Clonotypic Features of Rearranged Immunoglobulin Genes Yield Personalized Biomarkers for Minimal Residual Disease Monitoring in Multiple Myeloma

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BACKGROUND: Due to improved treatment, more patients with multiple myeloma (MM) reach a state of minimal residual disease (MRD). Different strategies for MM MRD monitoring include flow cytometry, allele-specific oligonucleotide–quantitative PCR, next-generation sequencing, and mass spectrometry (MS). The last 3 methods rely on the presence and the stability of a unique immunoglobulin fingerprint derived from the clonal plasma cell population. For MS-MRD monitoring it is imperative that MS-compatible clonotypic M-protein peptides are identified. To support implementation of molecular MRD techniques, we studied the presence and stability of these clonotypic features in the CoMMpass database.

METHODS: An analysis pipeline based on MiXCR and HIGH-VQUEST was constructed to identify clonal molecular fingerprints and their clonotypic peptides based on transcriptomic datasets. To determine the stability of the clonal fingerprints, we compared the clonal fingerprints during disease progression for each patient.

RESULTS: The analysis pipeline to establish the clonal fingerprint and MS-suitable clonotypic peptides was successfully validated in MM cell lines. In a cohort of 609 patients with MM, we demonstrated that the most abundant clone harbored a unique clonal molecular fingerprint and that multiple unique clonotypic peptides compatible with MS measurements could be identified for all patients. Furthermore, the clonal immunoglobulin gene fingerprints of both the light and heavy chain remained stable during MM disease progression.

CONCLUSIONS: Our data support the use of the clonal immunoglobulin gene fingerprints in patients with MM as a suitable MRD target for MS-MRD analyses.

Introduction

Multiple myeloma (MM) is a plasma cell malignancy characterized by the clonal expansion of plasma cells that predominantly reside in the bone marrow. This population of malignant plasma cells secrete a characteristic monoclonal immunoglobulin (Ig), termed M-protein, which is used as biomarker for the diagnosis and disease monitoring of patients with MM. Improved treatment strategies for MM have led to an increased percentage of patients with MM who have minimal residual disease (MRD), in which disease activity can no longer be detected using routine M-protein diagnostics in blood and/or urine due to lack of analytical sensitivity. Therefore, novel methods to measure MRD are required that can accurately detect the myeloma disease activity beyond stringent complete remission (1).

The M-protein consists of unique variable domains that are generated by immunoglobulin V(D)J-gene rearrangements and somatic hypermutations at both the heavy and light chain gene loci in the clonal plasma cells (2, 3). This unique molecular fingerprint serves as a biomarker to detect MRD in the bone marrow of patients with MM using allele-specific oligonucleotide–quantitative PCR (ASO–qPCR) or next-generation sequencing (NGS) (1, 4). Bone-marrow biopsies are often needed, but these analyses are less suitable for dynamic MRD monitoring with sampling at multiple timepoints. More recently, mass spectrometry (MS) methods performed

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on peripheral blood have been developed as a less invasive complementary assay to monitor MRD (5–7). These ultra-sensitive blood-based MS-MRD assays quantify the M-protein by measuring the unique peptides derived from the variable region of the M-protein (clonotypic peptides) by liquid chromatography–tandem MS (LC–MS/MS) (8, 9). ASO–qPCR, NGS, and MS-MRD all rely on the ability to identify patient-specific V(D)J-gene rearrangements and somatically hypermutated gene sequences. A second requirement of these techniques is that the unique fingerprint remains stable over time [i.e., no ongoing somatic hypermutations within the V(D)J region during MM disease progression]. For MS-MRD, a third requirement is that at least one MS-compatible unique clonotypic peptide that contains at least one amino acid substitution compared to the predicted germline sequence should be present within the M-protein unique molecular fingerprint sequence.

The aim of this study was to further support the concept of using unique clonotypic fingerprint approaches to assess MRD in MM, via investigation of the analytical specificity and the stability of the clonal V(D)J region and the availability of clonotypic peptides. To this end, we performed a large-scale and unbiased analysis of the Multiple Myeloma Research Foundation CoMMpass database, the most comprehensive sequence database of patients with MM. This database comprises genomic profiling data of a large cohort of MM cases at diagnosis and follow-up points, which includes RNA-sequencing (RNA-seq) datasets of the purified $CD138^+$ plasma tumor cells. Based on the available transcriptomic datasets of 609 unique MM cases, we established the clonal fingerprint and the number of unique candidate clonotypic peptides in the M-protein sequence for each individual patient. Furthermore, we assessed the stability of the clonal fingerprint during MM disease progression, based on 136 paired sequences of 36 patients with MM for whom RNA-seq data were available for both the primary and relapsed tumor in the CoMMpass database.

