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KVANTIFIKACIJA SERUMSKOGA M-PROTEINA METODOM IMUNOSUPTRAKCIJE

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QUANTIFICATION OF SERUM M-PROTEIN BY IMMUNOSUBTRACTION METHOD

DOCTORAL DISSERTATION

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SAŽETAK

Prisutnost M-proteina u serumu karakterizira stanje monoklonske gamapatije. Koncentracija M-proteina izravno korelira s veličinom tumorske mase, osim u vrlo rijetkim slučajevima nesekrecijske bolesti. Detekcija, tipizacija i kvantifikacija M-proteina nužne su za postavljanje dijagnoze, procjenu rizika progresije i praćenje uspješnosti terapije. Ograničenja postojećih metoda kvantifikacije serumskoga M-proteina (denzitometrijska i imunokemijska) razlog su kontinuiranih istraživačkih napora u osmišljavanju novih postupaka koji bi unaprijedili dijagnostiku i praćenje kliničkoga stanja bolesnika s monoklonskom gamapatijom. Cilj je ovoga istraživanja bio razviti i evaluirati osmišljeni računski postupak za kvantifikaciju serumskoga M-proteina primjenom laboratorijskih rezultata kvalitativne metode imunosuptrakcije. Istraživanje je obuhvatilo 133 uzorka bolesnika s monoklonskom gamapatijom klinički praćenih u Kliničkom bolničkom centru Zagreb. U svim je uzorcima provedena elektroforeza serumskih proteina, imunofiksacija i imunosuptrakcija te određena koncentracija ukupnih proteina i pojedinih razreda imunoglobulina G, A i M. Amplituda vrška M-proteina nativnoga uzorka i globulinske krivulje nakon imunoprecipitacije prepoznate su kao ključni parametri u laboratorijskim rezultatima imunosuptrakcije. Podatci o amplitudi krivulja su, uz koncentraciju ukupnih proteina, razreda imunoglobulina uključenoga u sintezu M-proteina i globulina, poslužili kao temelj osmišljenih računskih modela. Utvrđena je vrlo dobra povezanost deriviranih varijabli AD1nIg, D1Ig, D2Ig i D1nIg temeljenih na podacima iz imunosuptrakcijskog elferograma s rezultatima uspoređivanih metoda. Podatci iz imunosuptrakcijskog elferograma o albuminskoj frakciji nisu pridonijeli jačini povezanosti rezultata deriviranih varijabli s rezultatima postojećih metoda. Dokazana je veća preciznost određivanja koncentracije M-proteina primjenom deriviranih varijabli u odnosu na denzitometrijsku metodu i nije ovisila o koncentraciji, prisutnosti poliklonske pozadine niti migracijskom položaju M-proteina.

U ovom je radu istražena i opisana mogućnost umjeravanja rezultata imunosuptrakcije. Najusporediviji rezultati s postojećim metodama dobiveni su s rezultatima derivirane varijable D1Ig uz najbolje rezultate procjene preciznosti i točnosti. Dobiveni rezultati upućuju na zaključak da su u računskim modelima za kvantifikaciju M-proteina amplitude krivulja na mjestu vrška M-proteina u imunosuptrakcijskom elferogramu objektivan i vrijedan mjerljivi parametar neovisan o prisutnosti pozadine. Evaluacijom deriviranih varijabli pokazano je da je njihovom primjenom moguće unaprijediti dijagnostiku i praćenje kliničkoga stanja bolesnika s monoklonskom gamapatijom.

Ključne riječi: kapilarna elektroforeza; M-protein; imunosuptrakcija; gamapatija; multipli mijelom; elektroforeza serumskih proteina, imunokemijski test.

