Phenotypic identification of subclones in multiple myeloma with different chemoresistant, cytogenetic and clonogenic potential

Running title: Phenotypic characterization of MM clonal diversity

Authors: *Teresa Paíno [1,2], *Bruno Paiva [3], José María Sayagués [2,4], Ines Mota [4], Tiago Carvalheiro [5], Luis Antonio Corchete [1,2], Irene Aires-Mejía [6], José J. Pérez [6], Maria Luz Sanchez [4], Paloma Barcena [4], Enrique M. Ocio [1,2,6], Laura San-Segundo [1,2], María E. Sarasquete [2,6], Ramón García-Sanz [2,6], Maria-Belen Vidriales [2,6], Albert Oriol [7], Miguel-Teodoro Hernández [8], María-Asunción Echeveste [9], Artur Paiva [5], Joan Blade [10], Juan-Jose Lahuerta [11], Alberto Orfao [2,4], María-Victoria Mateos [2,6], Norma C. Gutiérrez [2,6] and Jesús F. San-Miguel [3] on behalf of the GEM (Grupo Español de MM)/PETHEMA (Programa para el Estudio de la Terapéutica en Hemopatías Malignas) cooperative study group

Author Affiliations: (1) Centro de Investigación del Cáncer, Instituto de Biología Molecular y Celular del Cáncer/Consejo Superior de Investigaciones Científicas-Universidad de Salamanca (IBMCC, USAL-CSIC), Spain; (2) Instituto de Investigación Biomédica de Salamanca (IBSAL), Salamanca, Spain; (3) Clínica Universidad de Navarra; Centro de Investigaciones Médicas Aplicadas (CIMA), Pamplona, Spain; (4) Servicio General de Citometría y Departamento de Medicina, Universidad de Salamanca, Spain; (5) Centro do Sangue e da Transplantação de Coimbra, Instituto Português do Sangue e da Transplantação, Coimbra, Portugal; (6) Hospital Universitario de Salamanca, Salamanca, Spain; (7) Hospital Germans Trias i Pujol, Badalona, Spain; (8) Hospital Universitario de Canarias, Sta Cruz de Tenerife, Spain; (9) Hospital de Donostia, San Sebastian, Spain; (10) Hospital Clínic, IDIBAPS, Barcelona, Spain; (11) Hospital 12 de Octubre, Madrid, Spain.

*These authors contributed equally to this work

Corresponding author:

Jesus F. San Miguel, M.D; Ph.D.

Clinica Universidad de Navarra; Centro de Investigacion Medica Aplicada (CIMA)

Av. Pio XII 36, 31008 Pamplona, Spain

Phone: +34 948 296 296

Fax: +34 948 296 386

e-mail: sanmiguel@unav.es

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Abstract

Knowledge about clonal diversity and selection is critical to understand multiple myeloma (MM) pathogenesis, chemoresistance and progression. If targeted therapy becomes reality, identification and monitoring of intraclonal plasma-cell (PC) heterogeneity would become increasingly demanded. Here, we investigated the kinetics of intraclonal heterogeneity among 116 MM patients using 23-marker multidimensional-flow-cytometry (MFC) and principal-component-analysis, at diagnosis and during minimal-residual-disease (MRD) monitoring. Distinct phenotypic subclones were observed in 35/116 (30%) newly-diagnosed MM patients. In 10/35 patients persistent MRD was detected after 9 induction cycles, and longitudinal comparison of patient-paired diagnostic vs. MRD samples unraveled phenotypic clonal tiding after therapy in half (5/10) of the patients. After demonstrating selection of distinct phenotypic subsets by therapeutic pressure, we investigated if distinct FACS-sorted PC subclones had different clonogenic and cytogenetic profiles. In half (5/10) of the patients analyzed, distinct phenotypic subclones showed different clonogenic potential when co-cultured with stromal cells, and in 6/11 cases distinct phenotypic subclones displayed unique cytogenetic profiles by iFISH, including selective del(17p13). Collectively, we unravel potential therapeutic selection of pre-existing diagnostic phenotypic subclones during MRD monitoring; because phenotypically distinct PCs may show different clonogenic and cytogenetic profiles, identification and follow-up of unique phenotypic-genetic myeloma PCs subclones may become relevant for tailored therapy.