Materials and Methods

THE COMMPASS DATABASE

The Multiple Myeloma Research Foundation provided access to the data from the CoMMpass study on the NIH dbGAP platform (accession: phs000748.v6.p4) (10). At the time of analysis, 609 RNA-seq datasets on newly diagnosed patients with MM were available to identify clonotypic peptides. For 36 patients with MM, RNA-seq data were also available from relapsed disease to analyze clonal Ig-fingerprint stability.

IDENTIFICATION OF CLONAL FINGERPRINT AND CLONOTYPIC PEPTIDES FROM COMMPASS DATABASE

RNA-seq data in the Multiple Myeloma Research Foundation CoMMpass database was used as a source for the clonal assembly and the identification of clonotypic peptides for each MM patient. Raw data files (Fastq) were obtained from Sequence Read Archive files and used with the MiXCR software package v.3.03 (11). The MiXCR analyses involved 4 steps: alignment, assembly, contig assembly, and export of clone assemblies. These steps of the analysis were run on a high-performance Linux cluster. The resulting data was further processed using custom scripts in R (v.3.5.3). Details on MiXCR analysis parameters, and any additional code involved is available (12).

After clone assembly by MiXCR, the most abundant clone assembly was determined for the heavy and light chain of each patient. The nucleotide sequences of the clone assemblies were translated by HIGH-VQUEST on the IMGT platform using default parameters to identify amino acid substitutions compared to their germline database (13–15). Clone assemblies that contained nonproductive rearrangements shorter than 50 amino acid length or imputed inserts were omitted from further analyses. Isoleucine to leucine and leucine to isoleucine alterations were removed due to their equal mass and thus inability to distinguish between these 2 amino acids with our fragmentation technique (collision induced dissociation). In silico proteolytic digestion was performed to determine the peptide sequences of the clone assembles with either trypsin (cleavage after lysine or arginine, except when followed by a proline), LysC (cleavage after lysine) or GluC (cleavage after aspartate or glutamate). Subsequently, MS-compatible peptides were selected based on length (5–30 amino acids) and location in sequence (nonterminal). MS-compatible peptides that were exclusively present in the sequence of the clone assembly, and thus contained amino acid alterations compared to its predicted germline sequence, were labeled as clonotypic peptides. To determine the number of unique clonotypic peptides per patient, the clonotypic peptides from both the heavy and light chain were considered. All in silico digestions and subsequent analysis were performed with in-house available scripts.

DETERMINE THE STABILITY OF CLONAL IG FINGERPRINT DURING MM DISEASE PROGRESSION

From the CoMMpass database patients were selected with RNA-seq datasets from both the primary and relapsed tumor. These paired RNA-seq datasets were processed with MiXCR and HIGH-VQUEST as described previously. To distinguish dominant from background sequences, assembled clones with <250 were

omitted from further analysis. To determine the stability of the clonal molecular fingerprint, pairwise Basic Local Alignment Search Tool analysis was performed for each primary-relapse pair of each patient on both the nucleotide sequences and translated amino acid sequences.

VALIDATION OF THE ALGORITHM TO IDENTIFY CLONOTYPIC PEPTIDES FROM RNA-SEQ DATA IN MM CELL LINES

Details regarding materials and methods are provided in the online Data Supplement. In short, clonotypic peptides were identified from RNA-seq data of MM cell lines using the algorithm described before. The clonotypic peptides were synthesized as stable isotope labeled standards. Targeted LC–MS/MS was performed on supernatants of the cultured MM cell lines to validate the identified clonotypic peptides as MS-MRD biomarker.

Results

ANALYSIS PIPELINE FOR CLONE ASSEMBLY AND THE IDENTIFICATION OF CLONOTYPIC PEPTIDES

To reconstruct the unique Ig-clonotype and identify clonotypic peptides from the variable region of the monoclonal immunoglobulins (M-protein), we designed an analysis pipeline (Fig. 1). First, we selected RNA-seq datasets obtained from bone-marrow derived $CD138^+$ plasma cells of a cohort of 609 newly diagnosed patients with MM present in the CoMMpass database. For each patient the most abundant heavy (IGH) and light chain (IGK/IGL) clone assembly was determined, which were clearly distinguishable from the polyclonal background in the $CD138^+$-enriched fraction (Fig. 2, A). One of the most common rearranged variable genes within the cohort of 609 patients with MM was *IGKV1-5*03*, which was present in 36 myeloma samples and which displayed a unique clonal fingerprint for each patient (Fig. 2, Supplemental Tables 1 and 2). One of the most common Ig heavy chain V genes was *IGHV3-33*01/3-33*06* ($n = 21$), which also showed a unique clonal fingerprint for each patient (Supplemental Fig. 1, Supplemental Table 3). The combination of V(D)J-gene rearrangements and somatic hypermutations resulted in a 100% unique clonal fingerprint in all 609 patients with MM (data not shown).

