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Evaluation of laboratory diagnostic tests for light-chain clonality and bone marrow findings in AL amyloidosis

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Background
Light-chain amyloidosis (AL) is the most common form of systemic amyloidosis. This study aimed to evaluate the usefulness of laboratory tests for light-chain clonality and bone marrow (BM) findings in AL amyloidosis.

Methods
We retrospectively enrolled patients newly diagnosed with AL amyloidosis on pathological examination who underwent a BM biopsy. Laboratory test data for light-chain clonality were collected and compared. Amyloid deposits were identified with H&E, Congo red, and PAS stains.

Results
We reviewed 98 patients with AL amyloidosis. Light chain clonality (λ, 64 cases; κ, 34 cases) was detected by serum immunofixation electrophoresis (IFE) (63.3%), urine IFE (70.8%), serum protein electrophoresis (PEP) (44.9%), urine PEP (44.8%), serum free light chain (SFLC) ratio (79.5%), and BM immunohistochemistry (IHC) (85.7%). Flow cytometric (FCM) assay identified aberrant BM plasma cells in 92.9% of cases. BM amyloid deposits were identified in 35 of the 98 cases (35.7%); 71.4% (25/35) were Congo red-positive, and 100.0% (35/35) were PAS-positive.

Conclusion
Laboratory tests for detecting light-chain clonality in AL amyloidosis in order of sensitivity include FCM assay for aberrant plasma cells, IHC for light chains on BM biopsy or clot section, SFLC ratio, and serum and urine IFE. Congo red staining of BM samples remains an important tool for identifying amyloid deposits in BM. Periodic acid-Schiff (PAS) staining can be useful in diagnosing some cases of Congo red-negative amyloidosis.

Key Words Light-chain (AL) amyloidosis, Monoclonality, Bone marrow findings, Serum free light chain ratio, Immunohistochemistry, Flow cytometry

INTRODUCTION

Light-chain (AL) amyloidosis is caused by the secretion of intact or fragmented abnormal immunoglobulin light chains by monoclonal plasma cells. In AL amyloidosis, light chains produced by monoclonal plasma cells are deposited in various organs and tissues, including the heart, kidneys, gastrointestinal tract, peripheral or autonomic nervous system, and bone marrow (BM), and form beta-pleated sheets [1, 2]. AL amyloidosis is the most common form of systemic amyloidosis [1], with a reported incidence of one case per 100,000 people [3]. AL amyloidosis should be ruled out in patients with monoclonal gammopathy, symptoms of autonomic or sensory neuropathy, unexplained fatigue, or edema [2]. AL amyloidosis should also be suspected in patients with non-diabetic nephrotic syndrome, non-ischemic cardiomyopathy, or hepatomegaly [4].

Confirmation of amyloidosis requires a tissue biopsy to identify amyloid deposits [2], and the diagnostic biopsy site for AL amyloidosis is typically the abdominal subcutaneous fat pad or BM [1]. Even when both the BM and fat pad

are negative, there remains a 15% chance that amyloidosis will be detected on a biopsy of the involved organ [2]. Once a histological diagnosis of amyloidosis is made, subsequent typing for AL amyloidosis depends on the identification of monoclonal light chains using serum and/or urine immunofixation electrophoresis (IFE) [3]. The evaluation of AL amyloidosis includes identifying the underlying clonal plasma cell disorder by BM aspirate and biopsy evaluation and laboratory tests, including serum and urine protein electrophoresis (PEP), IFE, and serum free light chain (SFLC) ratio [1, 2]. Flow cytometric evaluation of plasma cells in BM aspirates is also useful. This study aimed to evaluate the usefulness of laboratory tests for light-chain clonality and bone marrow (BM) findings in AL amyloidosis.

