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Combined Selection System to Lower the Cutoff for Plasma Cell Enrichment Applied to iFISH Analysis in Multiple Myeloma[1]

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Abstract

Multiple myeloma (MM) is a very heterogeneous disease, characterized by multiple cytogenetic aberrations on plasma cells (PC) that have been traditionally used to predict the outcome of the disease. A mayor issue on the analysis of PC is the sometimes low infiltration of these cells in the bone marrow that hampers cytogenetic studies. To solve this problem we have optimized a selection strategy based on PC immunomagnetic isolation that has allowed us to lower to 1% the minimal PC infiltration requirement without loss of purity, enabling to perform genetic analysis. In this study, we have analyzed 153 bone marrow samples of patients suspected of MM, collected from February 2015 to May 2017 by the Genetics service of the Complejo Hospitalario de Navarra. Clinical characteristics of the patients and PC immunophenotyping, conventional cytogenetics and interphase fluorescence *in situ* hybridization (iFISH) analyses have been assessed on these samples. In our cohort 90% of the samples had cytogenetic abnormalities, among them 50% presented immunoglobulin rearrangements, 41.9% showed 1q gains, 29.7% showed 1p deletions and 33% presented TP53 deletion.

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Introduction

Multiple Myeloma (MM) is the second most common blood cancer in the world, there are approximately 229,460 people living with myeloma worldwide, 114,250 new cases are diagnosed yearly (International Myeloma Foundation-IMF). According to the American Cancer Society, approximately 30,280 new cases of multiple myeloma (17,490 men and 12,790 women) will be diagnosed in 2017, and around 12,590 people (6660 men and 5930 women) will die from this disease. According to the latest data from the IMF, incidents are 1.5 more likely in men than in women; it is most frequently diagnosed in 65–74 year-olds, and twice as common in people of African descent.

MM represents 10% of all hematologic malignant neoplasms. In lymphoproliferative disorders of bone marrow, it represents 42% of B-cell tumors and affects mainly older patients in their sixth or seventh decades of life, where, according to the IMF, death rates have been falling on average 0.8% yearly.

Based on the synthesized monoclonal immunoglobulin, there are different MM subtypes: IgG, IgA and/or Bence-Jones are the most frequently observed subtypes (90%) [1]. IgM and IgE MM are extremely rare (0.5%) and are associated with aggressive clinical course [2,3]. IgD variant MM constitutes ≤2% of all MM cases, displays generally an aggressive phenotype and is usually characterized by poor prognosis [4].

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Although MM is more manageable each day, and current treatments can extend the life of many patients, it is still an incurable disease with a median survival of two to three years [5]. It's a malignant neoplasm, made up of plasma cell (PC) clones, characterized by plasmacytosis in the bone marrow, production of monoclonal proteins, osteolytic bone lesions, renal disease, anemia, hypercalcemia and immunodeficiency, which not only exert a negative effect on patients' quality of life, but also decrease their survival time. That's why its diagnosis causes both in the patient and in his family environment, an important degree of suffering, not only in the physical dimension typical of the symptoms of the disease, but also in the psychological aspect, facing an aggressive, progressive and incurable disease.

Although the diagnosis of MM is simple, it could initially cause confusion, given the clinical heterogeneity of the MM. In fact, it's not uncommon to find cases close to the diffuse limit between MM and other gammopathies (Monoclonal Gammopathy of Uncertain Significance-MGUS, amyloidosis, etc.). Thus, in 2003, the International Myeloma Working Group published the criteria that allow unifying the diagnosis of MM [6]. According to it, for the diagnosis it is necessary to perform several tests or studies that include, among others: electrophoretic parameters (monoclonal immunoglobulin, detection of serum free light chain), biochemical parameters (β-2-microglobulin, albumin...) immunophenotype, DNA content, cytogenetics and interphase fluorescence *in situ* hybridization (iFISH). However, the genetic background of the disease is highly heterogeneous, as evidenced by the current numerous studies about Next-Generation Sequencing in MM, such as the three main studies [7–9] analyzing large cohorts of MM patients by means of Whole-Genome Sequencing and Whole-Exome Sequencing where authors conclude that examination of mutational signatures of patients over time indicate that relative contribution of diverse mutational processes are involved in the generation of the mutational repertory in MM and may change over time [10]. Due in part to its highly heterogenous genomic nature, the design of effective treatments that increase overall survival is very complex. In the clinical practice, flow cytometry, cytogenetics and molecular biology analysis are essential for diagnosis and correct determination of the individual prognostic factors of each patient with MM.

