Leukemia
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BRIEF COMMUNICATION

Multiple myeloma gammopathies

Assay to rapidly screen for immunoglobulin light chain glycosylation: a potential path to earlier AL diagnosis for a subset of patients

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Systemic light chain (LC) amyloidosis (AL) is a protein misfolding disease in which a monoclonal immunoglobulin LC self-aggregates to form insoluble amyloid fibrils, which deposit in different organs and impairs the physiology of organs [1]. Researchers postulated that glycosylation also has a pathogenic effect on LCs and glycosylated LCs could be more prone to be amyloidogenic [2]. Pathogenic glycosylation of proteins has been implicated in various hematological malignancies, often with prognostic implications [3]. However, in comparison with other diseases, glycosylation of LCs has been relatively underinvestigated in AL. This is largely due to the lack of a high-throughput procedure to facilitate rapid analysis of LC glycosylation.

Previous studies using immuno-enrichment-based matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), termed MASS-FIX, demonstrated that M-protein mass distributions from AL patients often had an additional "polytypic-like" pattern along with the diagnostic monoclonal LC [4]. Further studies using high-resolution liquid chromatography-based MS (LC-MS) suggested that posttranslational modifications such as glycosylation on LCs could produce this "polytypic-like" pattern in AL patients [5]. Using MS approaches, we provide the most comprehensive mapping of LC glycosylation in AL reported to date.

We obtained serum or plasma samples from total 311 patients and arranged them into two cohorts. The first cohort had 157 AL previously untreated patients who had their amyloid protein sequenced by LC-MS/MS [1]. The second cohort included 154 patients who had previously untreated AL ($n = 32$) or another diagnosis such as multiple myeloma ($n = 54$), Waldenstrom macroglobulinemia ($n = 8$), monoclonal gammopathy of undetermined significance ($n = 57$), and other plasma cell disorder (PCD) [4] (Table 1).

The immuno-enrichment was performed as previously described by adding 10 µL aliquot of serum to 20 µL agarose beads coupled with one of the single-domain antibodies specific for heavy chain (HC) of IgM, IgA, and IgG, and LC of κ or λ constant domains (Thermo Fischer Scientific), washed, reduced, spotted on MALDI target plate (Bruker), and analyzed separately on MALDI-TOF-MS (Microflex LT, Bruker) [4, 6]. The spectra from each five immuno-enrichment of each sample were overlaid and LC m/z distribution was visually inspected for the presence of peaks in $[M + 1\,H]^{1+}$ and $[M + 2\,H]^{2+}$ using Flex Analysis software (Bruker), and categorized patient's monoclonal LC as either "suspected-glycosylation or "no glycosylation" based on observed peak patterns.

Deglycosylation was executed to confirm suspected-glycosylation in monoclonal LC. Briefly, 10 µl of serum of suspected-glycosylation sample was mixed with 20 µl of either κ- or λ-specific beads, and incubated for 45 min at room temperature. After washing with phosphate-buffered

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Table 1 Distribution of MASS-FIX patterns for LCs in patients with AL vs. non-AL and serum-free LC measurements for glycosylated vs. non-glycosylated LC Shouldn't data in columns be cnetered like their headers are

	κ Clone	λ Clone	All patients
Sample type	**Suspected glycosylation by MASS-FIX**		
AL, *n*/*N* (%)	20/61 (32.8)	13/128 (10.2)	33/189 (17.5)
Non-AL, *n*/*N* (%)	3[a]/81 (3.7)	2[b]/41 (4.9)	5/122 (4.1)
P-value	< 0.001	NS	*P* < 0.001
Odds ratio	12.68	2.20	4.95
	Median FLC measurement, mg/dL (IQR)		
Glycosylated ($n = 36$)	34.4 (4.1–90.5)	50.8 (13.9–79)	–
Non-glycosylated ($n = 252$)	16.5 (3.1–90.5)	13.5 (3.8–45.1)	–
p-Value	NS	0.02	

[a]Diagnoses of these three patients included plasma cell leukemia ($n = 1$) and multiple myeloma ($n = 2$)

[b]Diagnoses of two patients were one each of MGUS and multiple myeloma.