Introduction

The discovery and clinical incorporation of drugs with new mechanisms of action has led to high response rates in multiple myeloma (MM) patients.(1, 2) Albeit the better quality and significantly prolonged duration of response achieved after therapy, the vast majority of patients ultimately relapse; (3) thereafter, overall survival will mostly depend on the sensitivity of residual/resistant myeloma plasma cells (PCs) to salvage therapy. Such observations indicate that while the majority of tumor cells are initially chemosensitive, a minimum number would be chemoresistant and responsible for tumor regrowth after variable periods of time, frequently in the absence of response to new lines of therapy.(4) Altogether, these findings point out to the existence of different subsets of myeloma PCs with unique patterns of chemoresistance. Accordingly, intratumoral genetic heterogeneity has not only been recently demonstrated in newly-diagnosed MM patients (5-9) but also at relapse, where the subclonal diversity of myeloma PCs often differs to that observed at diagnosis.(4-6, 10, 11) However, no information exists on the subclonal distribution of myeloma PCs after response to first-line therapy where, particularly among patients in serological response, only low levels of minimal residual disease (MRD) tumor cells persist.

Multidimensional-flow-cytometry (MFC) is a readily available method to identify clonal PCs through aberrant antigenic expression profiles (12), even when these are present in low numbers (i.e.: MRD). Furthermore, MFC has the advantage that myeloma PCs can be sorted according to their specific phenotypic profiles, which allows the possibility to perform additional studies on highly-purified tumor PCs. Based on all the above, MFC could represent a useful technique to identify in individual patients distinct phenotypic myeloma PC subclones potentially coexisting at diagnosis and during MRD monitoring.

Here, we used MFC to analyze intratumoral phenotypic heterogeneity in a series of 116 newly-diagnosed MM patients. Additionally, we evaluated *in vivo* drug sensitivity of distinct phenotypic myeloma PC subclones coexisting in individual

patients at diagnosis, through the phenotypic analysis of longitudinal patient-paired diagnostic vs. MRD samples. Finally, we demonstrated that distinct phenotypic myeloma PC subclones often display different cytogenetic profiles and clonogenic potential.

Material and methods

Multidimensional flow cytometry (MFC) immunophenotyping. Approximately 400µL of EDTA-anticoagulated BM aspirated samples from 116 newly-diagnosed MM patients included in the GEM2010 trial were immunophenotyped using an 8-color direct immunofluorescence stain-and-then-lyse technique, with four different combinations of monoclonal antibodies (MoAb): [Pacific Blue (PacB), Pacific Orange (PacO), fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5), PE-cyanin 7 (PE-Cy7), allophycocyanin (APC), alexafluor 700 (AF700)]: i) CD29, CD45, CD11a, β7, CD79b, CD49d, CD19, CD38; ii) CD11c, CD45, CD41a, CD49e, CD33, CD117, CD19, CD38; iii) CD20, CD45, CD81, CD54, CD138, CD56, CD19, CD38, and; iv) HLA-DR, CD45, CD44, CXCR4, CD27, CD28, CD19, CD38. For the remaining MM patients (n=18), a simplified immunophenotypic BM screening was performed following the EuroFlow guidelines and antibody panels.(13) Data acquisition was performed for approximately 10^6 leukocytes/tube in a FACSCantoII flow cytometer (Becton Dickinson Biosciences – BDB – San Jose, CA) using the FACSDiva 6.1 software (BDB). Monitoring of instrument performance was performed daily using the Cytometer Setup Tracking (CST; BDB) and rainbow 8-peak beads (Spherotech, Inc; Lake Forest, IL) after laser stabilization, following the EuroFlow guidelines (14); sample acquisition was performed only in case of longitudinal instrument stability. Per protocol, newly-diagnosed MM patients included in the GEM2010MAS65 trial [which investigates sequential chemotherapy with 9 cycles of bortezomib, melphalan, prednisone (VMP) followed by 9 cycles of lenalidomide, low-dose dexamethasone (Rd) (n=56), vs. alternating cycles of VMP and Rd up to 18

cycles (n=60)] had BM samples studied after 9 induction cycles to monitor MRD levels; whenever persistent myeloma PCs were detected (MRD-positive), exactly the same immunophenotypic method performed at diagnosis was repeated for the characterization of the chemoresistant clone after therapy. In all patients, samples were collected after informed consent was given by each individual, according to the local ethical committees and the Helsinki Declaration protocol.

