Clonotypic Light Chain Peptides Identified for Monitoring Minimal Residual Disease in Multiple Myeloma Without Bone Marrow Aspiration

H. Robert Bergen, III,1* Surendra Dasari,2 Angela Dispensieri,3,4 John R. Mills,4 Marina Ramirez-Alvarado,1,5 Renee C. Tschumper,5 Diane F. Jelinek,5 David R. Barnidge,4 and David L. Murray4

BACKGROUND: Analytically sensitive techniques for measuring minimal residual disease (MRD) in multiple myeloma (MM) currently require invasive and costly bone marrow aspiration. These methods include immunohistochemistry (IHC), flow cytometry, quantitative PCR, and next-generation sequencing. An ideal MM MRD test would be a serum-based test sensitive enough to detect low concentrations of Ig secreted from multifocal lesions.

METHODS: Patient serum with abundant M-protein before treatment was separated on a 1-dimensional SDS-PAGE gel, and the Ig light-chain (LC) band was excised, trypsin digested, and analyzed on a Q Exactive mass spectrometer by LC-MS/MS. We used the peptide’s abundance and sequence to identify tryptic peptides that mapped to complementary determining regions of Ig LCs. The clonotypic target tryptic peptides were used to monitor MRD in subsequent serum samples with prior affinity enrichment.

RESULTS: Sixty-two patients were tested, 20 with no detectable disease by IHC and 42 with no detectable disease by 6-color flow cytometry. A target peptide that could be monitored was identified in 57 patients (91%). Of these 57, detectable disease by LC-MS/MS was found in 52 (91%).

CONCLUSIONS: The ability to use LC-MS/MS to measure disease in patients who are negative by bone marrow–based methodologies indicates that a serum-based approach has more analytical sensitivity and may be useful for measuring deeper responses to MM treatment. The method requires no bone marrow aspiration.

Multiple myeloma (MM)6 is characterized by amplification of a single plasma cell clone producing a monoclonal Ig. The clone proliferates in the bone marrow and can result in bone destruction. The monoclonal Ig produced can circulate as intact Ig or, in 20% of cases, as free light chain (FLC) only, with no heavy chain (H). The annual incidence of MM is 4–5 per 100,000 and accounts for 1% of all cases of cancer (2).

In 97% of patients with MM, electrophoresis of serum protein (SPEP) or urine protein (UPEP) results in a large spike (M-spike) appearing in the γ, β, or α-2 region of the densitometer tracing, corresponding to the Ig protein produced by the clone (1). Three percent of patients have no apparent M-protein in serum or urine as measured by electrophoresis. There are numerous treatment options for patients with MM, including high-dose chemotherapy with hematopoietic cell support, but the disease is considered incurable. Treatments have improved considerably over the past decade, and a higher percentage of patients are achieving complete response (CR), but virtually all these patients relapse, suggesting either that current CR criteria are not analytically sensitive enough to detect the deeper response required for an eventual cure or that therapies are not effective in killing bone marrow clones. With improving therapy, there needs to be an equally sensitive method to routinely identify these deeper responses. Minimal residual disease (MRD) is a
measure used to estimate the number of cancer cells remaining after treatment.

The International Myeloma Working Group has issued guidelines for determining the response to treatment and when a relapse has occurred (3, 4). Current methods to measure MRD include immunofixation (IFE), immunohistochemistry (IHC), FLC ratios, real-time quantitative PCR (RQ-PCR), multiparametric flow cytometry (MFC), and next-generation sequencing (NGS) (3, 5, 6). Most patients with MM after treatment have residual disease that is below the diagnostic sensitivity of these assays and will eventually relapse (7).

RQ-PCR is a highly sensitive technique but requires patient-specific primers (6, 8–16). More recently, NGS has been found to be as sensitive as RQ-PCR and does not require the patient-specific reagents (6). RQ-PCR, MFC, and NGS are all analytically sensitive, and in prospective studies immunophenotypic CR and molecular CR are both predictive of survival outcomes (5, 17). Currently, the most analytically sensitive techniques available for measuring MRD are immunohistochemistry, RQ-PCR, MFC, and NGS, all of which require bone marrow aspiration. This is an invasive procedure, and although considered safe, it is painful and more expensive (> $5000) than a venous blood draw.

An ideal MM MRD test would be a serum-based test sensitive enough to detect low concentrations of secreted Ig. Such a serum-based test would be less invasive than bone marrow aspiration and would have the potential to detect the presence of patchy or focal disease that bone marrow aspiration might miss. Several groups have shown previously that clonotypic peptides specific to the complementarity determining region (CDR) of the M-protein obtained from serum can be used to monitor MRD (18–20). Additional work showed that the accurate molecular mass of LCs from intact M-proteins isolated from serum could be used to monitor MRD in MM patients who were negative by SPEP and IFE (21).