For MRD assessment by MS, it is required that MS-compatible peptides can be generated by protease digestion of the patient-specific Ig amino acid sequence. We used HighV-QUEST, which performs both translation of the nucleotide sequence and alignment to their germline database. In silico proteolytic digestion of the assembled clone and closest germline amino acid sequence was performed as described in the Materials and Methods section. The peptides that were exclusively present in the assembled clone sequence and contained at least one amino acid

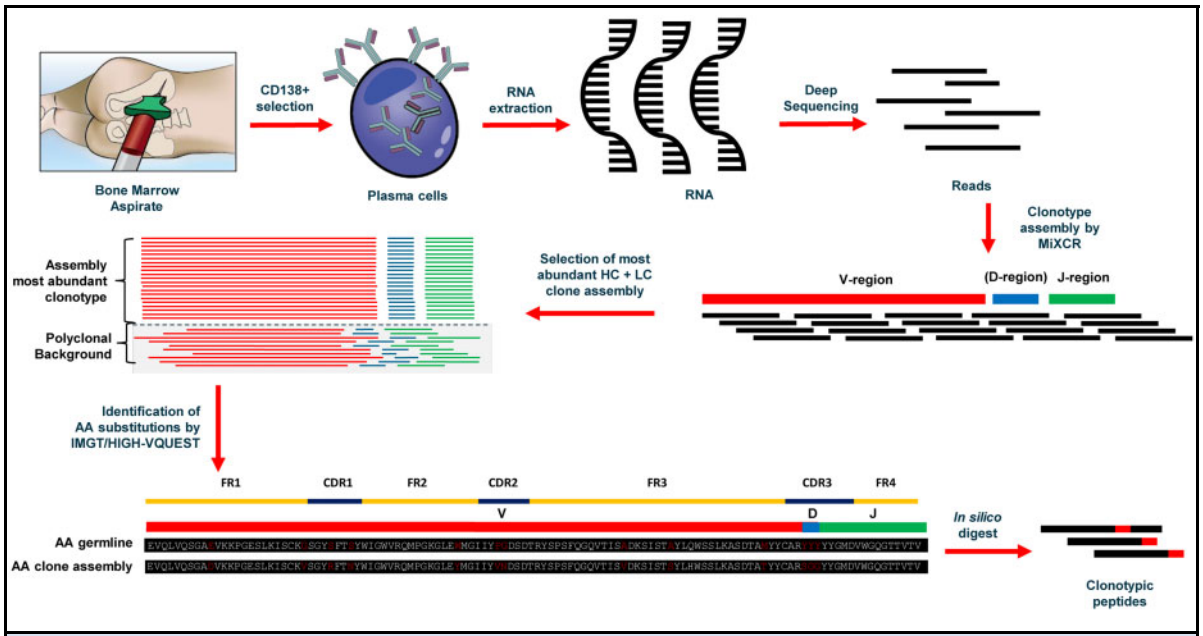

Fig. 1. Schematic representation of the clonotypic peptide selection pipeline of the M-protein based on transcriptomic datasets. AA, amino acid; CDR, complementary-determining region; FR, framework; HC, heavy chain; LC, light chain.

