Multiparameter Flow Cytometry Identification of Neoplastic Subclones: A New Biomarker in Monoclonal Gammopathy of Undetermined Significance and Multiple Myeloma

Fabián Tarín^a Francisco López-Castaño^a Carmen García-Hernández^a
Paola Beneit^b Héctor Sarmiento^a Pablo Manresa^a Olga Alda^a
Blanca Villarrubia^a Margarita Blanes^c Javier Bernabéu^c Carmen Amorós^d
Sara Sánchez-Sánchez^e Carmen Fernández-Miñano^f Francisco De Paz^a
José Verdú-Belmar^a Pascual Marco^a Estella Matutes^g

^aDepartment of Hematology, Hospital General Universitario de Alicante, Alicante, Spain; ^bDepartment of Hematology, Hospital Universitario San Juan de Alicante, San Juan de Alicante, Spain; ^cDepartment of Hematology, Hospital General Universitario Virgen de la Salud, Elda, Spain; ^dDepartment of Hematology, Hospital Virgen de los Lirios, Alcoy, Spain; ^eDepartment of Hematology, Hospital Marina Baixa, Villajoyosa, Spain; ^fDepartment of Hematology, Hospital Vega Baja, Orihuela, Spain; ^gDepartment of Hematology, Hospital Clinic de Barcelona, Barcelona, Spain

Keywords

MGUS · Multiple myeloma · Flow cytometry · Neoplastic subclones

Abstract

Multiparameter flow cytometry (MFC)-based clonality assessment is a powerful method of diagnosis and follow-up in monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM). However, the relevance of intraclonal heterogeneity in immunophenotypic studies remains poorly understood. The main objective of this work was to characterize the different immunophenotypic subclones in MGUS and MM patients and to investigate their correlation with disease stages. An 8-color MFC proto-

col with 17 markers was used to identify the subclones within the neoplastic compartment of 56 MGUS subjects, 151 newly diagnosed MM patients, 30 MM subjects in complete remission with detectable minimal residual disease, and 36 relapsed/refractory MM patients. Two or more clusters were observed in >85% of MGUS subjects, 75% of stage I MM patients, and <15% in stage III. Likewise, a significant correlation between the dominant subclone size, secondary cytogenetic features, and changes in the expression of CD27, CD44, and CD81 was detected. The loss of intraclonal equilibrium may be an important factor related with kinetics and risk of progression not well considered to date in MFC studies. The MFC strategy used in this work can provide useful biomarkers in MGUS and MM.

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Introduction

In recent years, multiparameter flow cytometry (MFC) has considerably increased its diagnostic relevance in monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM). This technology allows the distinguishing of clonal neoplastic versus normal polyclonal plasma cells (PCs) with great sensitivity and specificity [1]. MFC-based clonality assessment is the most stringent method of diagnosis and follow-up, and provides very useful information to assess the risk of progression from MGUS to MM [2]. MFC also plays a fundamental role in the diagnosis of amyloidosis [3], staging of solitary bone plasmacytoma [4], evaluation of minimal residual disease (MRD) [5] and prognosis [6], and identification of new promising therapeutic targets in PC dyscrasias [7].

Intratumor heterogeneity is a widespread phenomenon of critical importance to understanding MGUS and MM pathogenesis [8–11]. The presence of subclonal clusters with different immunophenotypes and genotypes, and probably different sensitivity or resistance to treatment, has been clearly demonstrated [8–10]. However, the relevance of this finding in MFC studies remains poorly understood. The main objective of this study was to characterize the different immunophenotypic subclones in MGUS and MM patients, investigating their correlation with disease stages and whether the subclones remained stable or changed along the disease course.

Patients and Methods

Between March 2013 and June 2017, we used an 8-color MFC protocol to analyze the intratumoral phenotypic heterogeneity of 56 MGUS subjects, 151 newly diagnosed MM patients, 30 MM patients who reached complete remission with detectable MRD (CR/MRD+), and 36 relapsed/refractory MM patients defined according to IMWG criteria [12]. Likewise, comparison of the number and characteristics of subclones was made between the time of diagnosis and the first response evaluation, both in relapsed/refractory MM patients and CR/MRD+, to see possible changes in their immunophenotype.

