tract 34.5, heart 24.1, lung 10.3, bone marrow 6.9, lower urinary tract 3.4, bronchi 3.4, nerve 3.4, carpal tunnel 3.4, thyroid gland 3.4
AH	10 (1.4)	5 (3.5)	5 (0.9)	10	69.8 ± 8.5	50	kidney 100, bone marrow 60, heart 10, GI tract 10, nerve 10, carpal tunnel 10, eye 10
Aβ2M	6 (0.8)	1 (0.7)	5 (0.9)	5	63.7 ± 12.2	66.6	kidney 33.3, carpal tunnel 33.3, heart 16.7, nerve 16.7, muscle 16.7, skin/subcutaneous tissue 16.7, tongue 16.7, salivary gland 16.7
AGel	2 (0.3)	0 (0.0)	2 (0.3)	2	45.5 ± 9.2	0	kidney 100
ACys	1 (0.1)	0 (0.0)	1 (0.2)	1	62	100	GI tract 100
AApoAI	1 (0.1)	0 (0.0)	1 (0.2)	1	42	100	kidney 100, liver 100, testis 100
AFib	1 (0.1)	0 (0.0)	1 (0.2)	1	40	0	heart 100, kidney 100
AANF	1 (0.1)	0 (0.0)	1 (0.2)	1	NA	NA	heart 100
Aβ	1 (0.1)	0 (0.0)	1 (0.2)	0	81	100	brain 100
Undetermined	6 (0.8)	3 (2.1)	3 (0.5)	2	60.4 ± 13.0	33.3	heart 33.3, GI tract 33.3, kidney 16.7, skin/subcutaneous tissue 16.7

AL: immunoglobulin light chain amyloidosis, ATTR: transthyretin amyloidosis, wt: wild type, v: variant, AA: amyloid A amyloidosis,
AH: immunoglobulin heavy chain amyloidosis, Aβ2M: β2-microglobulin amyloidosis, AGel: gelsolin amyloidosis,
ACys: cystatin C amyloidosis, AApoAI: apolipoprotein A I amyloidosis, AFib: fibrinogen α amyloidosis,
AANF: atrial natriuretic factor amyloidosis, Aβ: Aβ amyloidosis, LC-MS/MS: liquid-chromatography tandem mass spectrometry,
GI: gastrointestinal, NA: data not available

Table 2. Results of IHC and LC-MS/MS analyses in whom LC-MS/MS was performed

			Immunohistochemical reaction for our routine antibodies				LC-MS/MS
Patient	Age	Sex	anti-κ	anti-λ	anti-TTR	anti-AA	results
1	43	F	−	−	−	−	ALκ
2	70	F	−	−	−	−	AH
3	78	M	−	−	−	−	AH
4	40	F	−	−	−	−	AFib
5	73	F	−	−	−	−	Aβ2M
6	NA	NA	−	−	−	−	ALκ
7	41	M	−	−	−	−	Aβ2M
8	75	M	−	−	−	−	ALκ
9	67	F	−	−	−	−	ALκ
10	15	M	−	−	−	−	ALκ
11	59	M	−	−	−	−	Aβ2M
12	70	M	−	−	−	−	Aβ2M
13	41	F	−	−	−	−	undetermined
14	71	M	−	−	−	−	ALκ
15	39	F	−	−	−	−	AGel
16	62	M	−	−	−	−	ACys
17	42	M	−	−	−	−	AApoAI
18	69	M	±	−	−	−	ALκ
19	NA	NA	−	−	−	−	AANF
20	87	F	−	−	−	−	AH
21	72	M	−	−	−	−	Aβ2M
22	73	M	−	±	−	−	ALλ
23	71	M	−	−	−	−	ATTR V121A
24	NA	M	±	−	−	−	ALκ
25	70	M	+	+	−	−	ALλ
26	74	M	−	−	−	−	ALκ
27	NA	M	−	−	−	−	ALκ
28	62	F	−	−	−	−	AH
29	54	M	−	−	−	−	undetermined
30	52	F	−	−	−	−	AGel
31	71	F	−	−	−	−	AH
32	61	F	−	−	−	−	AH
33	67	M	−	−	−	−	AH
34	76	M	−	−	−	−	AH
35	61	M	−	−	−	−	AH
36	65	M	−	−	−	−	AH

IHC: immunohistochemistry, LC-MS/MS: liquid-chromatography tandem mass spectrometry,
AL: immunoglobulin light chain amyloidosis, AH: immunoglobulin heavy chain amyloidosis,
AFib: fibrinogen α amyloidosis, Aβ2M: β2-microglobulin amyloidosis, AGel: gelsolin amyloidosis,
ACys: cystatin C amyloidosis, AApoAI: apolipoprotein A I amyloidosis,
AANF: atrial natriuretic factor amyloidosis, ATTR: transthyretin amyloidosis, NA: data not available

Figure legends

Figure 1. Representative pictures of routine histopathological analysis

Sample organ types of pictures are as follows: A1 – 6, E1 – 6: kidney; B1 – 6, C1 – 6: heart; D1 – 6: digestive tract. Bars = 100 μm in A; 200 μm in B, C, D, and E. Areas positive on Congo red staining in each organ sample showed specific subtype-dependent positive reaction to immunostaining using anti-κ (A3), anti-λ (B4), anti-TTR (C5), and anti-AA (D6) antibodies. A representative case with negative reaction to all routine immunostaining (E1 – 6) was eventually confirmed to be AH amyloidosis by LC-MS/MS.