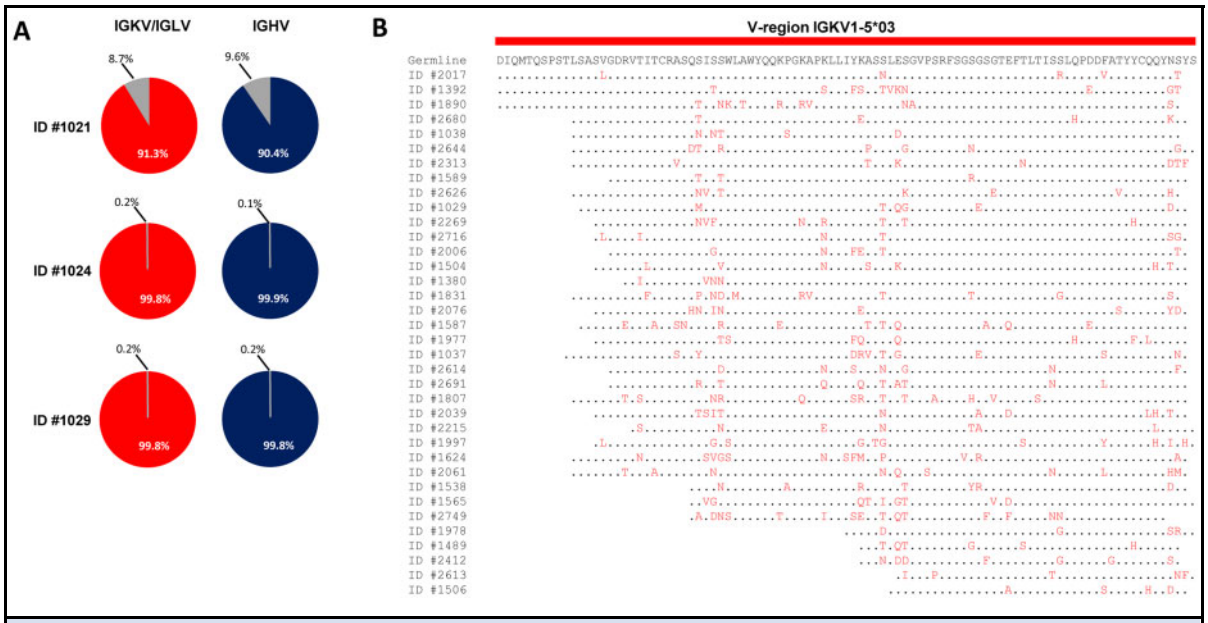

Fig. 2. (A) Abundance, in percentage of the total, of the most abundant light (red) and heavy (blue) chain clone compared to the polyclonal background (gray). (B) Uniqueness of a common V-region in the CoMMpass database. Amino acid alterations are depicted in red. IGHV, immunoglobulin heavy chain variable region; IGLV, immunoglobulin light chain lambda variable region; IGKV, immunoglobulin light chain kappa variable region.

alteration compared to the closest germline sequence were labeled as clonotypic peptides.

VALIDATION OF THE ANALYSIS PIPELINE BY DETECTING CLONOTYPIC PEPTIDES WITH MS

To investigate the validity of our analysis pipeline in its ability to define unique clonotypic peptides from RNA-seq datasets, and to detect these peptides by LC–MS/MS, a proof-of-concept study was performed. We used our analysis pipeline to predict the sequence of clonotypic peptides of 3 MM cell line models (RPMI8226, U266, and MM.1S) based on RNA-seq data. RPMI8226 and MM1.S secrete a lambda free light chain and U266 cells an IgE-lambda M-protein. We were able to identify at least one MS-compatible tryptic clonotypic peptide for each cell line. In RPMI8226 and MM1.S the clonotypic peptide originated from the light chain and in U266 the clonotypic originated from the heavy chain (Fig. 3, A and B). We subsequently developed targeted LC–MS/MS assays based on the predicted clonotypic peptides and measured these clonotypic peptides in culture medium supernatant of the cell lines. Since all 3 cell lines secrete a lambda light chain, we also traced a peptide from the constant region of the lambda light chain as a positive control. As expected, a specific signal of the relevant clonotypic peptide exclusively in the corresponding cell line was observed (Fig. 3, C), whereas the control-peptide from the constant region of the lambda light chain was detected in the supernatant of all 3 cell lines (Fig. 3, C). Although these measurements in a culture medium are different from tracking the M-protein in patient serum, our results indicate that our developed pipeline is capable of selecting MS-compatible clonotypic peptides from RNA-seq data, which can be used for targeted MRD-MS.

NUMBER OF CLONOTYPIC PEPTIDES PER PATIENT AFTER IN SILICO PROTEOLYTIC DIGESTION

For MS-based MRD monitoring, the presence of least one unique clonotypic M-protein peptide per patient is essential. To assess whether this requirement was met in our cohort of MM patients, we applied our analysis pipeline to the CoMMpass database. First, we determined the number of clonotypic peptides using in silico protein digestion of the assembled clone sequence with trypsin. This resulted in at least one clonotypic peptide in either the light or heavy chain in 97% of the patients ($n = 592/609$) with a mean of 4 (range 1–11) clonotypic peptides (Fig. 4, A). For 17 patients, no MS-compatible clonotypic peptides could be identified. These peptides were either too long or represented terminal sequences. Next, we assessed whether the use of an additional protease would increase the chance of identifying a MS-compatible unique clonotypic peptide.

