



OPEN Dynamic monitoring of M-protein quantification by immunotyping using capillary zone electrophoresis during the chemotherapy of patients with multiple myeloma

Zhongwei Jia & Qiong Lu

Dynamic quantification of monoclonal immunoglobulin proteins (M-proteins) by immunotyping using immunosubtraction (ISUB) through capillary zone electrophoresis (CZE) was performed to examine the efficacy of chemotherapy drugs in patients with multiple myeloma (MM). Twenty-one patients with eight different types of M-protein were analyzed, and M-protein quantification during chemotherapy regimens was dynamically monitored. For patients with M-protein identified by CZE, immunotyping by ISUB can accurately determine the percentage of M-protein. In this study, 15 of the 16 included patients with a definite diagnosis of MM were initially treated with bortezomib chemotherapy, and the treatment efficacy differed significantly among individuals. Three patients showed M-protein clearance, with the M-protein decreasing by more than 50% after the first course of treatment. Capillary-based immunotyping accurately determined the percentage of M-proteins. Dynamic monitoring of M-protein through immunotyping using ISUB can objectively and effectively aid in evaluating treatment efficacy. Clinically, chemotherapeutic drugs that reduce M-protein levels by more than 50% after a treatment course should be selected. The early detection of trace changes in M-protein levels is crucial for disease monitoring and medication guidance. Quantification of M-protein should be regularly undertaken in patients with MM.

Keywords Monoclonal Immunoglobulin proteins, Immunotyping, Immunosubtraction, Capillary zone electrophoresis, Multiple myeloma, Chemotherapy

Abbreviations

M-proteins	Monoclonal immunoglobulin proteins
ISUB	Immunotyping by immunosubtraction
CZE	Capillary zone electrophoresis
MM	Multiple myeloma
IMWG	International Myeloma Working Group
Ig	Immunoglobulin
ELP	Electrophoresis
FLC	Serum free light chain
IFE	Immunofixation electrophoresis
CR	Complete response
VGPR	Very good partial response

According to the International Myeloma Working Group (IMWG) guidelines, in addition to being a diagnostic criterion for multiple myeloma (MM), monoclonal immunoglobulin protein (M-protein) quantification can be used to evaluate treatment efficacy in patients^{1,2}. However, quantifying proteins has always been a problem in clinical practice. Although immunoglobulin quantitative detection has been widely used in clinical practice, the results include both monoclonal and polyclonal antibodies, and therefore, we cannot accurately assess the true level of an M-protein in patients. Particularly in patients with low levels of M-protein, quantitative

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immunoglobulin levels may be within the reference range, and there will be a large deviation in judging the M-protein level based on immunoglobulin quantitation. Agarose immunofixation electrophoresis (IFE) is a traditional method for the identification of M-proteins; however, it has only been reported qualitatively. For patients under treatment, the M-protein level may be in a downward trend, but the IFE may still be positive during monitoring. Therefore, based on the qualitative results of IFE, improvements from or ineffectiveness of drug treatment cannot be accurately assessed. However, immunotyping using immunosubtraction (ISUB) not only detects the presence of M protein but also quantifies the M protein in the capillary zone electrophoresis (CZE) image. This approach yields the percentage area of M-protein in the total protein, which when multiplied by the quantity of the total protein can quantify the M-protein^{3–5}. For the same patient, the position of M-protein in the CZE image before and after treatment is relatively fixed. Therefore, theoretically, if the area of the M-protein in the CZE image of the same patient after treatment is reduced, the drug treatment can be considered effective. In this retrospective study, we used ISUB immunotyping to quantitatively and dynamically monitor M-proteins in 21 patients for up to three years, to assess the practicability and its clinical value in evaluating the efficacy of chemotherapy.