MATERIALS AND METHODS

We retrospectively enrolled patients with newly diagnosed amyloidosis by pathologic examination between 2005 and 2015 who underwent a BM study based on clinical signs of suspected AL amyloidosis. During the 11-year study period, 108 patients were diagnosed with amyloidosis by pathological examination and underwent BM biopsy. Two patients were found to have inflammatory amyloidosis (AA), five showed no monoclonality, and three were excluded because of insufficient slides for evaluation. The remaining 98 patients with AL amyloidosis were reviewed.

This study was approved by the Institutional Review Board (IRB) of Asan Medical Center (IRB No. 2017-0423) and was performed in accordance with the Declaration of Helsinki.

To evaluate AL amyloidosis, laboratory tests used to detect light-chain clonality, including serum and urine PEP, IFE, SFLC ratio, flow cytometric (FCM) assay of BM aspirate, and immunohistochemistry (IHC) of BM biopsy or clot sections, were reviewed. PEP of serum and urine samples was performed using an epalyzer (Helena Laboratories, Beaumont, TX, USA). The IFE of the serum and urine samples was tested using HYDRASYS 2 SCAN (Sebia, Evry, France). The SFLC ratios were determined using a SPAPLUS (Binding Site, Birmingham, United Kingdom) device with a Freelite kit (Binding Site).

Wright-Giemsa-stained BM aspirate smears and hematoxylin- and eosin-stained BM biopsy or clot sections were reviewed. Congo red and periodic acid-Schiff (PAS) stainings were performed on BM biopsies or clot sections. Amyloid deposits were identified based on Congo red and PAS stainings. Amyloid deposits were defined as amorphous, waxy-appearing, pink material on H&E staining with 1) apple-green birefringence by polarized light microscopy in Congo red staining, 2) pink-to-red color under a transmitted-light microscope on Congo red staining, and 3) PAS-positive staining. Amyloid deposits were graded as 1+, blood vessels only; 2+, interstitial deposition of $\leq$2 high-power fields (HPF); and 3+, interstitial deposition of $>$2 HPF.

IHC of CD138, κ, and λ were also performed on BM biopsies or clot sections. Mouse monoclonal anti-CD138 antibodies (DAKO, Carpinteria, CA, USA) and rabbit polyclonal anti-immunoglobulin light-chain antibodies (DAKO) were used as primary antibodies. An OptiView DAB IHC Detection Kit (Roche, Basel, Switzerland) was used with hematoxylin for counterstaining. Plasma cell monoclonality was defined as a κ/λ ratio of more than 4 (κ clone) or less than 0.5 (λ clone) [5].

The FCM assay of BM plasma cells was performed using a flow cytometer (FACSCANTO II; BD Biosciences, San Jose, CA, USA) with a panel of CD56/CD19/CD45/CD38/CD138 monoclonal antibodies purchased from BD Biosciences. A total of 500,000 nucleated BM cells were acquired by the flow cytometer, and plasma cells positive for CD38 and CD138 were gated. Plasma cells showing aberrant expression of CD19 (dim or negative), CD45 (dim or negative), and/or CD56 (positive) were measured as percentages of the total plasma cells.

For statistical analysis, the Chi-squared test or Fisher's exact test was used for comparison of categorical data, and the Mann-Whitney U test was used for comparison of continuous data. Statistical significance was set at $P<0.05$. All statistical analyses were performed using SPSS, v.18.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Age, sex, and affected organs

The median age of patients with amyloidosis was 62 years (range, 40–82 yr); 56 were men and 42 were women. The organs affected by AL amyloidosis were the kidney (26), heart (24), gastrointestinal tract (17), liver (4), skin (4), lung (3), soft tissue (4), and others (5; bladder, prostate, paraspinal mass, rib, and tongue). Multiple organs $\geq$2 were affected in 11 cases.