AL, amyloidosis; IQR, interquartile range; FLC, free light chain; LC, light chain; MGUS, monoclonal gammopathy of undetermined significance; NS, not significant.

saline and water, the mixture were denatured and reduced with 100 µl of 2% SDS and 10 mM Tris (2-carboxyethyl) phosphine at 56 °C for 30 min with shaking. SDS was removed using DRS spin column (Thermo Fischer Scientific). The reduced LCs was treated with 1 µl of PNGase F (New England Biolab, Inc.) at 37 °C for 3 h with shaking. Reactions were stopped by adding 20 µl of 0.1% trifluoroacetic acid and analyzed on MALDI-TOF-MS.

The same suspected-glycosylation-LC samples were further analyzed on high-resolution LC-MS consisting of Orbitrap Elite (Thermo Fisher Scientific) coupled to an Ultimate 3000 HPLC system (Thermo Fisher Scientific) using ProSwift RP-4H capillary monolithic column (Thermo Fisher Scientific). The spectra were acquired in positive mode between 500 and 3000 m/z in Orbitrap, and analyzed using Xcalibur Qual Browser software (Thermo Fisher Scientific). The difference in mass of suspected-glycosylation-LC before and after PNGase F treatment was determined, and matched with mass of immunoglobulin *N*-glycans. All experiments were repeated in three replicates.

The differences between patient groups were calculated using Fisher's exact test (JMP 13 SAS, Carey, NC). The odds ratio for suspected-glycosylated-LC patient being AL was 12.68 for κ and 2.20 for λ (Table 1). This study was approved by the Mayo Clinic Institutional Review Board.

The mass spectra for all patients were classified into two mutually exclusive categories: suspected glycosylation ($n = 38$) and no glycosylation ($n = 273$) (Supplementary Figure 1). Suspected glycosylation LC presented as a broad peak in MS spectrum that was higher in mass than expected for LCs. Thirty-three percent of AL-κ patients had suspected-glycosylation pattern, compared with 10.2% of AL-λ patients. The rate of suspected-glycosylation LC among non-AL, κ, and λ patients was 3.7% and 4.9%, respectively (Table 1). Of the 38 patients with suspected-glycosylated LCs, 16 were LC-only patients and 22 patients had intact M-proteins along with their free LCs. Among the λ patients (Table 1), the λ free LC was higher in the suspected-glycosylated patients than the non-glycosylated patients, which could be a function of the assay's inability to detect glycosylation in a very small clone in a polyclonal background with the current immuno-enrichment methodology. Once LC beads become available as part of the pre-analytics and/or more urine samples are tested, there will likely be a greater ability to detect these patterns in smaller clones.

To confirm LC glycosylation, the subset of 21 κ (18 AL and 3 non-AL) and 9 λ (7 AL and 2 non-AL) suspected-glycosylation samples were analyzed using PNGase F on MALDI-TOF-MS. The LC spectra of all 30 samples shifted to a narrower and lower molecular mass after PNGase F as compared with native LC peak, in both $[M + 2\,H]^{2+}$ and $[M + 1\,H]^{1+}$ charged states, indicating *N*-glycosylation (Fig. 1a).

To further verify the MASS-FIX pattern represented *N*-glycosylation, 19 (13 κ and 6 λ) suspected-glycosylation-LC samples were studied by LC-MS using PNGase F; all samples demonstrated a shift toward lower molecular weight after PNGase F treatment, affirming that the broad peak patterns observed by MASS-FIX were a signature for glycosylated LCs. The difference in mass before and after PNGase F was matched with molecular weights of known *N*-glycans (Fig. 1b). Bi-antennary sialated *N*-glycan forms G2FNSA2 and G2FSA2 were observed in most cases, whereas others had fragments of these glycan groups (Supplementary Table 1).

As 158 patients had their LC gene sequence by tissue mass spectrometry [1], associations between immunoglobulin LC usage and the presence of glycosylation were sought. As shown in Fig. 1c, 41% of AL whose amyloid protein was of the KV1 gene family had glycosylated circulating LC. The κ-LCs derived from *KV1-33* and *KV1-39* were most represented (Fig. 1d). The λ gene family most represented was LV3, with *LV3-21* having the highest likelihood of being glycosylated.