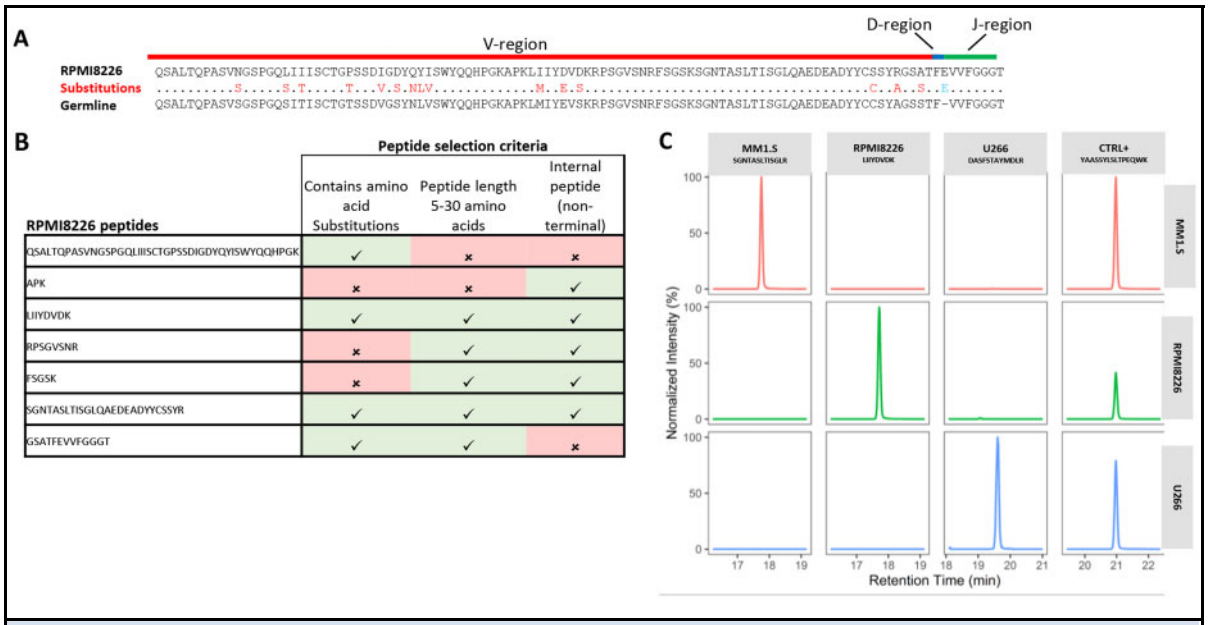

Fig. 3. (A) Alignment of RPMI8226 clone assembly to its closest germline. Highlighted in red are substitutions and in blue alterations. (B) Peptide selection based on given criteria. (C) LC–MS/MS analysis of the clonotypic peptides. Row-wise; respective peptide signals, column wise; measurement of the respective cell line. The *x* axis is retention time in minutes and the *y* axis is normalized to highest observed intensity.

To do so, we also applied in silico digestions with GluC and LysC proteases that display alternative cleavage site(s) to trypsin (Materials and Methods section), and combined the respective candidate clonotypic peptides with the tryptic digestion. We observed an additive effect, where the combination of these 2 independent digestions (Trypsin and GluC/LysC) led to a 100% success rate for the identification of clonotypic peptides in all patients (Fig. 4, A), with a mean of 9 clonotypic peptides per patient (range 1–23). From these results, we concluded that in most cases a tryptic digestion would be sufficient for the identification of M-protein specific clonotypic peptides. However, occasionally additional proteases will be required to generate sufficient clonotypic M-protein peptides for LC–MS/MS analysis.

FEATURES DEFINING THE IDENTIFICATION OF CLONOTYPIC PEPTIDES FROM RNA-SEQ DATASETS

We further characterized the clonotypic peptides and their relation to the clone assembly sequence to identify possible factors that can predict the number of tryptic clonotypic peptides per patient. The length of the sequences that could be assembled differed between clone assemblies and did not always cover the full length of the variable domain. The highest number of unique peptides could be identified in those patients with relatively long assembled sequences of the variable region (Fig. 4, B). Similarly, sequences with relatively more amino acid substitutions compared to the germline variant also corresponded to a higher mean number of unique clonotypic peptides that could be identified (Fig. 4, C).

We next investigated the location of the tryptic peptides in the rearranged V(D)J region to assess whether hotspot region(s) for clonotypic peptides could be identified. We selected clone assembly sequences with complete V(D)J-region sequences spanning from framework region 1 (FR1) to FR4 (Fig. 1). This resulted in the analysis of 482 clonotypic peptides derived from 102 heavy chain sequences and 241 clonotypic peptides from 93 light chain sequences. In general, clonotypic peptides for both the heavy and light chains could be observed across the entire variable region (Fig. 4, D). Clonotypic peptides derived from the heavy chain were most frequently derived from the middle part of FR3, followed by FR2. Alternatively, clonotypic peptides derived from the light chain were most frequently observed at the region FR2 and FR3. Taken together, these data show that the clonotypic peptides are observed in sequences located at both the variable complementary-determining region (CDR) and the more conserved FR regions, covering the full variable gene sequence.

Another requirement for clonotypic peptide-based M-protein diagnostics is compatibility of the identified clonotypic peptides with quantification by LC–MS/MS.