Generation of immunophenotypic protein expression profiles (iPEP) to assess the intratumoral PC architecture. To generate iPEPs, we used first those five parameters measured in common use for each sample aliquot (CD38, CD45, CD19, forward light scatter –FSC- and sideward light scatter –SSC-) to define the PC compartment. Then, the merge function of the Infinicyt software (Cytognos SL, Salamanca, Spain) was used to fuse the different data files corresponding to the 4 different 8-color MoAb combinations studied per sample, into a single data file containing all information measured for that sample.(15) For any single cell in each 8-color MoAb combination, this included data about those antigens that were measured directly on it and antigens which were not evaluated directly ("missing values") for that cell in the corresponding tube it was contained in. Then, the calculation function of the Infinicyt software was used to fill in those "missing values", based on the "nearest neighbor" statistical principle, defined by its unique position in the multidimensional space created by the five common (backbone) parameters (FSC, SSC, CD38, CD45 and CD19). Ultimately, an iPEP was generated for every single PC, which included all 23 phenotypic markers analyzed plus FSC and SSC as illustrated in Figures 1B and 1C.(14) Finally, BM clonal PCs were discriminated from normal PCs based on their aberrant phenotypic profiles as extensively described elsewhere.(12) Intraclonal heterogeneity within the tumor PC compartment was further investigated by principal component analysis (PCA), based on the 25 parameters evaluated and the automated population separator (APS; principal component 1 vs. principal component 2) graphical

representation of the Infinicyt software.(14) For the comparison between the whole diagnostic tumor PC bulk vs. MRD myeloma PCs, exactly the same methodology was used to generate the iPEP of chemoresistant (MRD-positive) cells, and PCA of merged patient-paired iPEP of baseline vs. MRD myeloma PCs.

Interphase Fluorescence in Situ Hybridization (iFISH) studies on phenotypic distinct myeloma PCs subclones. Phenotypically distinct myeloma PC subclones from 11 MM patients were FACS-sorted (FACSAria II, BDB; purity ≥97%) to investigate their specific cytogenetic profiles. FACS-sorted PC subclones were fixed in 3/1 methanol/acetic (v/v) and screened by iFISH for the presence of IGH translocations [t(4;14), t(11;14) and t(14;16)], del(13q14), del(17p13) and 1p/1q chromosomal alterations. A BX60 fluorescence microscope (Olympus, Hamburg, Germany) equipped with a 100X oil objective was used for the enumeration of hybridization spots per nuclei. A minimum of 100 interphase nuclei were analyzed per probe/probe combination, as described elsewhere.(16) Only those spots with a similar size, intensity, and shape were counted in areas with <1% unhybridized cells; doublet signals were considered as single spots. Deletion was defined by the loss of 1 or 2 fluorescence signals, whereas trisomy or hiperdiploidy was defined by gains of 1 or more fluorescence signals, each one in ≥10% of the nuclei. IGH translocations were considered as positive if present in ≥10% of the nuclei.

Colony assays. Identical numbers of cells from two coexisting phenotypically distinct myeloma PCs subclones from 10 patients with MM were FACS-sorted (FACSAria II, BDB; purity ≥97%) and plated into Methocult® media (StemCell Technologies, Grenoble, France) supplemented with 10% lymphocyte conditioned media plus 20ng/mL IL-6 (Peprotech, London, UK) and 20ng/mL IGF-1 (Prospec, Rehovot, Israel), and co-cultured with the human mesenchymal stem cell line immortalized by expression of the telomerase reverse transcriptase gene, hMSC-TERT (Kindly

provided by Dr. Dario Campana, Department of Oncology and Pathology, St Jude Children's Research Hospital, Memphis, TN, USA), at a 10:1 ratio. Cells were cultured at 37ºC in a 5% CO_2 humidified atmosphere. After 14 days of culture, colonies (≥40 cells) and clusters (10-39 cells) were scored. A negative control with the hMSC-TERT cell line alone and incubated under identical conditions was used along with all patient samples, such control cells systematically showing no colonies or clusters at day+14 in the 10 experiments performed.