Figure 2. Number of samples and distribution of amyloidosis subtypes according to organ

ALκ, κ-type immunoglobulin light chain amyloidosis; ALλ, λ-type immunoglobulin light chain amyloidosis; ATTRwt, wild-type transthyretin amyloidosis; ATTRv, hereditary transthyretin amyloidosis; ATTR unknown, genotype-unknown ATTR amyloidosis; AA, AA amyloidosis; AH, immunoglobulin heavy chain amyloidosis; others, other types of amyloidosis.

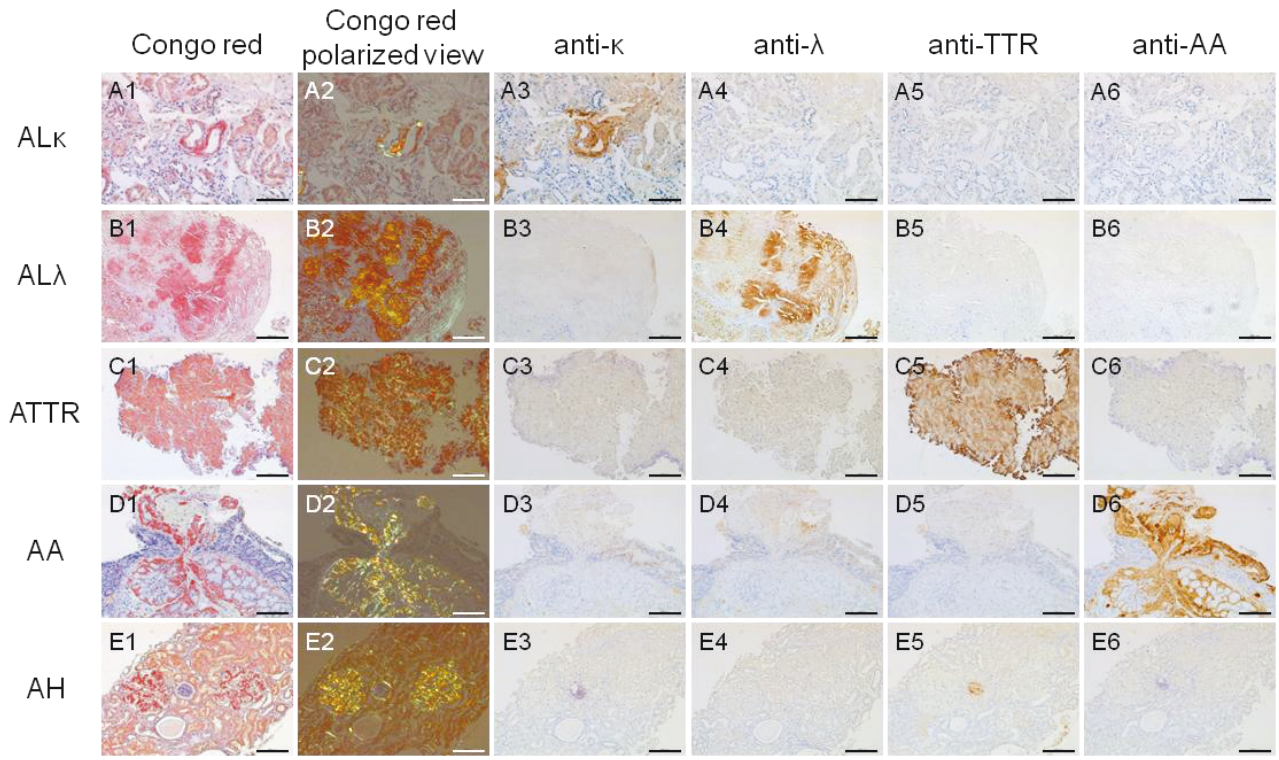
Congo red
Congo red polarized view
anti-κ
anti-λ
anti-TTR
anti-AA
ALκ
ALλ
ATTR
AA
AH
A1
A2
A3
A4
A5
A6
B1
B2
B3
B4
B5
B6
C1
C2
C3
C4
C5
C6
D1
D2
D3
D4
D5
D6
E1
E2
E3
E4
E5
E6