Methods

Patient selection

Sixteen patients with MM admitted to the Department of Hematology, the First Affiliated Hospital of Anhui Medical University from 2021 to 2024 were retrospectively examined, including three cases each of IgG κ type, IgG λ type, κ light chain-only type, λ light chain-only type, and two cases each of IgA κ type and IgA λ type. In addition, three patients with IgM κ small B-cell lymphoma and two with IgM λ Waldenstrom macroglobulinemia were also enrolled. The patient cohort consisted of 13 males and 8 females with a median age of 66.0 (55.0–72.0) y. All patients underwent pathological analysis of their bone marrow. The efficacy criteria for patients referred to the 2016 IMWG efficacy criteria⁶. Complete response (CR): Negative immunofixation (IF) on the serum and urine and disappearance of any soft tissue plasmacytomas, and <5% plasma cells in bone marrow aspirates. Very good partial response (VGPR) : Serum and urine M-protein detectable by immunofixation (IF) but not on electrophoresis (ELP) or $\geq 90\%$ reduction in serum M-protein plus urine M-protein level <100 mg per 24 h. Blood samples were analyzed by immunotyping (Capillarys 2 Flex Piercing; Sebia, Lisses, France), serum IFE (Hydrasys 2 Scan Focusing Analyzer; Sebia), and total protein quantitation (Cobas 8000 Series Modular Analyzer; Roche Diagnostics, Mannheim, Germany).

Immunotyping electrophoresis

Immunotyping was performed in four automated steps. First, 15 μ L serum sample was diluted with 285 μ L specific diluent preloaded in the antisera segment. Then, 15 μ L of the diluted serum was mixed with 20 μ L of specific antiserum. The prepared samples were injected by simultaneous aspiration into six capillaries at the anodic end, and the proteins were separated by ELP at high voltage. The separated proteins were detected at the cathodic end of the capillaries at 200 nm. Finally, superimposition of the ELP pattern with the antisera patterns (IgG, IgA, IgM, kappa and lambda) permits the characterization of the suspected monoclonal component.

Immunofixation electrophoresis (IFE)

IFE requires manual dilution and sample addition. According to the instructions provided by the manufacturer, 20 μ L of sample serum was mixed with 100 μ L of specific diluent for the IgG immunofixation lane, and 30 μ L of sample serum was mixed with 60 μ L of specific diluent for the ELP reference lane and the IgA, IgM, kappa and lambda immunofixation lane. Then, 10 μ L of properly diluted serum was added to the applicator wells, then the migration program was selected from the instrument menu. After electrophoresis, 8 μ L ELP fixative solution and individual specific antisera were added into the corresponding lanes using the dynamic mask with the instrument. The assay was then completed using incubation, staining, and decolorization steps. After electrophoresis, one track served as a reference, providing the complete electrophoretic pattern of the proteins in the sample. The remaining five tracks allow the characterization of the monoclonal component from its reaction, or lack thereof, with antisera against gamma (IgG), alpha (IgA), and mu (IgM) heavy chains and against free and bound kappa and lambda light chains. The immunofixed bands were then compared with the suspected bands in the reference pattern.

Statistical analysis

The experimental data were statistically analyzed using SPSS 26.0 software (IBM Corp., Armonk, NY, USA). A dynamic monitoring chart for M-protein during chemotherapy was prepared using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA).

Ethics approval and consent to participate

This study was approved by the Ethics Review Committee of the First Affiliated Hospital of Anhui Medical University, Anhui, China (reference number: PJ 2024-12-73) and was conducted in accordance with the guidelines of the Declaration of Helsinki. The need for individual patient informed consent was waived.

Results

M-protein identification by immunotyping using ISUB

The location and shape of the M-protein peak in the CZE images differed between patients (Fig. 1). Capillary immunotyping by ISUB can identify different types of M-proteins in patients and determine the exact location of the M-proteins on CZE images. The area of this position was marked with a tangent line using Capillarys 2