With these facts in mind, an analysis of specific CDR peptides unique to each patient’s M-protein analyzed in a highly sensitive manner from a venous blood sample would suffice to monitor MRD. Each patient’s clonal CDR-tryptic peptide is a MRD signature for that patient. Because deeper responses in treatment are correlated with survival outcomes, the ability to measure deeper responses will be necessary to eventually cure this disease (5, 17). Here, we outline an analytically more sensitive method to measure MM MRD: serum analysis of CDR peptides by use of only Ig LCs isolated and identified from the M-spike protein in a venous blood sample. DNA sequence information is not required.

**Methods**

**PATIENT SAMPLES**

We obtained serum or plasma from an archive of samples collected under Mayo Clinic institutional review board approval 13–000220 from the same patients before and after autologous stem cell transplant. All pretransplant samples had to have M-protein values ≥ 0.8 g/dL. The posttransplant serum and bone marrow aspirates were obtained within 5 days of each other, and no posttransplant samples had detectable disease (serum or urine IFE; bone marrow IHC or MFC).

**IDENTIFICATION OF TARGET Ig LC-VARIABLE REGION PEPTIDES**

Serum or plasma (0.5 μL) was separated on a reducing gel, and the Ig LC band was excised and analyzed by LC-MS/MS on a Q Exactive mass spectrometer (for details, see Supplemental Materials, which accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue1).

**SOFTWARE AND DATA ANALYSIS**

We identified clonotypic peptides by traditional database search strategies within PEAKS software (Bioinformatics Solutions) with PEAKS de novo, database, posttranslational modification (PTM), and Spider search algorithms, 4.5-ppm precursor mass tolerance, and 0.02-Da fragment ion tolerance. Carbamidomethylation was used as a fixed modification, and Met oxidation and deamidation of Asn/Gln were used as variable modifications. We used the augmented database of Dasari et al. (22) for the database searches. Base peak ion chromatograms in XCalibur Qual Browser 2.2 (Thermo Fischer Scientific) for each high M-spike sample were then interrogated. Abundant ions were matched to their corresponding mass and retention time in the Spider search results. We then mapped the resulting sequences corresponding to λ or κ LCs to known LC sequences with IMGT/Domain-GapAlign (23). Peptide sequences that contained all or part of CDRs 1, 2, or 3 were considered to be diagnostic for each patient and were monitored in subsequent patient samples to measure MRD. Peptide sequences covering the CDRs contain the hypervariable regions and thus offer unique targets for subsequent analysis.

**LC mRNA SEQUENCING**

To confirm that proteomics analysis alone provided authentic CDR tryptic peptides, we compared LC sequences obtained by proteomics with sequences obtained by Sanger-sequenced plasma cell LC mRNA isolated from bone marrow aspirate (see online Supplemental Materials for details).
**LC mRNA SEQUENCE ALIGNMENT**

The Sanger LC mRNA sequence of each patient was aligned to the public repertoire of the Ig LC sequences with the ImmunoGeneTics V-QUEST search feature (24). The alignment was configured to use human Ig-variable region sequences and restricted to use only the V and J regions of the LCs. The alignment automatically detected the CDR regions and any insertions and deletions in the mRNA sequence. The edited mRNA sequence was translated into a mature, full-length protein sequence, which was considered the patient’s M-protein sequence. Variable-region peptides obtained from the proteomics experiment were matched to these protein sequences with PEAKS software.

**MEASUREMENT OF MRD**

*Enrichment.* CaptureSelect anti-κ or anti-λ affinity matrix (Life Technologies) (75 μL) was added to a 10-μm fritted microspin column and washed 3 times with PBS. The resin was resuspended in 200 μL PBS, 50 μL serum or plasma was added, and the mixtures were incubated at 4 °C overnight with gentle inversion. The bound Ig was washed 2–3 times with 200 μL PBS, and the bound Ig material was eluted with 100 μL of 100 mmol/L glycine, pH 2.7, and collected in a microcentrifuge tube containing 6 μL of 1 mol/L Tris, pH 10.

*Electrophoresis.* Eluate (10 μL) was applied to a 10.5%–14% Criterion precast SDS-PAGE gel. After staining with BioSafe colloidal Coomassie, the LC band was excised and digested with trypsin.