Laboratory tests for light chain clonalities

Monoclonality (λ and κ) was identified by IHC on BM biopsies or clot sections or by serum and urine IFE and SFLC ratio; 65.3% of the patients (64/98) had lambda light-chain monoclonality and the rest had kappa light-chain monoclonality. Among the cases, AL amyloidosis without plasma cell myeloma (PCM) occurred in 44 (44.9%), PCM with AL amyloidosis in 49 (50.0%), non-secretory myeloma with AL amyloidosis in 2 (2.0%), plasma cell leukemia with AL amyloidosis in 1 (1.0%), and B-cell lymphoma with AL amyloidosis in 2 (2.0%) patients.

Monoclonality was identified by serum and urine PEP in 44.9% (44/98) and 44.8% (43/96) of all cases, respectively. Serum and urine IFE detected monoclonality in 63.3% (62/98) and 70.8% (68/96) of all patients, respectively. When both serum and urine IFE tests were considered, monoclonality was detected in 79.6% of cases (78/98). The median concentration of serum M protein and percentage of Bence Jones protein were 6.0 g/L (range, 1.0–62.0 g/L) and 9.6% (range, 0.2–79.5%), respectively. The SFLC ratio detected

monoclonality in 79.5% of cases (70/88). The combination of both serum and urine IFE and the SFLC ratio detected monoclonality in 89.8% of the cases (88/98).

IHC for light chains on BM biopsies or clot sections detected monoclonality in 85.7% (84/98) of the cases. The detected monoclonal light chains were concordant between the laboratory tests (IHC on BM biopsy or clot sections, serum and urine IFE, and SFLC ratio).

FCM assay of the BM aspirate detected aberrant plasma cells in 92.9% of cases (39/42). Aberrant plasma cells expressed CD38 and CD138, but unlike normal plasma cells, CD45 or CD19 was negative to dim or CD56 was positive. The median percentage of aberrant plasma cells among the total plasma cells was 88.5% (range, 10.5–99.9%). Table 1 shows the results of BM and laboratory tests according to BM involvement in 98 cases of AL amyloidosis. Only SFLC ratio abnormalities were significantly associated with BM amyloid deposition (P=0.032). Table 2 shows the discrepancies between the laboratory tests for detecting light-chain clonality. Patients with negative IFE showed a dominance of kappa clonality. Cases with a normal SFLC ratio and negative IFE revealed high IHC positivity rates of 83% (15/18) and 85% (17/20), respectively. All cases with negative IHC or FCM assay showed an abnormal SFLC ratio, and all cases with normal SFLC ratio and negative IFE showed aberrant plasma cells in the FCM assay. These findings suggest the high sensitivity of the SFLC ratio and FCM assay.

BM findings

Plasma cells comprised 10% or more of the BM cellularity in 53.1% of the cases (52/98), including 49 cases of PCM with amyloidosis, two cases of non-secretory myelomas with AL amyloidosis, and one plasma cell leukemia case, and comprised less than 10% of the cellularity in 46.9% (46/98) of the cases. A high plasma cell percentage (>10%) was not correlated with BM amyloid deposition (P=0.292) according to the Chi-squared test.

Amyloid deposition was identified in 35.7% of the BM specimens (35/98) (Table 3). The amyloid deposition grades were: 1+, 31.4% (11/35); 2+, 34.3% (12/35); and 3+, 34.3% (12/35). Patients with BM amyloid deposits showed a tendency towards greater lambda light-chain clonality (27/35, 77%) than those without amyloid deposits (37/63, 59%).

Congo red and PAS stainings were positive in 71.4% (25/35) and 100.0% (35/35) of the patients, respectively (Table 4). Three Congo red-stained BM specimens showed pink-to-red amyloid deposits under light microscopy but did not show apple-green birefringence under polarized-light microscopy. In these cases, the PAS stain was also positive. Ten cases were negative for Congo red staining, but positive for amyloid deposition on PAS staining. We did not find any association between the stainability of Congo red or PAS stainings with the amyloid deposit grades and plasma cell percentage in the BM.

Table 1. Bone marrow and related laboratory findings according to bone marrow involvement in 98 cases of AL amyloidosis.