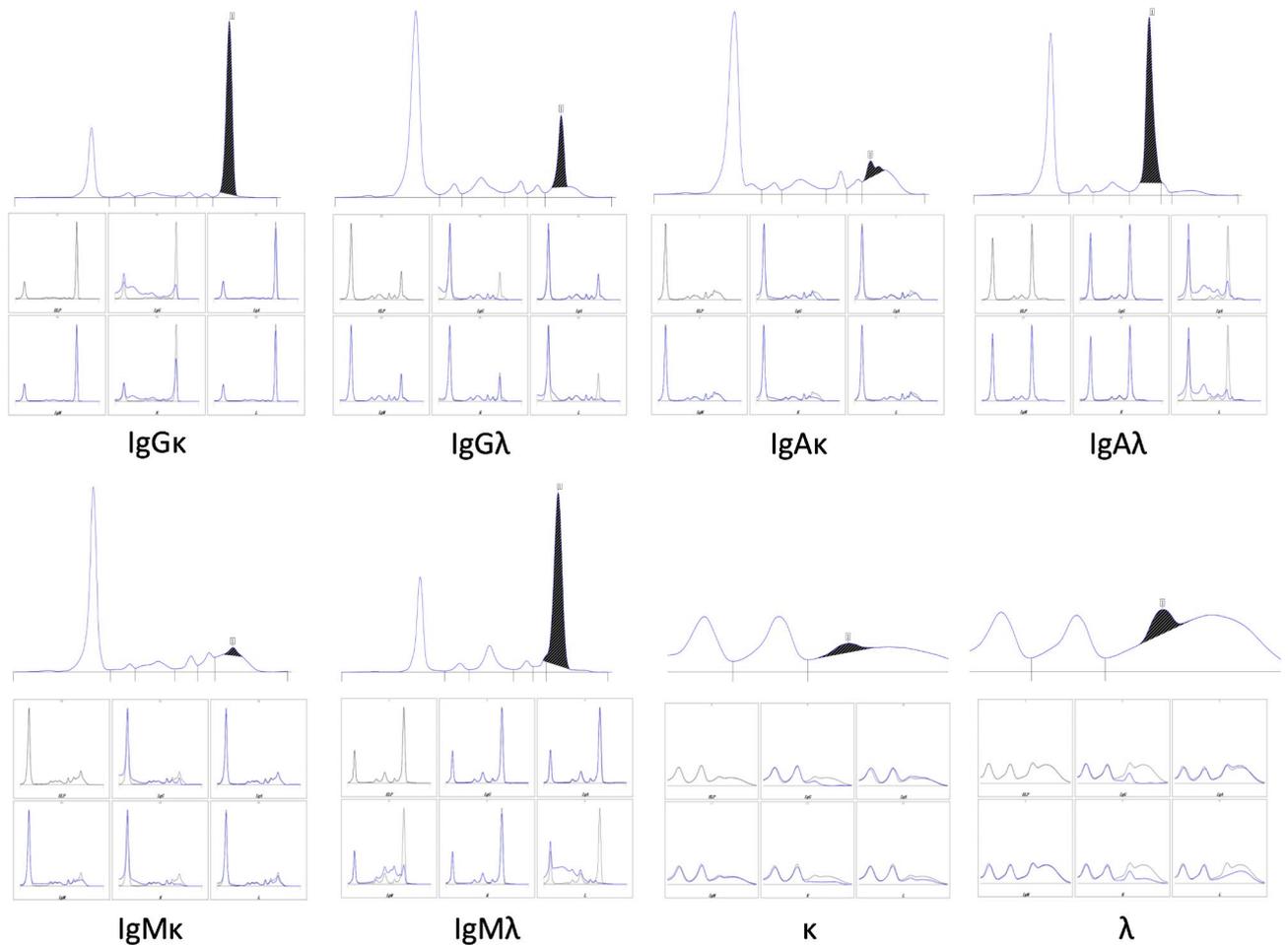


Fig. 1. M-protein identification by immunotyping through immunosubtraction. The shaded areas in the figure represent the areas where M-protein was present in the capillary zone electrophoresis map. Each panel represents the capillary zone electrophoresis image from different patients with the indicated type of M-protein.

software provided by the manufacturer. The software analyzed and calculated the percentage of the total area of the serum protein electrophoresis map in the shaded area.

Comparison of the results of immunofixation electrophoresis (IFE) and immunotyping by ISUB for M-protein in the β region

The IFE result of a patient with M-protein in the β region (Fig. 2A) is shown in the protein electrophoresis (ELP) lane in the figure. Here, M-protein overlapped with the normal proteins in the β zone and was difficult to distinguish, hence, the proportion of M-protein could not be determined. For the ISUB results in Fig. 2B, after incorporating antibody electrophoresis results compared with ELP, the location of the M-protein in the ELP could be determined more precisely. The area occupied by M-protein was accurately delineated by employing the software provided by the Capillaries 2 system (Fig. 2C).

Quantitative dynamic monitoring of M-protein to monitor the efficacy of drugs

Sixteen patients with MM were treated with four different chemotherapy drugs, and the observation duration for each patient ranged from three months to three years. Among them, 15 patients received bortezomib chemotherapy for the first time; however, the efficacy differed significantly. Assessment of M-protein after treatment completion showed that the M-protein level decreased in 12 patients and increased in three patients. Of the 12 patients with reduced M-protein levels, two showed no satisfactory decline upon subsequent treatment, while three had increased levels of M-protein after the first treatment, and the treatment of these five patients was replaced with daratumumab. M-protein decreased markedly after a course of daratumumab in four patients; contact was lost with the other patient. At the end of the observation period, seven patients achieved a CR or a VGPR. As a common feature, the level of M-protein decreased by more than 50% after the first treatment (Fig. 3; Table 1).