The results obtained with the use of conventional metaphase karyotype for the study of cytogenetic abnormalities was initially considered to have prognostic value, but according to the new genomic data published by different authors, is a technique with a very limited sensitivity. Nowadays, the presence of genetic abnormalities in PCs is considered an important prognostic factor. As a result, the application of molecular cytogenetic methods such as, comparative genomic hybridization or iFISH, the most useful clinical test for genetic aberration detection, is increasing the detection rate of chromosomal aberrations detected in MM [11].

The main chromosomal abnormalities that currently give clear information about the prognosis of the disease and the choice of the appropriate treatment has been described by several authors:

The t(11;14)(q13;q32) is the most common translocation in MM with a frequency of 15–20% based on iFISH and conventional cytogenetic analyses [12,13]. Del(17p) is associated with loss of the tumor suppressor gene TP53 located 17p13 and is considered one of the worst prognostic risk factors in MM [14]. Translocation t(4;14)(p16;q32) is seen approximately in 12–15% of MM and causes deregulation of the two proteins encoded on 4p16: fibroblast growth factor receptor 3 (FGFR3), an oncogenic receptor tyrosine kinase, and multiple myeloma SET domain (MMSET) protein [15]. This translocation has traditionally been regarded as a high-risk feature with early relapse and shortened survival [16,17].

Translocation t(14;16)(q32;q23) leads to the juxtaposition of the immunoglobulin heavy chain (IGH) and c-musculoaponeurotic fibrosarcoma (c-MAF) oncogene loci and is considered a high risk feature [18]. Abnormalities of chromosome 1 (loss of chromosome 1p and gain of chromosome 1q) generally occur at advanced stage of the disease and have shown to be correlated with poor prognosis [19]. The most frequent gains detected by iFISH involved chromosome 9 and 15, while the most common losses were those of chromosome 13 and chromosome X in females [20], trisomies of chromosome 6, 9 and 17 were associated with prolonged survival [21].

The commonly low median proportion of PCs, within the bone marrow aspirates experienced by the InterGroupe Francophone du Myeloma (IFM), LLR UK Myeloma Forum Cytogenetic Database and the Nordic Myeloma Study Group (NMSG), ranging from 1–20%, indicates that the iFISH technique cannot be performed directly as in other hematologic malignancies. The PCs need to be selected, by flow cytometry or immunomagnetic-bead based PC sorting [22], providing a pure PC population that increase sensitivity of detection of these chromosomal aberrations.

In our study, the abnormalities previously described have been studied in a cohort of 153 patients where we have established the association of these abnormalities with the patient's clinical characteristics.

Materials and Methods

Patients

Bone marrow samples from patients suspected of MM were sent between February 2015 and May 2017, to the Genetics Service of the Complejo Hospitalario de Navarra during the routine diagnostic/follow-up procedure. An appropriate informed consent was signed by all patients. The diagnosis of MM was made according to the criteria in the myeloma management guidelines established by the International Myeloma Working Group [23,24].

Bone marrow aspirates were extracted from the iliac crest for blood smears, flow cytometry and cytogenetic analyses and collected into 2 sterile tubes, one of them with lithium-heparin and the other with ethylene-diamine-tetraacetic acid (EDTA) as anticoagulation agents.

Immunophenotype

A version of the panel suggested by the Euroflow Consortium for Plasma Cell Disorders [25] was used for the polychromatic flow cytometry analysis: Tube 1 contained CD38-FITC, CD56-PE, CD27-PerCPCy 5.5, CD19-PECy7, CD117-APC, CD81-APCH7, CD45-V450 and CD138-V500. Tube 2 was composed of CyIgλ-FITC, CyIgκ-PE, CD45-PerCPCy5.5, CD19-PECy7, CD10-APC, CD38-APCH7. All the monoclonal antibodies and fluorochromes were purchased from BD Biosciences. A FACSCanto™ II flow cytometer and FACS Diva 6.0 (BD Biosciences) were used for data acquisition and Infinicyt software (Cytognos) for analysis.

PCs were described as $CD38^+$ $CD138^+$. CD45 and CD19 were used to distinguish between normal and malignant PC, being positive on normal cells, and losing its expression on aberrant PC [26]. The rest of markers were used to assess the clonality and phenotype of PC.