Parameters	Bone marrow involved patients (N=35)	Bone marrow non-involved patients (N=63)	P
κ/λ clonality[a]	8/27	26/37	0.067
% plasma cells in bone marrow, mean (range)	8.4 (1.2–49.2)	11.0 (0.0–92.4)	0.360[b]
M-component detection by serum and urine protein electrophoresis	57.1% (20/35)	60.3% (38/63)	0.759
Monoclonality detection by serum and urine immunofixation electrophoresis	80.0% (28/35)	79.4% (50/63)	0.940
Abnormal serum free light chain ratio	91.2% (31/34)	72.2% (39/54)	0.032
Monoclonality detected by immunohistochemistry	82.9% (29/35)	87.3% (55/63)	0.547
Aberrant plasma cells by flow cytometric assay	93.3% (14/15)	92.6% (25/27)	1.000[c]

[a]The number of cases which showed κ or λ clonality. [b]The Mann-Whitney U test was used for this comparison. [c]Fisher's exact test was used for this comparison, and the Chi-squared test was used for other comparisons of categorical data.

Table 2. The discrepancies between laboratory tests detecting light-chain clonality and aberrant plasma cells by flow cytometric assay.

Cases	N	Plasma cell %	κ/λ clonality	IFE (+)	SFLC ratio (Abn)	IHC (+)	FCM assay (+)
IFE (-)	20	2.5 (0.8–52.0)	15/5	NA	10/16	17/20	3/3
SFLC ratio (N)	18	5.1 (1.2–26.8)	5/13	12/18	NA	15/18	7/7
IHC (-)	14	4.0 (0–18.8)	1/13	11/14	13/13	NA	1/3
FCM assay (-)	3	6.8 (3.2–9.6)	0/3	3/3	3/3	1/3	NA

Abbreviations: Abn, abnormal; FCM, flow cytometric; IFE, immunofixation electrophoresis; IHC, immunohistochemistry; N, normal; NA, not applicable; SFLC, serum free light chain.

Table 3. Amyloid deposition in bone marrow biopsies or clot sections in 98 cases of AL amyloidosis.

Amyloid deposit grade[a]	Cases (N=98)	% plasma cells, median (range)	λ (N=64)	κ (N=34)	*P*
Bone marrow					0.887[b]
0	63 (64.3%)	11.0 (0.0–92.4)	37	26	
1+	11 (11.2%)	5.6 (1.2–27.2)	8	3	
2+	12 (12.2%)	8.2 (3.6–49.2)	9	3	
3+	12 (12.2%)	14.3 (2.4–35.0)	10	2	

[a]Amyloid deposit grade: 1+, blood vessels only; 2+, interstitial deposition of ≤2 high-power fields; 3+, interstitial deposition of >2 high-power fields. [b]Fisher's exact test was used for this comparison.

Table 4. Amyloidosis detection methods and bone marrow findings in 35 cases of AL amyloidosis with bone marrow involvement.

N	22	3	10
Detection method			
Congo red stain by polarizing microscopy	Positive	Negative	Negative
Congo red stain by light microscopy	Positive	Positive	Negative
PAS stain	Positive	Positive	Positive
Amyloid deposit grade score[a]			
Median (range)	2+ (1+–3+)	2+ (1+–3+)	2+ (1+–3+)
% plasma cells in bone marrow			
Median (range)	7.6 (1.2–49.2)	18.0 (2.8–23.4)	8.2 (1.6–35.0)

[a]Amyloid deposit grade: 1+, blood vessels only; 2+, interstitial deposition of ≤2 high-power fields; 3+, interstitial deposition of >2 high-power fields.