Our strategy consisted in characterizing the clonal population through the EuroFlow PCD standardized panel [13], to later identify the subclones using four specifically designed 8-color combinations. For this, we used 17 monoclonal antibodies divided into 5 backbone markers placed in the same position to provide identical multidimensional localization of the target cells (CD38, CD138, CD19, CD81 and CD45) and 12 characterization markers (CD56, CD117, CD27, CD28, CD44, CD200, β_2 -microglobulin, CD33, CD150, CD86, CD272 and CD52). The files were first merged and

analyzed using new software tools based on principal component analysis and displayed by the automatic population separator (APS) graphical representation of the Infinicyt™ software (Cytognos). APS integrates all the parameters and defines the best possible separation between clusters, as well as the significance of each parameter in this separation.

Results

By analyzing the neoplastic compartment, two or more clusters were observed in the great majority (>85%) of MGUS patients (Fig. 1a, 2a). On the other hand, this condition was present in only 42% of MM patients ($p < 0.001$), with the most common finding being a dominant subclone that represented >75% of the neoplastic compartment (Fig. 1b, 2a). In both MGUS and MM, the top-parameters that best separated the different subclones were, in order of importance, CD81, CD56, CD44, CD28, CD27, CD117, CD19, CD200, CD45, CD138, and β_2 -microglobulin.

Differences were also seen according to the stage of the disease in newly diagnosed MM. Thus, advanced Durie-Salmon stages had fewer subclones detected ($p < 0.01$). Of stage I MM patients, 75% presented at least 2 subclones, as opposed to <15% in stage III. This occurs similarly with other staging systems, such as the ISS and the R-ISS (Fig. 2b). In relapsed and refractory MM patients the dominant subclone virtually approached 100% of neoplastic PCs (Fig. 1c, 2c).

The immunophenotypic characteristics of the dominant clone were not clearly different between MGUS and newly diagnosed MM. Likewise, differences were also not observed between the different MM subtypes or clinical stages. However, when we compared the diagnosis and relapse, 31 out of 36 patients showed shifts of intensity of one or more markers restricted to the dominant subclone. The most frequent changes were the loss of CD27 intensity (26 patients) and increase of CD44 (23 patients) and CD81 (21 patients). Interestingly, these changes were not observed in 26 out of 30 CR/MRD+ MM patients in whom an intraclonal equilibrium similar to MGUS was partially restored (Fig. 1d, 2c; $p < 0.01$).

Finally, in MM patients with secondary cytogenetic features (1q21+, 17p-, 6q-, and 8p-) [14] the dominant subclone was apparently higher compared to MM with only primary features (Fig. 2d; $p < 0.01$) and a particularly significant correlation between the percentage of PCs carrying 1q21+ and the dominant subclone size was demonstrated (Fig. 2e; $p < 0.01$).

Fig. 2. Number and size of the different subclones related to the disease stage and cytogenetic events. **a** MGUS versus MM. The presence of subclones is more frequent in MGUS than in MM patients ($\chi^2 = 244.145$, $p < 0.001$). **b** Different MM stages. The progression to advanced Durie-Salmon, ISS, and R-ISS stages was closely related to the decrease in the number of detectable subclones ($\chi^2 = 44.122$, $p < 0.01$). **c** Intracлонаl equilibrium in MGUS and MM. The number and size of subclones show relevant differences between MGUS and MM, probably reflecting the progressive loss of the intracлонаl equilibrium. Interestingly, most CR/

MRD+ patients can recover a “MGUS-like” pattern (ANOVA $F = 26.473$, $p < 0.01$). **d** Intracлонаl equilibrium and cytogenetics. In MM patients with secondary cytogenetic features, the dominant subclone size was higher compared to MM with only primary features, perhaps with the exception of the high-risk primary cytogenetic group (ANOVA $F = 19.294$, $p < 0.01$). **e** Intracлонаl equilibrium and cytogenetics. High correlation between the percentage of PCs carrying 1q21+ and the dominant subclone size ($R = 0.69$, $p < 0.01$).