Until now, a limited number of patients had been studied to investigate *N*-glycosylation of LCs in AL [7, 8]. In 2000, Stevens [9] summarized the glycosylation literature. Only 9 of 22 λ-LC proteins exhibiting potential glycosylation sites

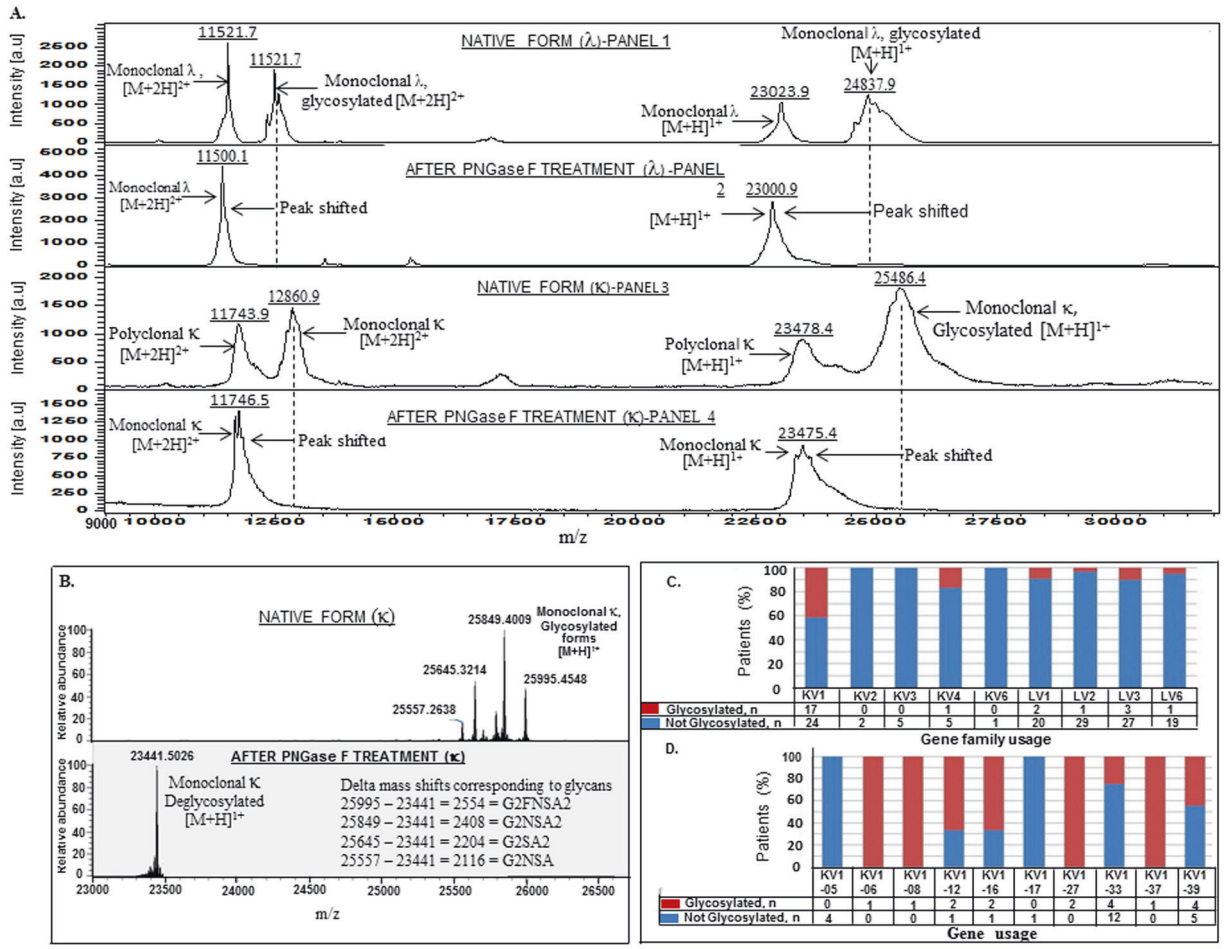

Fig. 1 **a** Deglycosylation of monoclonal light chain with PNGase F. Glycosylated monoclonal λ and κ, LC in native form (panel 1 and 3), and PNGase F treatment showing shift with reduced molecular weight toward unglycosylated form (panel 2 and 4). **b** Monoclonal κ, light chain glycosylation analysis by high-resolution LC-MS. There were at least four peaks representing different glycoforms of monoclonal κ light chain (upper panel). After PNGase F treatment, monoclonal κ light chain is resolved to a single peak of 23,441 Da (lower panel). The difference in molecular weight of peak before and after PNGase F was matched with molecular weight of different glycans as G2FNSA2, G2NSA2, G2SA2, and G2NSA mentioned in Supplementary Table 1. **c** Immunoglobulin LC gene usage based on the presence or absence of LC glycosylation. Gene family and gene usage was determined by bottom-up sequencing of amyloid from tissue biopsy [11]. By gene family: KV1 and KV4 gene families had highest rates of glycosylation. **d** A closer look at the KV1 gene family: 25% of *KV1-33* and 41% of *KV1-39* patients' monoclonal serum immunoglobulin light chains were glycosylated

were from AL patients. In contrast, 18 of 22 potentially glycosylated KV1, LCs were from AL patients despite the fact that germline KV1 genes do not encode for N-linked glycosylation motif (N-*x*-S/T) [9]. It is noteworthy that we have observed that 8.5% (81/948) of KV1 mRNA sequences of various PCDs including AL derived from a LC sequence database had an *N*-glycosylation motif [10]. These results suggest somatic hypermutation affinity maturation of κ-LCs generates *N*-glycosylation sites (N-*x*-S/T). *N*-glycosylation has been reported in κ-LCs from urine in two AL patients, who had consensus glycosylation sequence (N-*x*-S/T) in germline using chromatography and MALDI-TOF-MS [11], but chromatography is analytically complex, cumbersome, and time consuming, making it unsuitable for routine clinical use. In contrast, our novel MASS-FIX method easily and rapidly characterizes LC glycosylation.