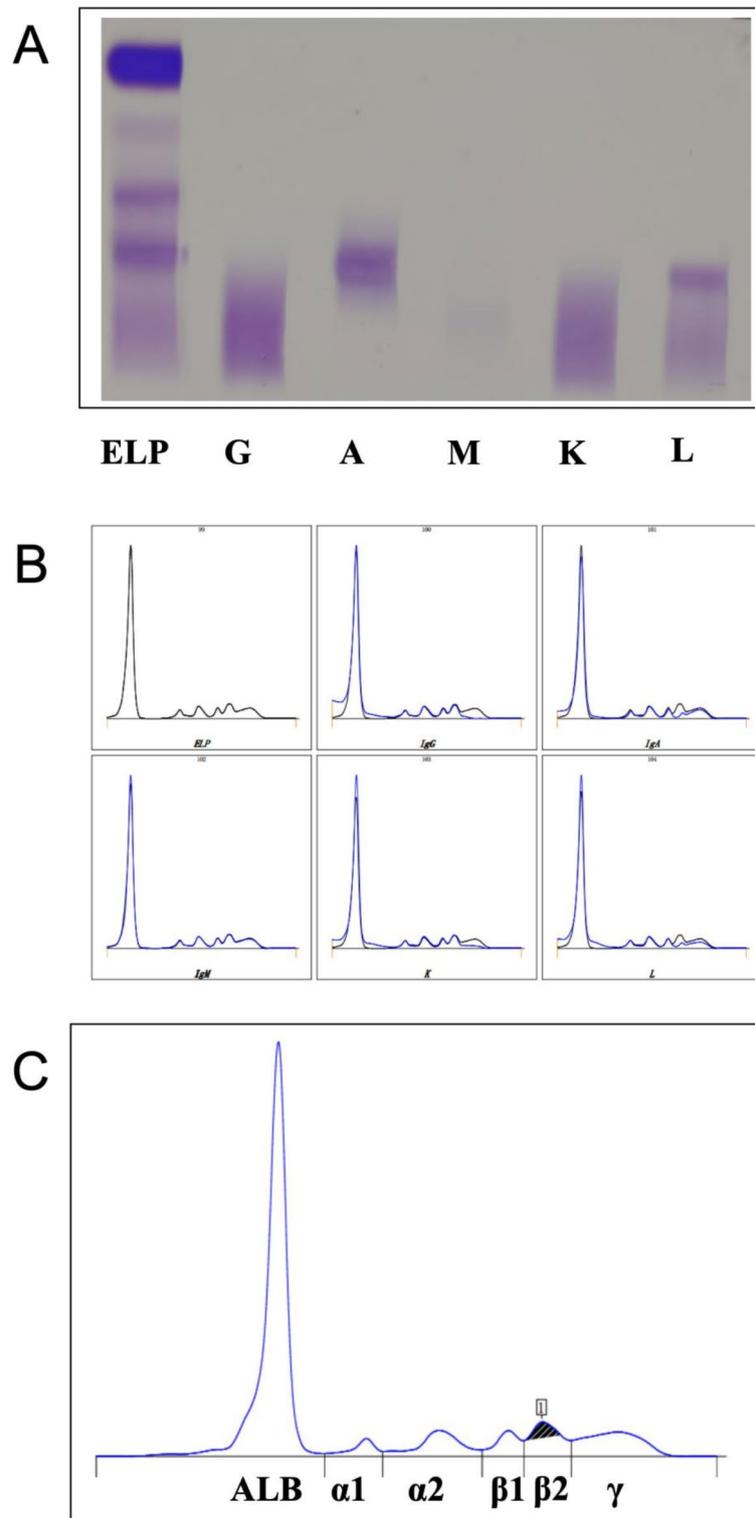


Fig. 2. Comparison of the results of immunofixation electrophoresis and immunotyping in the β region of M-protein (IgA λ). All three subfigures were derived from the same patient, a 77-year-old male, found during an inpatient examination in the Department of Nephrology. (A) Immunofixation electrophoresis (IFE). In the electrophoresis (ELP) lane, the M-protein is indistinguishable from the normal protein in the β zone; in addition, the color intensity in the antibody lane is higher than the same position in the ELP lane (for example, the color intensity is significantly higher in the IgG lane than that in the γ zone of the ELP lane). (B) Immunotyping using immunosubtraction. The electrophoresis pattern after adding antibodies was compared with that of ELP, and the position of M-protein in ELP can be distinguished. (C) Capillary zone electrophoresis, with the position of the M-protein identified in B marked by a tangent line.