Bone Marrow PC Sorting

The basal percentage of PC on bone marrow samples was assessed by flow cytometry. After defining a nucleated cell gate on FSC/SSC scattergram, PC were detected with a CD138-PE antibody (BD Biosciences). A FACSCanto™ II flow cytometer and FACSDiva 6.0 software were used for data acquisition and analysis. Automated PC sorting was performed only in those samples with a PC percentage higher than 1%.

Bone marrow mononuclear cells (BMMC) were filtrated through a 70 μm cell strainer and isolated by Ficoll-Paque density gradient centrifugation (GE Healthcare Bio-Sciences), counted in a Neubauer Hemocytometer using Türk staining (Sigma) and resuspended in AutoMACs Running buffer (Miltenyi Biotech).

Isolation was performed using anti-CD138-coated magnetic microbeads (Miltenyi Biotech). If BMMC numbers were lower than 20 million, all cells were incubated with 20ul of the microbeads. If BMMC numbers were higher, only 20 million were used for isolation. After the incubation, isolation was carried out on an AutoMACS Pro Separator (Miltenyi Biotech) according to the following scheme: When basal PC percentage was higher than 10%, a possel program was used; if the percentage was between 5 and 10% 2 consecutive possel programs were performed; when lower than 5% a possel followed by a possel_s program was performed.

Purity of the eluted positive fraction was confirmed by flow cytometric analysis based on CD138 expression.

Conventional Cytogenetics and iFISH

Karyotype analyses were performed on bone marrow lithium-heparin samples following standard cytogenetic guidelines. After a 72 h culture, cells were treated with colcemid (Gibco), and standard GTG banding was performed on 20 metaphases were analyzed by a genetist on each case.

For iFISH analyses, cells were selectioned from bone marrow EDTA samples and fixed with Carnoy's solution. Hybridization was carried out using HYBrite (Vysis) and the following panel comprising the probes IGH/FGFR3 t(4;14)(p16;q32); IGH/MAFB t(14;20)(q32;q12), P53 (TP53) and CKS1B/CDKN2C(P18) (Cytocell Aquarius) and IGH/MAF t(14;16)(q32;q23) (Vysis) testing respectively (4;14) translocation, (14;20) translocation, 17p13 deletions, 1q gains and 1p deletions, and (14;16) translocation. iFISH were evaluated by a geneticist with a 100× objective fluorescence microscope Nikon E400 (single, dual and triple emission filters). Images were captured using a FISH Image System with CoolCube CCD camera and Isis 5.5.4 software (MetaSystem). The cut-off level was established following EMN recommendations [22], 10% for fusion and breakapart probes and 20% for numerical aberrations.

Statistical Analysis

The description for quantitative variables was made with measures of central tendency and dispersion: mean (standard deviation) or median (IQR). Categorical variables were reported as N (%). Differences between PC numbers were compared with paired-t-Student analysis. Differences between categorical variables were compared using contingency coefficients. Statistical analysis was performed using SPSS 20 software package. Results were considered statistically significant at $P < .05$.

Results and Discussion

Patient Characteristics

The study comprised 153 samples, from consecutive patients suspected of MM at the Complejo Hospitalario de Navarra. The median age of the patients was 70 (19) being males 49.7% of the patients. 96 samples (62.7%) were newly diagnosed and 57 samples (37.3%) belonged to followed-up patients. According to the criteria of the International Myeloma Working Group, 108 samples (70.6%) were considered as MM, 27 samples (17.6%) corresponded to patients with MGUS and 9 samples (5.9%) corresponded to other PC disease (4 plasmacytoma, 3 plasma cell leukemia, 1 amyloidosis and 1 Waldestrom macroglobulinemia). Nine samples did not belong to any PC disease. MM has been described to evolve from premalignant plasma cell disorders (PPCD) [27–29]. In fact, according to the literature, more than 90% of MM is preceded by MGUS [27,28]. In our cohort, however, only 20 samples out of 108 (18.5%) corresponded to patients that had previously been diagnosed with a PPCD (11 evolved from MGUS, 8 had plasmacytoma, and 1 had an amyloidosis). To a certain extent, this circumstance is expectable, as many PPCD remain asymptomatic and are only discovered in relationship to unrelated symptoms or laboratory abnormalities.

The clinical characteristics of MGUS and MM patients analyzed are summarized in Table 1. The incidence of IgG was higher on MGUS samples when compared to MM. No Bence-Jones subtype was detected on MGUS samples; while on MM, IgA and Bence-Jones were similarly distributed. Regarding light chain distribution, κ type was predominant in both MGUS and MM. Slightly higher frequency of κ light chain was observed on MGUS compared to MM. Accordingly, λ type was more frequent on MM than on MGUS. It should be noted that around 40% of immunoglobulin information of MGUS samples was missing on the patient medical records. Nonetheless, these results are in line with the ones reported by other groups [30,31].