*LC-MS/MS.* We analyzed the LC digest by nano-LC-MS/MS as described in online Supplemental Materials. We prepared an extracted ion chromatogram (XIC) corresponding to the parent ion mass of the CDR-tryptic peptide previously identified and compared the integrated area and associated MS/MS spectra to the original pretreatment sample.

*IHC and MFC.* IHC of bone marrow aspirates followed standard clinical practice. MFC was performed with the method of Morice et al. (25).

**Results**

Samples from 8 MM patients (4 κ, 4 λ) with plasma cell mRNA Sanger sequencing and associated serum/plasma samples were initially analyzed. Fig. 1 shows serum or plasma from these patients with large M-spike separated by SDS-PAGE. The bands corresponding to Ig LC were isolated, reduced, alkylated, and analyzed by nano-LC-MS/MS. Fig. 2 shows the base peak ion chromatogram of the 4 κ patients. Online Supplemental Fig. 1 shows the base peak ion chromatogram for λ patients. In each case, high-intensity peaks corresponding to constant- or framework-region–tryptic peptides could be identified (PEAKS) in each patient with similar retention times and identical precursor mass. Additionally, close observation of these chromatograms indicated unidentified peptide peaks with intensity comparable to the constant- and framework-region peptides but with variable retention times. By use of the Spider function within PEAKS, these peptides were matched by homology to CDR-tryptic peptides in the IMGT database with Domain-GapAlign and to the sequences obtained by translating Sanger-sequenced plasma cell–isolated mRNA from the same patients. Online Supplemental Fig. 2 indicates the 8 LC sequences obtained for 8 MM patients.

Mass/retention time pairs corresponding to CDR-variable region–tryptic peptides could be used to monitor MRD in subsequent serum samples, thus eliminating the need for bone marrow aspiration. As proof of principle, we monitored the CDR-tryptic peptides from 2 additional patients with serial blood samples over the course of treatment for MM after an initial affinity purification of the appropriate Ig class (κ or λ). Fig. 3 and online Supplemental Fig. 3 compare the SPEP-reported concentrations and the LC-MS/MS–integrated areas of CDR-variable region–tryptic peptides from the 2 patients over time. The LC-MS/MS methodology recapitulated the SPEP results; however, 5 of the time points yielded either small (June 16, 2006) or no M-protein by SPEP and IFE (Fig. 3). In contrast, Fig. 4 shows the XIC of the 853.95 m/z ion of the CDR peptide (see online Supplemental Fig. 4 for CDR confirmation) from the same patient samples at the 5 time points indicated in
Fig. 3. The signal-to-noise ratio (S/N) and area counts indicated that the new MS method had a considerably higher analytical sensitivity to detect disease, even though 4 time points were negative by SPEP/IFE. Importantly, the results follow the disease trend reported by SPEP.

To measure the potential analytical sensitivity of this workflow, we purified Ig from a high M-spike sample and prepared a dilution curve into healthy serum. Fig. 5 shows the linearity of the workflow when the area under the curve from a CDR-region peptide was integrated and plotted against concentration with the MRD measurement technique outlined here. The lowest concentration prepared was 0.0001 g/dL, for which the S/N was still 1000. In contrast, the SPEP LOQ was approximately 0.2 g/dL. The protocol described here yielded an assay 2000 times more sensitive than SPEP without any optimization.

We evaluated how large an M-spike was necessary to identify a target CDR-tryptic peptide from blood. On-line Supplemental Fig. 5 is a scatterplot of 49 high M-protein (baseline) patient samples vs SPEP protein concentrations, indicating a threshold of \( \geq 0.8 \) g/dL M-protein for target peptide identification.

We next tested samples from 57 patients who had a baseline (high M-protein) and a serum sample that had been acquired \( \leq 5 \) days from bone marrow aspiration with either negative serum and urine IFE and \(<5\%\) plasma cells in bone marrow by IHC (18 patients) or no detectable plasma cells by 6-color flow cytometry (39 patients). Target peptides were identified in the baseline sample and subsequently monitored in the sample acquired with no detectable disease by IHC and MFC. All of these samples had no detectable MRD by the reference method. However, when the serum was analyzed by LC-MS/MS with the target peptides identified from the high M-spike sample, \( \geq 91\% \) of the IHC and MFC samples had detectable...
MS signal, still indicating the presence of disease (18/18 patients and 34/39 patients).