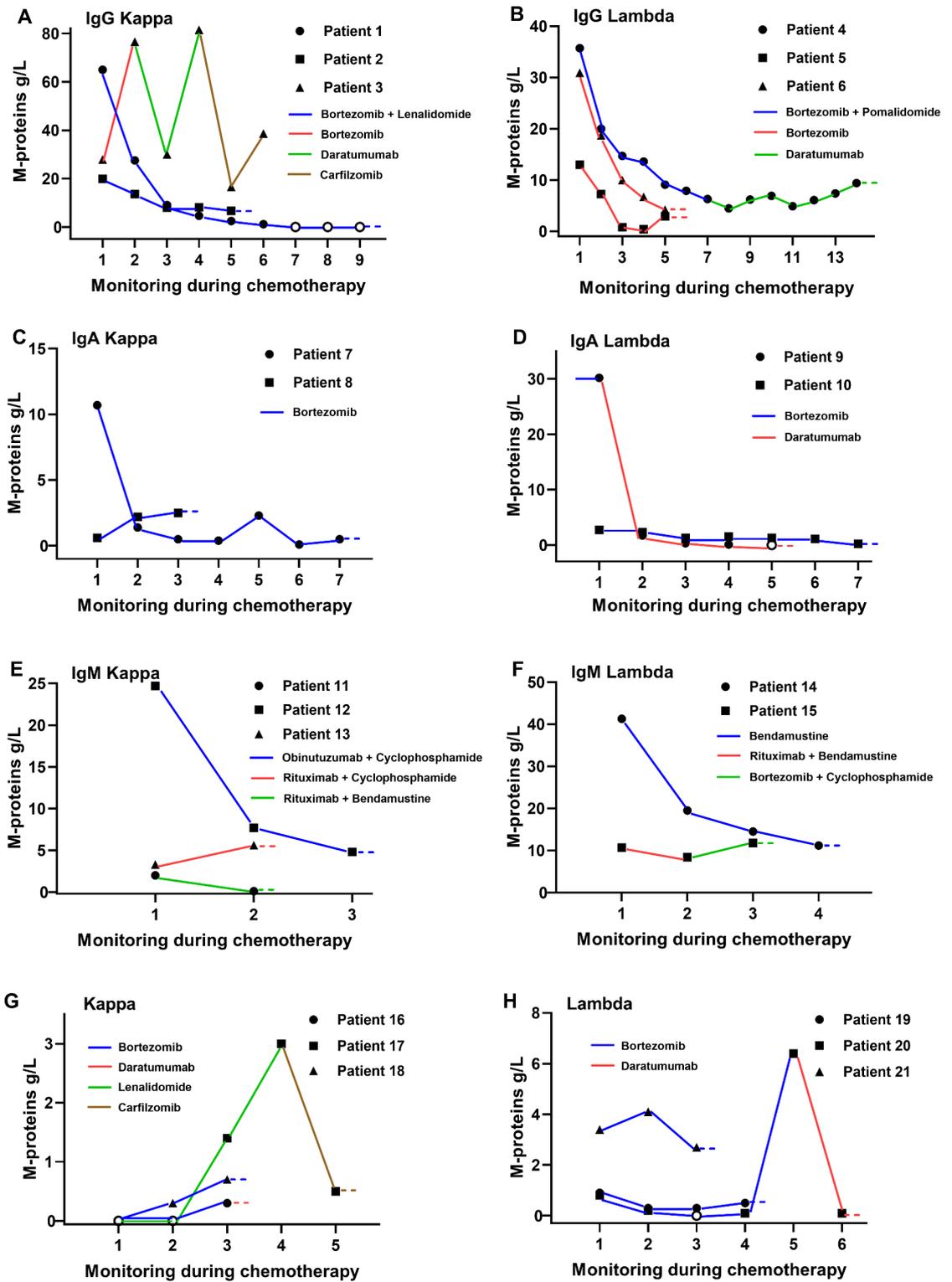


Fig. 3. Quantitative monitoring of changes in M-protein levels in patients during chemotherapy. In the same figure panel, different colored lines between the M protein monitoring points of patients represent different chemotherapy regimens.