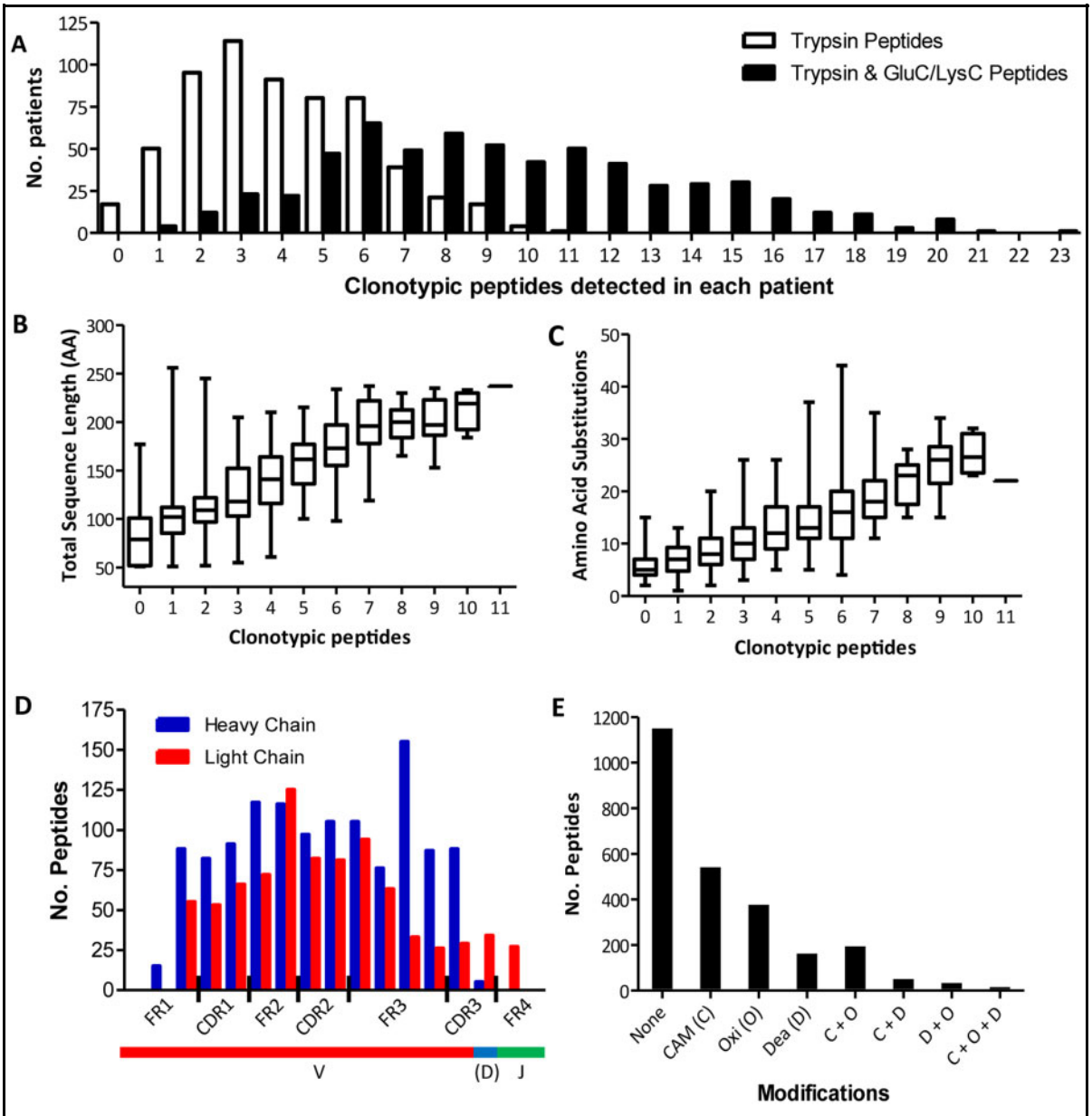

Fig. 4. (A) Clonotypic peptides per patient with trypsin or combined with LysC/GluC. Number of clonotypic peptides versus total sequence length (B) and amino acid alterations (C). (D) Peptide-localization per variable region of the heavy ($n = 482$) and light ($n = 241$) chains. (E) Number of modifications in 2521 clonotypic peptides. CAM, carbamidomethyl modification; CDR, complementary-determining region; FR, framework; Oxi, oxidation modification; Dea, deamidation modification.

Spontaneous chemical modification of a peptide can challenge their quantification by LC–MS/MS due to an induced mass shift, which may not be accounted for in the targeted analysis (16). Peptides prone to such modifications should therefore be avoided for reliable quantitation. Most modifications are oxidation of methionine or deamidation of asparagine/glutamine followed by a glycine or proline. Besides these modifications, cysteine is typically modified with carbamidomethyl to disable disulfide bridges from forming. We investigated to what extent these modification-prone amino acid residues were present in the in silico tryptic clonotypic peptides (Fig. 4, E). In total, 2521 clonotypic peptides were identified in 609 patients. From the 2521 clonotypic peptides, 1150 peptides (46%) contained no modification-prone amino acids, 1079 peptides (43%)

harbored one modification type, and 292 peptides (11%) showed 2 or 3 modification types. In conclusion, 88% of 2521 peptides contained either zero (preferred) or one (acceptable) potential modification sites, and were suitable for LC–MS/MS analysis.