DISCUSSION

Regarding the diagnosis of AL amyloidosis, identifying the underlying clonal light chain is essential [6]. Clonal light chains can be detected using serum and urine PEP, IFE, the SFLC ratio, FCM assay, IHC, or even mass spectrometry. Mass spectrometry is the most sensitive and specific tool for subtyping amyloidosis [1, 6], although it is not usually used to detect clonal light chains. Another method for subtyping amyloidosis is IHC for light chains, amyloid A protein, and transthyretin in the biopsied tissue. When using IHC on biopsied tissue for amyloid subtyping, laboratory tests used to detect monoclonality, such as serum and urine IFE and SFLC ratios, are useful tools for achieving definite subtyping, especially in AL amyloidosis [7, 8].

In AL amyloidosis, the median concentration of the serum M protein is 14.0 g/L, which is much lower than that in PCM (usually >30 g/L) [1]. Serum M protein or urine Bence Jones protein are often not detectable by serum and urine PEP, [6] because of their low sensitivity for detecting light-chain clonality. In contrast, monoclonality was detected in more than 80% of patients with AL amyloidosis by serum and urine IFE [3, 6, 9], which is similar to our results. These results indicated that serum and urine IFE, but not PEP, are useful tools for detecting monoclonality in patients with AL amyloidosis.

Previous studies have shown that the SFLC assay can detect monoclonality in more than 75% of cases, and when combined with IFE, its sensitivity for detecting monoclonality increases to 99% [1, 6, 10, 11], which is concordant with our results. The SFLC ratio is also a useful and sensitive tool for identifying underlying clonal light chains in patients with AL amyloidosis.

IHC for light chains in BM biopsies usually reveals monoclonal plasma cells. However, in cases with small numbers of clonal plasma cells, they may be masked by normal polyclonal plasma cells [1, 12, 13]. The reported detection rate of plasma cell dyscrasia in AL amyloidosis by IHC on BM biopsies is 60–69% [12, 14], which is lower than that in our results. In PCM, clonal plasma cells are usually detected using IHC for light chains [1, 15, 16]. In our study, a high proportion of patients was diagnosed with PCM accompanied by AL amyloidosis. This may have contributed to the high sensitivity of IHC for detecting monoclonality in BM biopsies or clot sections.

Few studies have reported the utility of FCM assay for AL amyloidosis. The reported sensitivity of FCM assays for detecting monoclonal plasma cells in AL amyloidosis was 97–100%, which is similar to our results [17-19]. Because of its high sensitivity, FCM assay of BM aspirates may be a useful tool for evaluating AL amyloidosis.

It is known that the detection of small numbers of clonal plasma cells may be masked and challenging with IHC staining [1, 12, 13]. Light-chain monoclonality was not detected using IHC in 14 cases. Most of these patients had a low percentage of plasma cells. Among these cases, plasma cells comprised more than 10% of the BM cellularity in only

one case (18.8%). In this case, light-chain monoclonality was detected only by the SFLC ratio. Also, among these 14 cases, 13 cases showed λ clonality by other laboratory tests. Schönland *et al.* [7] reported that the weakness of IHC can result from prominent variable domains of λ light chains.

It was reported that approximately 12% of patients with AL amyloidosis displayed normal SFLC ratio at diagnosis [20-22]. In the present study, light-chain clonality was not detected using the SFLC ratio in 18 cases. Among them, λ clonality (13 cases) was detected more than κ clonality (5 cases) by other laboratory tests. In these cases, nearly all the λ clonality was detected by IFE with the exception of one case. Singh [22] reported that when comparing IFE to SFLC ratio, false-negative rates of SFLC ratio were higher in λ chains than in κ chains.

Interestingly, among the 20 cases where light-chain clonality was not detected by IFE, many of the cases (75.0%) showed κ clonality. Serum IFE missed about 20% of patients with plasma cell disorder who showed involved serum free κ above the expected IFE threshold, while free λ was more reliably detected [23].