Results

Intratumoral phenotypic heterogeneity in MM. Aberrant phenotypes by MFC are a well-established surrogate marker for the presence (and degree) of clonality among BM PCs in MM. Here, we took advantage of novel bioinformatics tools to determine the immunophenotypic protein expression profiles (iPEP) of BM clonal PCs from 116 newly-diagnosed MM patients enrolled in the GEM2010 trial by a 23-color flow approach, and to investigate the presence of intratumoral PC heterogeneity using PCA. Based on the operator-independent automated population separator (APS) (Figure 1A) tool and a limit of detection of 5% subclonal PCs within the tumor PC compartment, the presence of ≥2 phenotypic myeloma PC subclones (Figures 1B and 1C) was detected in 35 of the 116 (30%) patients (representative examples are shown in Figures 1A and 1D). Although the panel of MoAb covered a wide range of proteins including integrins, adhesion molecules, activation and maturation-related markers, it should be noted that the top-three markers which contributed the most to dissect PC intratumoral heterogeneity were CD56, CD27 and CD81, all these markers being consensually used to screen for clonal PCs at baseline and MRD stages. Other relevant markers are listed in Figure 1E, and detailed description of the intratumoral composition on each of the 35 patients is shown in Supplementary Table 1.

Distinct phenotypic myeloma PCs subclones show different chemoresistant profile. In order to evaluate the *in vivo* chemoresistant profile of different phenotypic subclones of myeloma PCs coexisting in a patient, we performed longitudinal comparisons between the diagnostic (baseline) tumor subclones and the chemoresistant myeloma PCs that persisted after treatment (MRD+), in patients that showed baseline phenotypic heterogeneity at the intratumoral myeloma PC level (Figure 2). From the initial 116 cases included in the GEM2010 trial, 35 had >1 phenotypic subclone at diagnosis and therefore qualified for the present sub-analysis. In 10 of these 35 cases persistent MRD was detected after 9 cycles of treatment, and the iPEP of MRD clonal PCs was determined in these cases. After merging the iPEP of baseline vs. MRD myeloma PCs, we noted that in 5 of the 10 patients chemoresistant MRD cells scattered throughout the whole baseline tumor bulk (Figures 2A-2E); by contrast, in the other five patients clonal selection upon therapeutic pressure was observed, with MRD myeloma PCs clustering within specific baseline subclones (Figures 2F-2J). For example, in one patient (97832; Figure 2H) showing a predominant ($CD33^{het}/CD56^{+}$) and a minor clone ($CD33^{+}/CD56^{-}$) only the former persisted after 9 cycles of alternating Rd-VMP; by contrast, in another case (99140; Figure 2J) it was the minor clone present at diagnosis ($CD27^{+}/CD138^{+}$) that remained after chemotherapy. These results demonstrate that distinct phenotypic myeloma PCs subclones coexisting in individual patients at diagnosis frequently harbor specific chemoresistant profiles.

Distinct phenotypic myeloma PCs subclones show different clonogenic potential. The coexistence of distinct phenotypic myeloma PCs subclones with different chemoresistant profiles prompted us to investigate if such phenotypic subclones could also harbor different clonogenic potential. For this purpose, identical numbers (≤500000) of FACS-sorted distinct myeloma PCs subsets from 10 patients were cultured with hTERT stromal cells. In 8 of the 10 cases, colonies and/or clusters

were observed after 14 days of culture (Table 1), and in 5 of these 8 patients (cases 105321, 107234, 109027, 112061 and 114053), one phenotypic subclone of myeloma PCs had >3-fold more clonogenic potential than its paired myeloma PC subset. Minor differences were observed for patients 106344 (6 clusters for $CD45^+$ subclone *versus* 3 clusters for $CD45^-$ subclone) and 110803 (2 clusters for $CD81^+$ subclone *versus* 4 clusters for $CD81^-$ subclone), whereas virtually the same clonogenic potential was found for the two phenotypic subclones from patient 115085 (36 colonies for CD117+ subclone *versus* 35 colonies for CD117- subclone). Noteworthy, no clear association was found between the pattern of expression of specific markers and an increased or decreased clonogenic potential; for example, tumor growth of myeloma PCs from patient 105321 (Figure 3A) was apparently restricted to the FACS-sorted $CD45^+$ subset, whereas in patient 107234 (Figure 3B) the clonogenic potential of $CD45^+$ tumor cells was 3-fold inferior than that of the $CD45^-$ subclone.