Several studies have described carbohydrate moieties in their LC of patients with PCDs; [12] it was recognized that 4% (3/71) of Bence Jones proteins from myeloma patients' urine and 11% (2/18) of LCs from multiple myeloma serum had carbohydrate moieties attached [13]. Prior studies identified sialic acid, *N*-acetyl-glucsosamine, *N*-acetyl-galactosamine, and other "neutral sugars" attached to LCs [13]. Approximately 15% of immunoglobulin LCs in circulation have been shown to have oligosaccharides [13]. In the present study, the most common *N*-glycans were found to be G2FNSA2 and G2FSA2 as bi-antennary and sialated, which supports the previous study that LCs are highly sialylated [14, 15]. Despite the disparity of frequency of *N*-glycosylation for κ and λ-LCs, the composition of *N*-glycans did not appear to be different.

In summary, we identified *N*-glycosylation in the serum LCs of one-third of κ-AL and 10% of λ-AL patients. The novelty of our work is the application of a quick, inexpensive, high-throughput method to identify LC *N*-glycosylation, i.e., MASS-FIX. Although this finding will apply to only 11% of AL cases (one-third of AL cases are κ and one-third of κ-AL are glycosylated), we anticipate that over the next decade, MASS-FIX will replace immunofixation as a screen for monoclonal proteins, providing both information on isotype for all patients and AL risk in a subset. Moreover, with better immune-enrichment, the twofold higher rate of glycosylation observed in λ-AL cases over other PCDs may become significant, further increasing the value of this method to prompt clinicians to have a higher suspicion for AL in an even higher proportion of patients.

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Author contributions: SK performed the experiments and drafted the manuscript. SK, AD, DLM, and SD designed, analyzed, interpreted the data, and edited the manuscript. BM performed the LC-MS experiments. PM had performed the experiments on the 154 patients from a prior report. TK and SD had performed the analyses of tissue amyloid as per a prior publication. Other authors contributed intellectual content and review of this manuscript.

Compliance with ethical standards

Conflict of interest DLM and SD have financial interest in the MASS-FIX technology used in this study. All other authors declare that they have no conflict of interest.

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