The mean value of PC infiltration assessed by flow cytometry was 10.78% and 1.34% for MM and MGUS respectively, while the mean percentage of PC assessed by morphology was higher in both groups (22.5% and 3.21%), as previously described [32]. As expected, the median value of PC infiltration was higher on MM than on MGUS. It is interesting to remark that in our cohort, 48.14% of MGUS samples showed lower percentages than 1, being the highest PC infiltration value 5.5%. Regarding MM, 35% of samples had percentages higher than 10% (including 3 samples with more than 60% cells being PCs), 47.6% of the samples showed PC percentages between 1 and 10%. The samples with a PC value lower than 1% were 17.4%.

Table 1. Patient Characteristics

		MGUS	MM
Age		70 (23)	72 (17)
Male gender		12 (44,4%)	54 (50%)
Heavy chain disease	G	15 (75%)	62 (61.4%)
	A	5 (25%)	20 (19.6%)
	Free light chain	0	18 (17.6%)
	Other	0	2 (2%)
	Missing	7	6
Light chain disease	K	11 (68.8%)	57 (58.2%)
	L	5 (31.2%)	41 (41.8%)
	Missing	11	10
Durie-Salmon score (D-S)	III	-	42 (58.3%)
	II	-	23 (31.9%)
	I	-	7 (9.7%)
	Missing	-	36
PC by morphology (%)		3.21 (2.39)	22.54 (21.72)
PC by flow cytometry (%)		1.33 (1.43)	10.78 (15.6)
Cytogenetic abnormalities		7 (50%)	70 (90.3%)

CD138 Basal Levels Determine the Strategy Used for Optimal PC Enrichment

The European Myeloma network suggested already a decade ago that genetic studies of plasma cell diseases should always be performed on enriched samples [33] to increase the sensitivity to detect important chromosomal alterations. Multiple methods have been proposed, being CD138$^+$ immunomagnetic selection the most practical and widely available. Since then, no standard selection strategy has been defined, and each group establishes its own procedure. We established a selection scheme based on our previous experience with the technique.

Usually, other groups perform AutoMACs selection according to the manufacturer's instructions, without bearing in mind the percentage of PC in the sample [34,35]. In our hands, the percentage of PC in the samples turned out to be critical for cell selection. However, immunophenotype data are not always available at the time of the selection, and waiting for this datum implies loss of cell viability. Thus, we considered using CD138 as unique PC marker, optimizing at the same time the protocol for PC sorting. To be sure that it could be used one strategy instead of the other, paired measurements of PC numbers of 20 samples were performed (CD138 or immunophenotype panel). The data did not show any statistical difference (P = .254) so we incorporated this basal measurement to the selection protocol. However, for clinical purposes, the immunophenotype analysis remains as the reference value, as it is more accurate, and it includes other information apart from PC percentage.

As described in Materials and Methods, we applied different selection programs depending on the basal PC percentage. The limit for CD138$^+$ selection was established at 1%. According to it, we processed 92 samples: 35 samples under 5% PC, 22 samples with PC percentage between 5 and 10% and 35 samples with a PC percentage higher than 10%. The purity of the selected samples was 94% (5.26), 97% (2.44) and 96.2%, (3.77) respectively, with a mean purity higher than 95% (4.36) which is similar to what is found in other studies [36–38] ranging from 90 to 97%. Especially remarkable is the fact that we are capable of obtaining very pure PC from weakly infiltrated samples (1–5% PC). Regarding the cell recovery, we obtained from each range 0.32 (0.28), 1.07 (0.54) and 3.71 (4) $x10^6$ cells per sample on average. We were able to perform cytogenetic analysis from 93 out of 95 samples. On the remaining 2 samples the analysis failed due to low numbers of PC present in the bone marrow. It is important to remark that, while other groups argue that a minimum of $8x10^5$cells is needed for iFISH analysis [37], in this study we were able to perform iFISH analyses with as little as $1x10^5$ selected cells. Bearing in mind both the purity and the recovery rates achieved, we can affirm that the procedure applied in our study allows us to perform iFISH analysis in samples that would otherwise not be useful. This is especially noteworthy on patients with MGUS, as detection of abnormalities and early treatment can be decisive for not developing MM.