STABILITY OF THE CLONAL IG FINGERPRINT DURING MM DISEASE PROGRESSION

A crucial aspect for applying the clonal Ig fingerprint as biomarker for MS-based MRD monitoring in patients with MM is the stability of the variable Ig-region sequence during disease progression. To test clonal Ig stability, we selected RNA-seq datasets from patients in the CoMMpass database from whom both the primary and relapsed tumor data was available. In total, we analyzed 136 paired sequences (72 light chain and 64 heavy chain paired sequences) of 36 MM patients, and assessed the range of these alignments (Fig. 5, Supplemental Tables 4 and 5). For some cases, the clone assembly sequence of either the primary or relapsed tumor extended beyond the sequence of its counterpart (Fig. 5, A, ID #1783, ID #1401). These additional amino acids were excluded for analysis. We also observed full overlapping sequences in which the assembled clone sequences of the primary and relapsed tumor were of the same length (Fig. 5, A, ID #1700). Alignment of the assembled clones comparing primary and relapsed MM revealed a 100% amino acid sequence identity for both heavy chain and light chain clonotypes (Fig. 5, B and C). Furthermore, silent nucleotide mutations were also not observed (data not shown).

We concluded that the amino acid and nucleotide sequence of the entire variable region of both the heavy and light chain of the M-protein, and thus also the sequence of derived clonotypic peptides, were not altered during disease progression for these patients. As such, ongoing somatic hypermutations in the Ig genes were therefore rare events in MM patients, indicating that the clonal Ig region could be considered a reliable biomarker target for long-term MRD assessment in MM.

Discussion

MM pathogenesis is characterized by a progressive stepwise acquisition of somatic gene mutations, that further evolve after MM diagnosis and during treatment. This results in a complex genomic architecture with domination of the fittest myeloma subclone (17, 18). ASO–qPCR and NGS methods to measure MRD in bone marrow (1) and blood-based MS-MRD assays (5–7) all rely on a unique and stable clonal Ig fingerprint. This obviously triggers the question how well-suited the rearranged clonal Ig genes in myeloma cells are to MRD testing in patients with MM.

Based on the RNA-seq data of 609 MM patients in the CoMMpass database, we showed that the Ig clone signature was unique and harbored multiple MS-

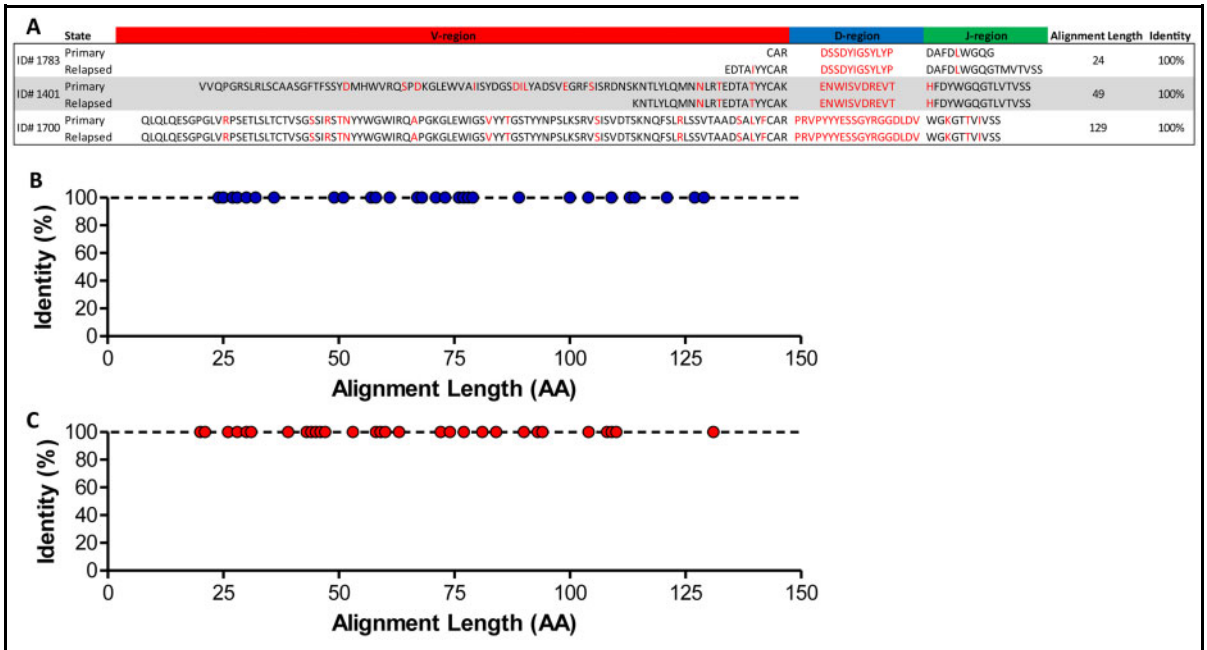

Fig. 5. (A) The range of findings for the sequence comparison between primary and relapsed clonal Ig sequence. (B, C) Comparison of clone assembly sequence for the heavy and light chain variable region between primary and relapse cases. The *x* axis represents the alignment lengths in amino acids and the *y* axis the percentage of the sequence identity. AA, amino acid.

suitable clonotypic peptides for each patient. Based on 136 paired sequences of 36 patients with MM with disease relapse, we demonstrated that the clonal Ig-rearrangements were stable during MM progression. These findings support the use of the clonal Ig fingerprint in patients with MM as suitable MRD target for MS-MRD analyses, since it constantly identifies the myeloma clone responsible for relapse.