Tables 1 and 2 summarize the characteristics of the 131 CDR tryptic peptides identified. Of the 57 patients negative for MRD by IHC and MFC, 41, 63, and 27 target peptides were associated with CDR 1, 2, and 3, respectively. Patients with ≥2 CDR-target peptides comprised 75% of all patients. With regard to uniqueness, of the 131 CDR-tryptic peptides identified, 54% were unique, 12% were found in 1 additional patient, 15% in 2–9 additional patients, and 18% in 10–49 additional patients. Sequence uniqueness by CDR was 66%, 52%, and 41% for CDR 1, 2, and 3, respectively.

Discussion

Our results indicate that LC CDR-region tryptic peptides can be identified from serum (<1 µL) after isolation of the LC band from a 1-dimensional SDS-PAGE gel separation. The protocol described here does not seek complete sequencing of the LC clone, which could be afforded only after digestion with multiple proteases, but only to identify a unique LC CDR-tryptic peptide distinctive to the plasma cell clone for subsequent monitoring. The M-spike concentration must be above the polyclonal background for a CDR-tryptic peptide to be identified from the polyclonal background. We preliminarily identified this threshold as >0.8 g/dL in an analysis of 49 MM patients from a previous study. Seven additional patients met our ≥0.8 g/dL M-protein threshold, but target peptides were not identified. Four patients had SPEP values between 0.8 and 1.1 g/dL, indicating that our previously defined threshold of 0.8 g/dL was too low for all patients. Two patients had SPEP values of 4.49 and 2.8 g/dL, but visual inspection of the SDS-PAGE gels indicated discrepancies between SPEP and our stained gels. One patient (3.6 g/dL) had abundant prominent constant/framework peptides but no abundant-variable tryptic peptide. Digestion with GluC (cleaves at Glu) yielded similar results (data not shown).

Once the peptide has been identified for a given patient, the technique can detect a target CDR peptide down to at least 0.0001 g/dL based on our dilution curve. A lower threshold would be possible by additional purification, but this has not been examined since the current protocol is already more sensitive than IHC and MFC. The CDR-tryptic peptide is identified by a combination of (a) relative ion abundance compared with constant-region peptides (either κ or λ), (b) sequence information as provided by Spider software within PEAKS, and either (c) BLAST homology search to ascertain homology with known LC-variable region sequences or (d) submission of a LC-variable protein sequence to IMGT DomainGapAlign. We are currently working with commercial soft-
ware (Pinnacle, Optys Tech Corp.), which has integrated most of these tasks.

Once treatment for MM has commenced, the M-spike protein serum concentration decreases and recedes into the polyclonal background until relapse. In a recent study of 126 patients by Song et al. (26), an almost equal number presented with focused (plasma cells segregated to one locale) vs diffuse/variegated MM (26, 27). Focused MM would not be identified in a bone marrow aspirate unless located at the site of aspiration. Whether the disease is diffuse/variegated or focal at diagnosis—or more importantly, at supposed remission—our technique should allow for detection potentially beyond what a bone marrow aspirate can provide. This fact, plus the additional analytical sensitivity of LC-MS/MS, may contribute to our ability to accurately diagnose disease in >91% of patients reported to be in CR by serum and urine IFE and bone marrow IHC and/or MFC. These observations point to a major advantage of the methodology outlined here in that—just like MRI, positron-emission tomography, and computed tomography—both focal and diffuse/variegated MM should be detected when the plasma cell Ig clone is secreted into the circulation, requiring neither initial nor subsequent bone marrow aspiration.

In all 8 patients with Sanger sequence data, a tryptic peptide covering ≥1 CDR was identified by proteomics. Online Supplemental Table 1 shows juxtaposed CDR-tryptic sequences obtained by proteomics and DNA. Subtle differences between DNA sequences and the MS2-interpreted spectra are noted. Identification of CDR region–tryptic peptides from all 8 DNA-sequenced, 18 IHC, and 39 MFC patients was accomplished without knowledge of the DNA-derived sequences, indicating that the workflow is robust.

![Fig. 4. Extracted ion chromatogram of m/z 853.95 for a CDR peptide from a MM patient at various time points.](image)

Each chromatogram normalized to 100%. Integrated MS1 peak area and S/N, SPEP, and IFE results are noted in the figure and correspond to the time points in Fig. 3. Note the extremely strong signal at all time points examined when IFE is negative.

An analytically sensitive blood-based test for MRD in MM, as shown here, would have a considerable impact in reducing invasive bone marrow aspirations and the associated costs of bone marrow sampling. The results shown here indicate that the test will have greater analytical sensitivity than SPEP, IFE, IHC, and MFC in detecting MRD. In the analysis of our IHC and MFC samples, all but 5 IHC samples still had detectable disease by LC-MS/MS, where IHC and MFC indicated no disease.