Discussion

MM is a malignant disease characterized by the abnormal proliferation of clonal plasma cells and is the second most common malignancy in the blood system, predominantly affecting older people, and remains incurable⁷⁻⁹. For patients with relapse and drug resistance, unused regimens, including drugs with new mechanisms of action, should be actively selected¹⁰. In recent years, several new chemotherapeutic drugs have been marketed

Patient number	Sex	Age (years)	Newly diagnosed at the first monitoring	Interval between the monitoring point and the previous one (days)						
				1	2	3	4	5	6	7
1	F	66	Yes	0	37	33	35	29	34	30
	The 8th to 9th monitoring			45	29					
2	F	50	No	0	44	27	35	38		
3	M	55	No	0	140	160	173	79	96	
4	F	56	No	0	37	40	45	46	36	28
	The 8th to 14th monitoring			40	35	35	99	36	43	46
5	F	55	Yes	0	42	229	107	152		
6	M	79	No	0	32	65	29	33		
7	M	63	Yes	0	28	31	154	88	98	55
8	M	55	No	0	52	35				
9	M	77	No	0	41	84	21	122		
10	M	74	No	0	28	38	76	53	71	104
11	M	68	Yes	0	301					
12	M	72	Yes	0	61	30				
13	M	79	Yes	0	70					
14	F	72	Yes	0	58	31	29			
15	M	60	Yes	0	101	140				
16	M	53	Yes	0	367	116				
17	F	67	No	0	407	343	176	43		
18	M	70	No	0	169	221				
19	F	66	Yes	0	47	31	35			
20	M	53	Yes	0	32	17	105	510	43	
21	F	56	Yes	0	100	106				

Table 1. The 21 patients in Fig. 3 and the time interval for monitoring M-protein during chemotherapy. Age was recorded when first monitored.

for the treatment of MM^{11,12}, and the choice of chemotherapeutic drugs for patients with MM is increasing. However, evaluation of the effectiveness of chemotherapy drugs in individual patients requires a practical approach. Patients with MM require long-term chemotherapy to achieve maximum remission and prolonged progression-free survival, necessitating a reliable and practical detection method to evaluate treatment efficacy. The effectiveness of previous rounds of chemotherapy is the key to determining whether to continue the same regimen.

M-protein is a monoclonal antibody secreted by the abnormal monoclonal plasma cells. When these abnormal monoclonal plasma cells are targeted and die under the action of chemotherapy drugs, the production of M-protein correspondingly decreases^{13,14}. Therefore, dynamic quantification of M-protein can be used for evaluating the efficacy of chemotherapy drugs in the treatment of patients with MM. Although quantitative detection of immunoglobulins is widely used in clinical practice, elevated levels of immunoglobulin alone cannot prove the presence of M-protein because this elevation in levels could be the product of reactive proliferation of polyclonal plasma cells. Even when immunoglobulin quantitative results are normal, low concentrations of M-protein may also be present. In particular, in patients with MM, chemotherapeutic drugs may inhibit normal immunoglobulin production, and the combined levels of immunoglobulin quantification (M-protein plus normal immunoglobulin) may fall within the normal reference range. Therefore, immunoglobulin quantification alone cannot evaluate efficacy in patients with MM with low concentrations of M-protein. Nonetheless, lower levels of M-protein can be detected by IFE.

IFE is a traditional approach employed for M-protein identification¹⁵. In this study, we observed a significantly higher color intensity in the antibody lane than in the ELP lane, as shown in Fig. 2. We hypothesized that because the serum was initially electrophoresed in separate lanes, the ELP lane received the fixed solution after electrophoresis, whereas the antibody was added to the antibody lane and incubated. Subsequently, proteins that did not undergo an antigen-antibody reaction were eluted and stained. The staining solution stained the M-protein, as well as the protein bound to in the labeled antibody; hence, the color intensity in the antibody lane was significantly higher than that in the ELP lane. Therefore, the color intensity of the M-protein in the antibody lane, visualized by the optical density scanner, showed a significant increase in the M-protein content in the ELP lane.

The advantage of immunotyping using ISUB over IFE is that the former is performed after adding specific antibodies to the serum. This ensures that only the serum components that react with the corresponding antibodies are visualized, and the immune complexes that form migrate to the albumin region for enhanced identification of the components. Comparison of electrophoresis results pre- and post-addition of antibodies can clarify the location of the M-protein and allow determination the proportion of the area of the M-protein

region. The ISUB process is fully automated, in contrast to the IFE method, which involves manual steps, thus the ISUB method enhances the repeatability of the test results.