Our data are in line with a study showing that the dominant MM clone retains a unique and stable CDR3 sequence of the IGH gene (19). The V(D)J-rearrangements and somatic hypermutations are unaffected by the selection pressure induced by treatment and disease progression. Here, we demonstrate stability of the clonal Ig fingerprint extending beyond the CDR3 and also to be retained in the light chain gene. This indicates that the full variable Ig region of both the heavy chain and the light chain can be targeted for MRD assessments. The stable Ig fingerprint we observe in MM strongly contrasts with B-cell acute lymphoblastic leukemia, in which continuous Ig gene rearrangements occur throughout the disease course in approximately 30–40% of the patients (20, 21).

Due to improved efficacy of novel treatments an increased percentage of patients with MMs reach a state of MRD, where the disease load is below the detection level of conventional M-protein diagnostics (22, 23). In the current International Myeloma Working Group guidelines, the methods to assess MRD in patients with MM are all performed on bone-marrow aspirates (1). However, due to the burden this imposes on the patients, less invasive alternatives would be preferred, such as monitoring MRD in peripheral blood. Furthermore, MRD monitoring in peripheral blood would allow for serial MRD measurements. Therefore, LC–MS/MS techniques have been developed based on the unique clonotypic features of the M-protein in serum (5). The ability to select MS-suitable clonotypic peptides in each patient with MM is crucial for wide-scale implementation of MS-MRD assays.

Earlier studies performed in small cohorts with fewer than 10 patients with MM have shown that a unique clonotypic peptide can be detected in each patient (8, 9). Here, we showed that clonotypic peptides could be identified in all 609 patients with MM and that most of these peptides are suitable for LC–MS/MS analysis. The algorithm we developed to identify clonotypic peptides in silico from RNA-seq data was successfully validated in 3 MM cell lines.

In the large-scale CoMMpass database study, a mean of 4 tryptic clonotypic peptides were identified in each patient with MM. The use of alternative nontryptic proteases can further increase the number of identified unique clonotypic peptides per patient. It is important to be aware that new challenges can be introduced when using alternative endoproteinases. For example, the lack of basic residues in peptides can hinder ionization and digestion efficiency may vary. The availability of multiple candidate clonotypic peptides per patient allows for the selection of the most optimal peptides that have the highest analytical sensitivity when analyzed by LC–MS/MS and lack modification-prone residues. The clonotypic peptides could be observed across the entire Ig variable region, thus for optimal peptide selection it is advisable to sequence the full Ig variable region. Our data are in line with a study concluding that clonotypic peptides are distributed throughout the variable region of the light chain and are most frequently identified in the CDR2 > CDR1 > CDR3 (24). For a robust and confident M-protein quantification by the MS-MRD assay, it is ideal to select a clonotypic M-protein peptide from both the heavy and light chain. On the one hand, this provides a quality check on the measurement; on the other hand, the M-protein can be quantified based on the mean of both clonotypic peptides. If both clonotypic peptides are measured, it becomes feasible to also monitor disease activity in patients with free light chain escape (25). We estimate the time required for in silico clone assembly based on RNA-seq data, peptide selection, and start of peptide testing by LC–MS/MS to be 1 week.

The current analysis pipeline was constructed to extract clonotypic peptide sequences based on RNA-seq datasets. In theory, this approach could also be applied to other types of input data. For example, whole-genome sequencing, whole-exome sequencing datasets, as well as amplicon-based NGS applications (26–28) also harbor the information regarding the sequence of the functional immunoglobulin gene rearrangements within the clonal plasma cells. Unlike RNA-seq datasets, genomic DNA-based datasets also contain intronic regions and the nonfunctional rearrangements of the variable region of B-cell receptor, which have to be accounted for when analyzing these datasets.

In conclusion, our results show that the monoclonal Ig clone signature is unique and harbors multiple MS-suitable clonotypic peptides for each patient with MM. Furthermore, the clonal Ig-rearrangements of both the heavy chain and light chain Ig loci are stable during MM disease progression. These data further support the use of the clonal Ig fingerprint in MM patients as a suitable MRD target for MS-MRD analyses.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: MM, multiple myeloma; Ig, immunoglobulin; MRD, minimal residual disease; ASO–qPCR, allele-specific

oligonucleotide–quantitative PCR; NGS, next-generation sequencing; MS, mass spectrometry; LC–MS/MS, liquid chromatography–tandem mass spectrometry; RNA-seq, RNA-sequencing; IGH, immunoglobulin heavy chain; IGK, immunoglobulin light chain kappa; IGL, immunoglobulin light chain lambda; FR, framework; CDR, complementary-determining region.

Human Genes: IGHV, immunoglobulin heavy chain variable region; IGLV, immunoglobulin light chain lambda variable region; IGKV, immunoglobulin light chain kappa variable region.

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