Our initial results are profound but require additional validation. The following will need to be addressed for translation of the methodology to the clinical laboratory.

First, additional data are required to determine whether a single CDR tryptic peptide is sufficient to characterize the clone or whether >1 peptide is required for monitoring. A “unique” tryptic peptide covering the CDR 3 region would offer the most reliable unique variable-region peptide, since it results from the recom-
combination of the VJ regions. In our data set, by abundance, we identified more CDR 2 > CDR 1 > CDR 3 target peptides. By sequence, more CDR 1 peptides were unique. The production of an appropriate CDR 3 target-tryptic peptide depends on the lysine and arginine locations in the LC sequence. Although the majority of CDR sequences identified were unique (54%) and not found in the rest of our patient cohort, it stands to reason that recombination events would produce identical copies of the same sequence in the polyclonal background. Monitoring multiple targets would provide confidence that the clonal Ig is being targeted and not another Ig in the polyclonal background and is an example of “precision medicine” (28). Where ≥2 targets were identified, all were always present in the posttreatment samples. Additionally, although this work focused on the LC, it could easily have been expanded to include heavy-chain CDR peptides or alternate proteases to capture ≥2 CDR peptides. Alternatively, as in Barnidge et al. (20), the clone could be identified by Sanger or NGS DNA sequencing and appropriate peptides/proteases identified a priori, subsequently requiring only a single bone marrow aspiration.

Second, the majority of clinical tests are not as informatically and computationally intensive as this test. However, we have already shown that clinical tests that are computationally equivalent can be translated to the

![Dilution curve of purified M-spike protein spiked into healthy serum at various concentrations and analyzed by the proposed method.](image)

Our lowest concentration was 0.0001 g/dL and still had S/N 1000.

<table>
<thead>
<tr>
<th>Table 1. Distribution by CDR of the 131 CDR tryptic peptides identified from MM patients.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDR</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
</tbody>
</table>

*a Sequence not found in any other patient, including the polyclonal background.

<table>
<thead>
<tr>
<th>Table 2. Additional patients in whom the same sequence was found in all patients, including the polyclonal background.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Additional patients, n</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2–9</td>
</tr>
<tr>
<td>10–49</td>
</tr>
</tbody>
</table>
clinical laboratory (29). We have successfully performed the database searches and de novo searches on both cluster-based and dual-processor workstations. After all the data are collected, they must be collated and analyzed to accept only potential target peptides that reach a minimum abundance threshold relative to the constant-region peptides. Some type of homology search (BLAST) must be performed to confirm that the de novo sequences belong to the LC variable–region protein family and then a CDR region. Pinnacle currently provides the majority of this functionality, which makes this easy and intuitive.

Third, a threshold needs to be defined for CR by LC-MS/MS. In 52 of 57 patients (91%) in CR by IHC and MFC, a clone was still detected by LC-MS/MS. Possible solutions might include the following: (a) defining a threshold ratio between the target peptide and an exogenous peptide standard; (b) defining a threshold ratio between the target peptide and a constant-region peptide; and (c) having a signal below a defined S/N. We are currently in the process of ascertaining a robust threshold.

Finally, optimization of some sample handling steps (e.g., SDS-PAGE) may be required. The results shown here were accomplished with only the tools at hand and took as little as 24 h. Simple modifications such as solution digests of intact immunoglobulins isolated by ammonium sulfate fractionation may provide comparable results.

Our previous work demonstrated that LCs derived from intact M-proteins can be used to successfully identify a monoclonal LC in serum at concentrations <0.8 g/dL (20). Both the intact method and the clonotypic-peptide–monitoring method outlined here show great promise for measuring MRD in MM, and neither requires a bone marrow aspirate. Continued optimization, validation, and comparison of these methods to the current gold standard techniques will further our knowledge of where this workflow fits in the clinician’s toolkit for measuring MRD in MM patients.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

Employment or Leadership: S. Dasari, Mayo Clinic.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: H.R. Bergen, National Cancer Center of NIH (award R21CA187462) National Center for Advancing Translational Science (Clinical and Translational Science Award UL1 TR000135), Mayo Center for Individualized Medicine, Gordon and Elizabeth Gilroy, the Jabbs Foundation, and the Robert A. Kyle Hematologic Malignancies Program.
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: The authors acknowledge the help of Diana L. Ayechart in manuscript preparation. The authors also thank Ben Maddox for acquiring the LC-MS/MS data, Christine Charlesworth for running the SDS-PAGE gels, and the rest of the Mayo Proteomics Core technical staff for all their assistance.

References