SUMMARY

Introduction

The presence of monoclonal protein (M-protein) is a characteristic of monoclonal gammopathy, which is commonly associated with malignancies, such as multiple myeloma (MM), Waldenström macroglobulinemia, and amyloidosis. The finding of serum M-protein without clinical indicators of the disease is characterized as an asymptomatic condition named monoclonal gammopathy of undetermined significance (MGUS). Except in very rare cases of nonsecretory disease, M-protein concentration directly correlates with tumor mass. Detection, typing, and quantification of M-protein are necessary for diagnosis, assessment of the risk of progression, and monitoring of the treatment outcome. The shortcomings of existing densitometric and immunochemical methods for measuring serum M-protein encourage ongoing research efforts to develop novel procedures that will improve the management of patients with monoclonal gammopathy. Considering that M-protein is produced by tumor-altered cells, the unpredictability of its structure represents an additional challenge in measuring the concentration.

One of the most used approaches for M-protein quantification is to measure the concentration of the immunoglobulin class involved in the synthesis of M-protein (Ig_{invl}) using turbidimetry or nephelometry. This approach frequently yields overstated monoclonal immunoglobulin concentration. Densitometry is a method that enables quantifying monoclonal fraction in the standard electropherogram (EPG). If M-protein is identified in the gamma globulin fraction, this model is appropriate, but method efficiency is limited by different factors, such as a high polyclonal background, the beta migrating position of M-protein, and the polymerization of immunoglobulin molecules. Furthermore, the use of two models, tangent skimming or perpendicular drop, which both lack objectivity, contributes to the variability of results.

Immunosubtraction is a fully automated method for characterizing M-protein. The method utilizes capillary electrophoresis in combination with immunoprecipitation. Antisera, used for immunoprecipitation, are coupled to sepharose beads that alter mobility in an electric field by binding to immunoglobulin molecules and forming immunocomplexes. Comparison of electropherograms of native serum and serum after immunoprecipitation (ISE-EPGs) enables detection and characterization of M-protein. In addition, the level of polyclonal background and comigrating beta fraction proteins can be assessed by comparing ISE-EPGs. Detected differences could be described by measured and derived variables.

This study aimed to develop and evaluate a novel approach for quantifying serum M-protein in patients with monoclonal gammopathy using laboratory data of qualitative immunosubtraction method. One of the goals of this dissertation was to demonstrate that the proposed derived models, based on immunosubtraction data, correlated with the results of EPG densitometric analysis and turbidimetric measurement of M-protein concentration in monoclonal gammopathy samples. Examining the analytical characteristics of the developed M-protein quantification model was one of the specific aims.

Materials and Methods

A total of 133 patient samples with monoclonal gammopathy were included. The concentrations of individual classes of immunoglobulins G, A, and M were determined in all samples, and capillary electrophoresis, immunofixation, and immunosubtraction were performed. To normalize patient data, the IF/IT Control (Sebia, Lysses, France) sample in which M-proteins IgGλ, IgAκ, and IgMλ were verified in the absence of a polyclonal background was employed. A perpendicular drop (PD) in points where the M-spike meets the polyclonal region, as well as a tangent skimming procedure (TS) that eliminates the polyclonal background and quantifies the M-spike above given points, were used for densitometrical quantification of M-protein. In IS-EPG, measurands were amplitudes of M-protein and albumin fractions. Assessed derived variables also included the immunoglobulin class involved in the synthesis of M-protein, total protein, and globulin data as follows:

AD – the difference between the amplitude of the M-protein spike of the native patient sample and the sample after immunoprecipitation generated from the IS-EPG;

$$AD = (M1 - M2)p$$

ADn – the patient sample AD and the control sample AD ratio;

$$ADn = \frac{(M1 - M2)p}{(M1 - M2)c}$$

AD1nIg – the product of ADn, the ratio of the total protein concentrations in the patient and control sample (TP_p/TP_c) and the concentration of the immunoglobulin class involved in the M-protein synthesis in the patient sample (Ig_{invl});

$$AD1nIg = \left[\frac{(M1-M2)p}{(M1-M2)c}\right] x \left(\frac{TPp}{TPc}\right) x\, Iginvl \quad \text{(g/L)}$$