Chromosomal Abnormalities Accumulate on Late Stage Patients

iFISH analyses were assessed in those patients in whom previous immunomagnetic selection had been performed. Studied aberrations were chosen based on their high-risk prognosis and included IGH translocations (4;14), (14;16) and (11;14), 17p deletion, and abnormalities in chromosome 1.

Karyotype studies are progressively losing its relevance for MM diagnosis, due to the low proliferation rate of PC. However, it is still valuable to detect numerical abnormalities (especially hyperdiploidy, defined as a karyotype with 48 to 74 chromosomes), and chromosome 13 abnormalities [33]. Studies on the karyotype normality of these samples were successfully performed in 88 samples (94.6%). Regarding MM samples, 33.8% of the samples showed an abnormal karyotype. These data are in concordance with other studies showing that between 30–50% of all MM tumors have aberrant karyotypes [33]. In addition to it, it has been described that around 50% of karyotypes with numerical and structural abnormalities show a hyperdiploid pattern [39,40]. In our cohort, hyperdiploidy was the most common numerical aberration, present in 13 samples (52% of the MM aberrant karyotypes). Although it has been proven that the percentage of hyperdiploid MGUS is similar to that one on MM [41], in our hands only 2 MGUS samples showed an alteration, consisting on a loss of the Y chromosome. This is probably due to the fact that MGUS samples present small percentages of clonal cells. Moreover, it was not possible to perform conventional karyotype analyses on 5 samples, due to the absence of metaphases in them. To try to solve the failure of metaphases, other methods for the study of the ploidy have been proposed, as indexes based on the presence of trisomies on specific chromosomes [41] or multiplex ligation-dependent probe amplification [42].

Chromosomal abnormalities involving IGH rearrangement are considered primary oncogenic events shared by MGUS and MM [43]. In our cohort, these alterations were present in 42 out of 90 samples (46.7%) analyzed, which reaches to 50% when only MM samples are analyzed. These data are in concordance with those found in other works [44–46]. IGH rearrangements can also be found in 28.6% of MGUS samples supporting the fact that IGH rearrangements appear on early stages of the disease. It is remarkable that no differences on the IGH rearrangement rate have been found between samples when increasing D-S stage (Table 2).

Deletion of chromosome 17p, which includes TP53 locus, has been described to be a secondary event on the development of the disease, only present on MM [43]. In fact, in our cohort 25 samples (33.3%) were positive for TP53 abnormality. As shown in Table 2, none of the MGUS samples was positive for TP53. Moreover, it has already been described that del(17)p frequency increases in late stages of the disease [43]. In fact, in our cohort, TP53 deletion was more prevalent on D-S III samples than on a D-S II.

1p gain and 1q deletion are also considered to have a poor prognosis, so they were also evaluated in these samples. We found that 62.1% of the MM samples had aberrations on this chromosome, being 1q gains more frequent than 1p deletions (41.9% versus 29.7%). Although 1q gains and 1p deletions have been described to occur frequently together [45], in our cohort only 9.5% of the samples with an aberration on chromosome 1 co-presented both events. Regarding MGUS, only 2 samples (15.4%) had any of the aberrations which can be explained by the fact that chromosome 1 alterations occur lately on the MM transformation.

Table 2. Chromosomal Abnormalities Classified by Disease Severity

	MGUS	D-S I/II	D-S III
IGH rearrangement	4 (28,6%)	13 (56.5%)	14 (45.2%)
p53	0 (0%)	7 (30.4%)	14 (45.2%)
1p	1 (7,1%)	6 (26.1%)	9 (31%)
1q	1 (7.1%)	10 (43.5%)	12 (40%)
Aberrant karyotype	2 (14.2%)	5 (21.7%)	14 (45.1%)

Regarding the presence of multiple aberrations on the same sample, among the MGUS group we found 6 samples containing a single aberration and 1 samples having 2 aberrations (in total 7 out of 14 had at least 1 aberration). On MM, only 9 samples out of 79 had a complete normal chromosome distribution, with 42 samples having more than 2 alterations. This fact would confirm the accumulation of mutations along the development of the disease.

In conclusion, in our group we have established a selection strategy that has enabled us to isolate PC and to successfully perform iFISH analysis on weakly infiltrated samples. Moreover, we have performed a concise description of the clinical characteristics of MGUS and MM patients in Navarra. In this cohort, high risk cytogenetic abnormalities were highly present among both MGUS and MM samples, being IGH rearrangements and 1q gains the most frequent aberrations.

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