The position of the M-protein in the ISUB images of the same patient is relatively fixed, and CZE, which has excellent repeatability, was used. Laboratory technicians can compare this test with previous tests, facilitating the observation of the dynamic change in the M-protein peak by comparing the figures before and after the test for the same patient. This comparison can distinguish subtle changes in protein area of the area of the protein. Immunotyping using ISUB to dynamically monitor M-protein can objectively assess the effect of chemotherapy and tumor cell residues and aid in monitoring the recurrence of MM.

Currently, several chemotherapeutic drugs are available for the treatment of MM, a few of which are monoclonal antibody drugs. Daratumumab is an anti-CD38 IgG κ monoclonal antibody, and, therefore, may interfere with the quantitative estimation of M-protein^{16,17}. Daratumumab has a relatively consistent molecular weight, leading to a relatively fixed position in the elimination stage of immunotyping maps and generally does not comprise > 1% of the total protein. We suggest that a patient's M-protein is quantified before the treatment cycle to avoid high blood concentrations of the monoclonal antibody drug, which may affect the quantitation results. The effect of drugs can also be eliminated through dynamic observation of changes in the M-protein peak type, location, and proportion.

In this study, immunotyping using ISUB at first diagnosis revealed that three patients with κ light chain-type MM did not exhibit M-protein. However, investigation revealed that the patients had abnormal serum free light chain (FLC), positive IFE, and multiple bone lesions. Theoretically, immunotyping using ISUB could distinguish the change in area of 0.1% protein, with the maximum detection sensitivity of about 100 mg/L¹⁸, whereas the 95% reference interval for kappa FLC is 3.3–19.4 mg/L¹⁹. Patients with MM having κ light chain only and whose M-protein was < 100 mg/L could not be detected due to detection limits. Concurrently, in the blood of patients with κ light chain-only type MM, there may be a large number of incomplete free κ light chain fragments, and the molecular weight of these incomplete κ FLC fragments is lower than that of κ FLC molecules (22.5kD)²⁰. Therefore, patients with κ light chain-only MM may have a high molar number FLC fragment molecules, but quantitation may be low. The low serum level makes it difficult to prove the presence of M-protein by immunotyping using ISUB. The large number of FLC molecules facilitates their detection by IFE (which is chromogenic to antigen-antibody complexes). Therefore, serum FLC and IFE are recommended for patients with κ light chain-only MM as indicators of diagnosis and efficacy evaluation.

This study shows that dynamic monitoring of quantitative changes in the magnitude of M-protein can be used as a basis for changing chemotherapy regimens. In our cohort, 16 patients with MM were treated with multiple combination chemotherapy regimens; five patients achieved CR, and three achieved VGPR after treatment with the first selected chemotherapy regimen. Monitoring of M-protein revealed that the M-protein levels in these eight patients decreased by > 50% after the first course of chemotherapy, and by 90% in some patients, while it did not reach 50% in patients with partial remission and other types of outcome (the remaining eight of 16 patients). If the M-protein level decreases by < 50% after the first course of treatment, adherence to the initial chemotherapy regimen may not yield a better effect, and a replacement regimen should be considered. We found that three of the five CR patients had relapsed at a later stage, with significant changes in their M-protein levels. Therefore, early detection of micro-changes in M-protein is crucial for monitoring the disease and guiding drug use, and M-protein quantitation should be regularly reviewed in the clinic.

Although new chemotherapeutic drugs for MM continue to be developed, their effects on individuals vary. Patients with the same type of MM exhibit different sensitivities to the same chemotherapeutic drugs. In addition, long-term use of chemotherapeutic drugs for the treatment of MM may have side effects. The efficacy observed in the most recent round of chemotherapy is key to deciding on continuation of the current regimen; therefore, an appropriate evaluation method is particularly important. Quantitative and dynamic measurement of M-protein by immunotyping using ISUB is a reliable and objective index for assessing the rational use of chemotherapy drugs in treating patients with MM. The assay requires only blood drawing and not bone marrow puncture and is thus easily accepted by patients. Based on the M-protein dynamic monitoring results, clinicians can objectively assess the effect of the last round of chemotherapy. This evaluation helps them decide whether to continue the original chemotherapy regimen or change to other treatment regimens, especially for patients with relapsed and refractory MM.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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References