AD2nIg – AD1nIg equation, in which the AD values are modified by the addition of the difference between the corresponding albumin fraction amplitude (in the patient and control

samples), generated from IS-EPG of the native patient sample and the sample after immunoprecipitation ($a1 - a2$);

$$AD2nIg = \frac{[(M1-M2)p+(a1-a2)p]}{[(M1-M2)c+(a1-a2)c]} \; x \left(\frac{TPp}{TPc}\right) x \; Iginvl \quad \text{(g/L)}$$

ADnG – the product of ADn and the globulin concentration (G, obtained by subtracting the albumin concentration from the total protein concentration);

$$ADnG = \left[\frac{(M1-M2)p}{(M1-M2)c}\right] x \; G \quad \text{(g/L)}$$

D1Ig – the ratio of the AD and M-protein amplitude of the native patient serum generated from IS-EPG multiplied by the concentration of the immunoglobulin class involved in the synthesis of the M-protein in the patient sample (Ig_{invl});

$$D1Ig = \left[\frac{(M1-M2)}{M1}\right] p \; x \; Iginvl \quad \text{(g/L)}$$

D2Ig – the D1Ig equation, in which the ratio of the AD and M-protein amplitude of the native patient serum generated from IS-EPG is modified by addition of the ratio of the difference between albumin fraction amplitude in the native patient sample and the sample after immunoprecipitation and albumin fraction in the sample after immunoprecipitation and multiplied by the concentration of the class involved in the synthesis of the M-protein in the patient sample (Ig_{invl});

$$D2Ig = \left[\left(\frac{M1-M2}{M1}\right) p + \left(\frac{a1-a2}{a2}\right) p\right] x \; Iginvl \quad \text{(g/L)}$$

D1nIg – the ratio of the AD and M-protein amplitude of the native patient serum generated from IS-EPG ratios, those obtained for the patient sample and the control sample multiplied by TP_p/TP_c and by the concentration of the immunoglobulin class involved in the synthesis of the M-protein in the patient sample;

$$D1nIg = \frac{\left[\frac{M1-M2}{M1}\right]p}{\left[\frac{M1-M2}{M1}\right]c} x \left(\frac{TPp}{TPc}\right) x \; Iginvl \quad \text{(g/L).}$$

The accuracy and measuring range were inspected using external quality control material (243 Gammopathies, INSTAND e.V. Gesellschaft zur Förderung der Qualitätssicherung in medizinischen Laboratorien e. V., Düsseldorf, Germany) as a reference material. Impact of background presence on accuracy of the proposed models was investigated by adding different amount of reference material in the serum samples with hypo-, normo- and

hypergammaglobulinemia. Precision testing was carried out in the serum samples, with four known variable factors in the M-protein quantification considered: migration pattern, polyclonal background, M-protein concentration, and gating method. The measurements were performed in hexaplicate and included two observers to inspect variation in gating strategy.