Discussion

Several studies have highlighted the value of the ratio between aberrant and total PCs to establish the differential diagnosis and risk stratification in MGUS and MM [2]. Additionally, recent studies suggest that the loss of intraclonal equilibrium may be an important factor related to kinetics and risk of MGUS and MM [9]. Our study demonstrates that the neoplastic compartment is also very heterogeneous, revealing important and additional information, since the loss of intraclonal equilibrium may be an important factor related to kinetics and the risk of progression not well considered to date.

The presence of a large dominant subclone is a usual finding in MM patients indicating the progressive loss of tumor heterogeneity. This phenomenon seems to characterize especially advanced stages and relapsed/refractory patients. The correlation between the dominant subclone size and the percentage of PCs carrying 1q21+ reinforces this observation and strongly suggests that in many cases this disease progression is probably related to a competitive mechanism of dominance of the 1q21+ subclone.

Likewise, other investigations have focused on the prognostic value of certain immunophenotypes, such as increased expression of CD19, CD20, CD44, and CD28, and lack of CD45, CD56, and CD117 [1, 6]. However, none of these markers have been proven by multivariate analysis to have independent prognostic significance. Interestingly, we have observed modifications in the expression of CD27, CD44, and CD81 throughout the evolution of the disease only in relapsed MM patients which are not present in residual cells from responsive CR/MRD+ patients. Changes in the expression of CD27, CD44, and CD81 are restricted to the dominant subclone and have been previously related with progressive disease [1, 6]. Monitoring of all different detected subclones can reveal early data related to the risk of the subclonal dominance and chemoresistance.

Some studies suggest that a better characterization of the neoplastic residual cells in CR/MRD+ MM patients may be relevant for clinical decisions [12]. Paiva et al. [15] provided very attractive data regarding the importance of the phenotypic and genetic characterization of these residual cells in order to understand chemoresistance. Our results show that a significant proportion of patients with CR/MRD+ can recover a subclonal pattern similar to MGUS. This new perspective can be useful to establish if different immunophenotypic features may identify high- or low-risk subclones.

Finally, the discovery of new targets for tailored therapy play a major role in MM, and tools for their fast and reliable detection are essential [1, 8]. Our results show that some makers (CD81, CD27, CD56, CD28, and CD44) have a variable expression and therefore a tendency to differentiate the subclones. Since they are probably implicated in the intraclonal heterogeneity, these findings must be considered when evaluating therapeutic strategies in order to avoid selecting initially minor subclones resistant to tailored therapy.

In conclusion, most of the existing diagnostic MFC algorithms based on “single-clone” models do not allow the ascertainment of the contributions of different subclones, implying a significant loss of relevant information. The MFC methodology used in our work allows the fast quantification and characterization of the different immunophenotypic subclones in MGUS and MM, and could modify the current paradigm of interpretation of the immunophenotypic features, providing new promising biomarkers in MGUS and MM.

Statement of Ethics

Subjects (or their parents or guardians) gave their written informed consent for inclusion in the study. The study protocol was approved by the research institute's committee on human research.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Fabián Tarín, principal investigator: study concept and design; preparation, creation, and presentation of the work. Francisco López-Castaño and Paola Beneit: preparation, creation and presentation of the work; data collection and analysis. Héctor Sarmiento, Pablo Manresa, Margarita Blanes, Blanca Villarrubia, Javier Bernabéu, Carmen Amorós, Sara Sánchez-Sánchez, Carmen Fernández-Miñano, Francisco De Paz, and José Verdú-Belmar: data collection and analysis. Olga Alda, Pascual Marco, and Estella Matutes: critical review for intellectual content.

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