1. International Myeloma Working Group. Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the international myeloma working group. *Br. J. Haematol.* **121** (5), 749–757 (2003). PMID: 12780789.
2. Rajkumar, S. V. et al. International myeloma working group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol.* **15** (12), 538–548 (2014).
3. Miyazaki, K. & Suzuki, K. Capillary electrophoresis/immunofixation as a better alternative to immunofixation for detecting and immunotyping serum monoclonal proteins in patients with Immunoglobulin light chain (AL) amyloidosis. *Amyloid* **23** (4), 221–224 (2016).
4. Cho, H. et al. Assessment of M-protein quantification using capillary electrophoresis and immunofixation-based integration in clinical samples with low M-protein concentrations. *Clin. Biochem.* **107**, 7–12 (2022).

5. Schroeder, L. F., Huls, F., Williams, C. L., Li, S. H. & Keren, D. F. A novel approach to estimating M-Protein concentration: capillary electrophoresis quantitative Immunosubtraction. *J. Appl. Lab. Med.* **2**, 914–919 (2018).
6. Kumar, S. et al. International myeloma working group consensus criteria for response and minimal residual disease assessment in multiple myeloma. *Lancet Oncol.* **17**, e328–e346 (2016).
7. Mukkamalla, S. K. R. & Malipeddi, D. Myeloma bone disease: A comprehensive review. *Int. J. Mol. Sci.* **22**, 6208 (2021).
8. Fan, H. et al. Monitoring minimal residual disease in patients with multiple myeloma by targeted tracking serum M-Protein using mass spectrometry (EasyM). *Clin. Cancer Res.* **30**, 1131–1142 (2024).
9. Alaterre, E. et al. Automated and simplified identification of normal and abnormal plasma cells in multiple myeloma by flow cytometry. *Cytometry B Clin. Cytom.* **94**, 484–492 (2018).
10. Dimopoulos, M. A., Richardson, P. & Lonial, S. Treatment options for patients with heavily pretreated relapsed and refractory multiple myeloma. *Clin. Lymphoma Myeloma Leuk.* **22**, 460–473 (2022).
11. Bhatt, P., Kloock, C. & Comenzo, R. Relapsed/Refractory multiple myeloma: A review of available therapies and clinical scenarios encountered in myeloma relapse. *Curr. Oncol.* **30**, 2322–2347 (2023).
12. Tanenbaum, B., Mielt, T. & Patel, S. A. The emerging therapeutic landscape of relapsed/refractory multiple myeloma. *Ann. Hematol.* **102**, 1–11 (2023).
13. Padala, S. A. et al. Epidemiology, staging, and management of multiple myeloma. *Med. Sci. (Basel)*. **9**, 3 (2021).
14. Barreto, I. V. et al. Kinase Inhibition in multiple myeloma: current scenario and clinical perspectives. *Pharmaceutics* **14**, 1784 (2022).
15. Thoren, K. L., McCash, S. I. & Murata, K. Immunotyping provides equivalent results to immunofixation in a population with a high prevalence of monoclonal gammopathies. *J. Appl. Lab. Med.* **6**, 1551–1560 (2021).
16. Rosenberg, A. S., Bainbridge, S., Pahwa, R. & Jialal, I. Investigation into the interference of the monoclonal antibody daratumumab on the free light chain assay. *Clin. Biochem.* **49**, 1202–1204 (2016).
17. McCudden, C. et al. Monitoring multiple myeloma patients treated with daratumumab: teasing out monoclonal antibody interference. *Clin. Chem. Lab. Med.* **54**, 1095–1104 (2016).
18. Jacobs, J. F. M. et al. An international multi-center serum protein electrophoresis accuracy and M-protein isotyping study. Part II: limit of detection and follow-up of patients with small M-proteins. *Clin. Chem. Lab. Med.* **58**, 547–559 (2020).
19. Katzmann, J. A. et al. Serum reference intervals and diagnostic ranges for free kappa and free lambda immunoglobulin light chains: relative sensitivity for detection of monoclonal light chains. *Clin. Chem.* **48**, 1437–1444 (2002).
20. Kleeberg, L. et al. Novel renal replacement strategies for the elimination of serum free light chains in patients with kappa light chain nephropathy. *Eur. J. Med. Res.* **14**, 47–54 (2009).

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Author contributions

Zhongwei Jia and Qiong Lu wrote the main manuscript text and prepared Figs. 1, 2 and 3. All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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