Figure 1

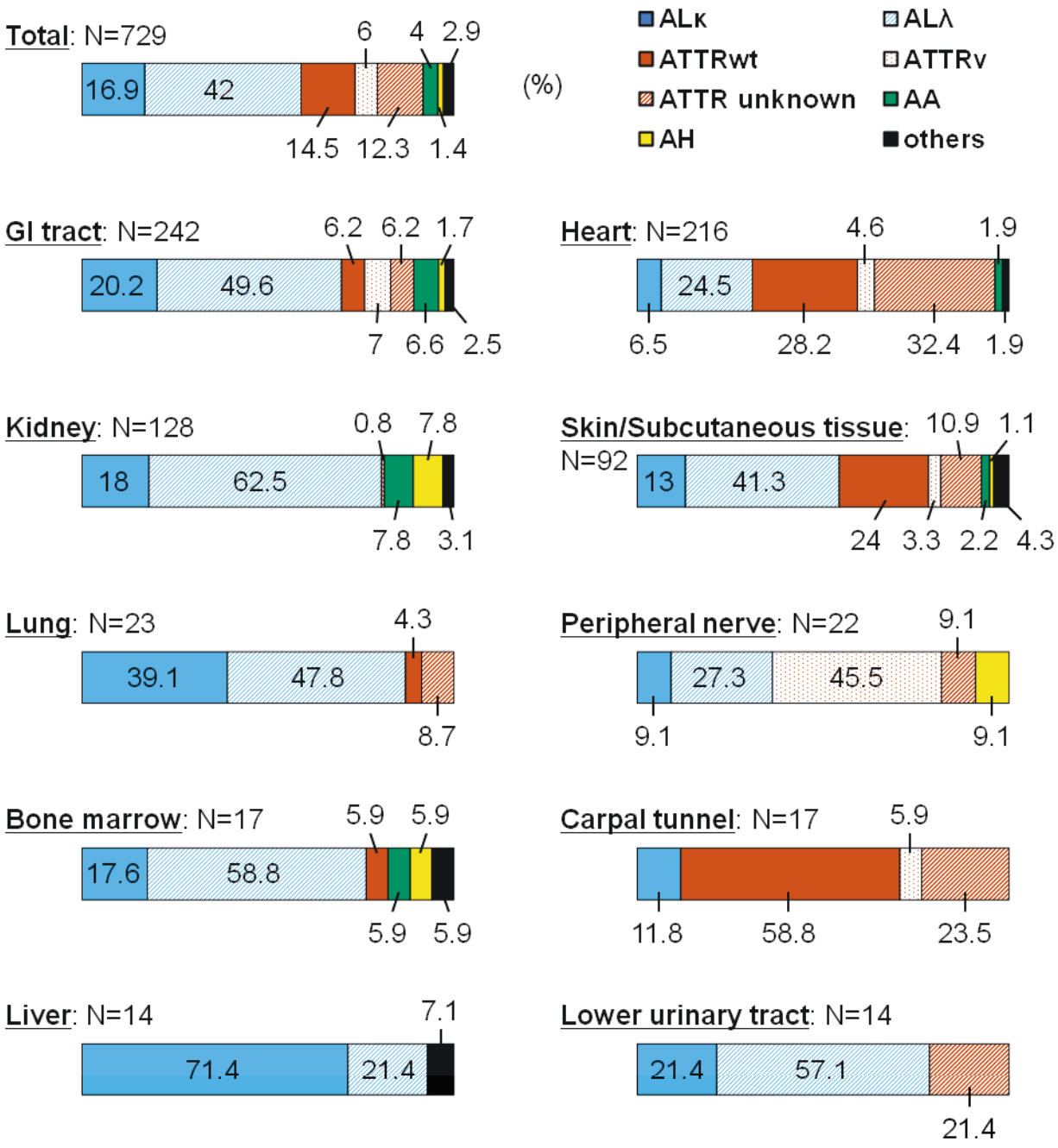
Total: N=729
16.9
42
14.5
12.3
1.4
6
4
2.9
(%)
ALκ
ALλ
ATTRwt
ATTRv
ATTR unknown
AA
AH
others
GI tract: N=242
20.2
49.6
7
6.6
2.5
6.2
6.2
1.7
Heart: N=216
6.5
24.5
28.2
32.4
1.9
4.6
1.9
Kidney: N=128
18
62.5
7.8
3.1
0.8
7.8
Skin/Subcutaneous tissue: N=92
13
41.3
24
3.3
2.2
4.3
10.9
1.1
Lung: N=23
39.1
47.8
8.7
4.3
Peripheral nerve: N=22
9.1
27.3
45.5
9.1
9.1
Bone marrow: N=17
17.6
58.8
5.9
5.9
5.9
5.9
Carpal tunnel: N=17
11.8
58.8
23.5
5.9
Liver: N=14
71.4
21.4
7.1
Lower urinary tract: N=14
21.4
57.1
21.4

Figure 2