In three cases, plasma cells showed a normal immunophenotype in the FCM assay, despite the high sensitivity for detecting aberrant plasma cells. All cases showed λ clonality, as detected by the SFLC ratio and IFE. Based on the fact that the immunophenotypic features of the monoclonal plasma cells in AL amyloidosis are similar to those of PCM [1], the same analysis method of FCM assay was used for the patients with AL amyloidosis and PCM in this study. However, two distinct populations of normal and malignant plasma cells were detected in AL amyloidosis compared to PCM. In addition, the surface antigen expression on monoclonal plasma cells in AL amyloidosis was different from that in PCM [19]. The differences in the immunophenotypic characteristics between AL amyloidosis and PCM or the different analysis methods might be the cause of the negative results of FCM assay in this study.

Amyloid deposition is reportedly found in the BM in a majority of AL amyloidosis cases [1, 3, 11]. A previous study identified BM amyloid deposits in 60% of patients, excluding those with PCM [3]. Petruzziello *et al.* [24] reported that amyloid deposits were found in the BM biopsies of patients with PCM in approximately 40% of cases, which was similar to our results. The differences between the study results can be attributed to the different proportions of patients with PCM in the study population. Other possible causes for the differences in results between studies include the definition of amyloid deposits, different stains, and specimen-processing methods.

Congo red fluorescence microscopy is an important tool for the detection of amyloid deposits and remains the gold standard for the diagnosis of amyloidosis [3]. However, the sensitivity of Congo red staining for detecting amyloidosis is related to the tissue source. The sensitivity of Congo red staining for amyloidosis is especially low in the BM (63%) compared to that in other tissues such as the kidney, liver, cardiac (87–98%), and rectal tissues (69–97%) [25, 26]. Congo red-negative amyloidosis has also been reported [25, 27, 28]. Bowen *et al.* [25] reported amyloid deposits in the BM that were negative on Congo red staining but were detected by mass spectrometry. Recently, the disadvantages of Congo red staining were revealed because it showed 24.3% false-negative and 4.2% false-positive rates in a study performed in 1964, and the causes of these false reactions were described [26]. They concluded that it is necessary to use IHC for confirmation, when amyloidosis is suspected in the presence of negative Congo red staining results.

BM amyloid deposition can also be detected by PAS staining using light transmission microscopy [3, 29]. PAS staining, which gives amyloid deposits a distinctive smudgy appearance in the BM, is often more reliable than Congo red staining for detecting amyloid deposits in AL amyloidosis [3]. We identified 10 cases of Congo red-negative and PAS-positive amyloid deposits, indicating that PAS staining may be useful in diagnosing some cases of Congo red-negative amyloidosis. However, to the best of our knowledge, there have been no reports on the sensitivity and specificity of PAS staining for detecting amyloid deposits in AL amyloidosis. For evaluating this, further studies using more accurate techniques for detecting amyloid deposits such as mass spectrometry will be required.

AL amyloidosis is highly suspected based on typical clinical findings and detection of monoclonality through laboratory testing [7]. However, hereditary amyloidosis may be incidentally present in patients with monoclonal gammopathy ranging from 3% to 10% [1, 7].

The limitation of this study is that there is little chance that patients with other subtypes of amyloidosis (such as hereditary amyloidosis) might have been included, because IHC on surgical biopsies was performed in only 53 cases, although all patients in this study were suspected to have AL amyloidosis based on their clinical signs and laboratory findings. To exclude hereditary amyloidosis, molecular tests targeting *TTR, FGA,* and *APOA1* may be helpful [7]. The most reliable methods for the diagnosis of AL amyloidosis are mass spectrometry and electron microscopy. However, these methods were not used in this study.

In conclusion, laboratory tests for detecting light-chain clonality, in order of sensitivity, include FCM assay of aberrant plasma cells, IHC for light chains on BM biopsy or clot sections, SFLC ratio, and serum and urine IFE. Congo red staining of BM samples remains an important tool for identifying amyloid deposits in BM. PAS staining can be helpful for diagnosing some cases of Congo red-negative amyloidosis.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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