Results

Monoclonal IgG was detected in 71%, IgA in 14% and IgM in 15% of the examined samples. Most of the IgG M-proteins were found in the gamma fraction (91%), IgA in the beta fraction (84%), and IgM in the gamma fraction (86%). Background, polyclonal or beta fraction proteins were found in 43% of the samples. The concentrations of the M-protein determined densitometrically were lower than those obtained turbidimetrically, with a mean difference of -44.69% (95%CI -53.25 to -36.02; P<0.001). Derived variables with included concentration of the immunoglobulin class involved in M-protein synthesis (AD1Ig, D1Ig, D2Ig, and D1nIg) achieved the strongest correlation results with both densitometric and immunochemical methods in the group of samples without the background (R_s for AD1nIg were 0.957 and 0.920 with P<0.001, for D1Ig 0.931 and 0.983 with P<0.001, for D2Ig 0.931 and 0.973 with P<0.001, and for D1nIg 0.957 and 0.983 with P<0.001). The variables with applied normalisation, AD1nIg and D1nIg, revealed a clear tendency of increasing difference in the results with the increase in M-protein concentration, especially above 20 g/L. Passing-Bablok regression analysis pointed out proportional and systematic differences in the AD1nIg results while the D1Ig and D2Ig variables achieved the most comparable results to both examined methods. When data were classified into two groups based on the background presence (polyclonal background or beta migrating M-protein) and compared to the densitometric results, the most comparable results were also noted in the D1Ig and D2Ig variables. Furthermore, the D1Ig results showed no proportional or systematic differences as compared to the results determined immunochemically in the samples without the background presence, (intercept 0.00 (95% CI -0.19 to -0.00), slope 1.00 (95% CI 1.00 to 1.01); P=n/a) and to densitometrically obtained results in the samples with the background (intercept 2.24 (95% CI -2.12 to -4.38), slope 1.19 (95 % CI 0.89 to 1.65); P=0.190). Accuracy testing showed a maximum bias of 11% in the D1Ig results. An experiment of adding different amounts of reference material to three different control serum samples with hypo-, normo- and gammaglobulinemia showed that the assessed models are not significantly affected by the background presence. Regardless of the M-protein concentration, polyclonal background or migration pattern, coefficients of variation (CVs) of the derived variables were lower (maximum 3.1%) than those obtained by densitometric

measurements (highest 37.7%). Among all evaluated approaches, the lowest CVs were observed in the patient sample with M-protein located in the gamma fraction (M-protein concentration 16.6 g/L by PD; 12.5 g/L by TS), in the absence of polyclonal background. In contrast to the densitometric approaches, in derived variables, there was no loss of precision in the patient samples with low M-protein concentration and pronounced polyclonal background.

Discussion

The impact of possible overestimation of the immunoglobulin class concentration due to the diversity in reactivity of the immune reagents with specific monoclonal protein amino acid sequences has not been eliminated in the proposed model, but it was minimised by visualisation of the M-protein in IS-EPG and inclusion of the total protein concentration in the presented model. The D1Ig results, which were not normalised, showed that the D1Ig variable and the PD approach can be used interchangeably in a group with the detected background. The assumption that the applied and only accessible material containing all three classes of M-proteins (IgA, IgG, and IgM) with no polyclonal background would improve the quantification model was not supported with conducted comparisons. We hypothesized that the low concentration of the M-proteins in the used material was the main limitation of normalised variables. Even though immunoprecitated complexes migrated in the albumin and prealbumin fractions, the albumin fraction data had no influence on the explored quantification strategy. Previously, two different strategies of the quantitative immunosubtraction approach were described. Schroeder et al. employed additional software to export IS-EPG results and Bergon and Miravalles estimated M-protein using polyclonal immunoglobulin heavy chain/light chain equivalency factors measured experimentally. Our results showed that the background, detected in 43% of our results, was not an uncommon occurrence and that it altered the relevance of using a universal approach for M-protein quantification, and had no or moderate impact on the D1Ig, D2Ig and D1nIg variables. The well-defined points in IS-EPG, baseline and spike amplitude, were used in this study to avoid the lack of objectivity, a well-known weakness of densitometrical approaches, due to the possibility of subjective M-protein spike demarcation.

Conclusions

For the first time, the concept of immunosubtraction result normalisation was described and studied in this research. The derived variable D1Ig delivered the most comparable results to both examined methods and had the best precision and accuracy testing results. The findings of this study implied that the amplitude of the curves at the M-protein spike point in IS-EPG were

an objective, background-independent, measurable, and relevant parameters for the M-protein quantification using the derived variables. The evaluation of the derived variables showed that their implementation could improve the follow-up of the patients with monoclonal gammopathy.

Keywords: capillary electrophoresis; M-protein; immunosubtraction electrophoresis; multiple myeloma; serum protein electrophoresis; immunoassay