Distinct phenotypic myeloma PCs subclones show different genetic profiles. The association observed between phenotypically distinct myeloma PCs subclones and different functional properties led us to investigate whether such phenotypic subsets also showed different genetic profiles. Using iFISH, we observed that in 6 out of 11 patients there was ≥1 cytogenetic alteration present in one of the FACS-sorted myeloma PC subsets while absent in the other (cases 100644, 100172, 381155, 380486, 113919, 114661; Table 2). In patient 100172 (Figure 4A) the $\beta7^+$ and $\beta7^-$ clonal PC subsets were both diploid for 1p, had gains at 1q, and harbored a t(14q32); by contrast, del(13q14) and del(17p13) were detected in 78% and 11% of $\beta7^-$ cells, respectively, but undetectable in $\beta7^+$ cells. In patient 380486 (Figure 4B), the $CD56^+$ and $CD56^-$ myeloma PC subsets had both trisomy of chromosome 1, del(14q32), and del(13q14); conversely, del(17p13) was observed in 60% of $CD56^+$ myeloma PCs, while undetectable in the $CD56^-$ subset. In fact, selective deletion of 17p13 in only one

of the two FACS-sorted clonal PC subsets was observed in 4/5 cases carrying this alteration (Table 2).

Discussion

The field of MM is experiencing unprecedented and exciting years of research and development. The existence of genetically defined patient subgroups with high-risk disease is well established (17, 18) and continuous efforts are currently being taken to identify therapeutic strategies to overcome the adverse prognosis of these genetic subsets.(19) Moreover, clonal heterogeneity reinforces the need for targeted therapies designed to eradicate all potentially coexisting subclones in an individual patient. Overall, this requires accurate and readily available technology in order to identify, characterize and monitor such coexisting tumor subclones.(20) Here, we used new 23-marker multidimensional flow cytometry to investigate in-depth the phenotypic diversity of myeloma PCs in individual patients. Additionally, we showed the effect of first-line therapy on clonal selection by looking at MRD cells, unraveling the existence of truly primary chemoresistant phenotypic subclones within the whole population of myeloma PCs. Lastly, we demonstrated that different phenotypic myeloma PC subclones may frequently show unique cytogenetic and clonogenic features.

After early observations (5, 6, 9) on individual and/or relatively small patient series by iFISH, copy-number variation analysis or whole genome sequencing showing the coexistence of multiple (~3) genetic subclones in newly-diagnosed MM patients, Lohr (10) and Bolli (4) have recently confirmed and expanded on pivotal mutation analysis (21) through integrated mutational, CNA and LOH profiles of PCs from large series of MM patients. Similar to previous observations, the latter authors confirmed that most patients harbored ≥3 detectable genetic subclones (beyond the major clone), some patients having as many as seven subclones.(10) That notwithstanding, it should be noted that MM intraclonal heterogeneity had been inferred for many years using less sophisticated and more readily available techniques such as the serum free light-

chains (the FLC escape phenomenon) (22) or iFISH.(23) In fact, both Keats (6) and Schmidt-Hieber (23) had shown that by iFISH it was possible to determine the relative distribution of different subclones of myeloma PCs coexisting in individual patients at diagnosis and/or after disease progression. Of note, we and others have previously shown that some specific cytogenetic alterations were associated with the presence of unique antigenic profiles (e.g.: CD20, CD28, or CD117) (24-28); however, none of these studies postulated if intraclonal heterogeneity was behind this correlation.

The multiparameter nature of flow cytometry (MFC) allows the measurement of several proteins (antigens) inside and on the surface of thousands to millions of single-cells per sample. Thus, MFC provides specific information about individual PCs which allows clear discrimination between normal/reactive and myeloma PCs, even when these are present at very low numbers (e.g.: MGUS or MRD patient samples). Here, we used the new analytic tools developed by the EuroFlow Consortium (14) to determine the iPEP of BM myeloma PCs, and to investigate on phenotypic grounds the intraclonal heterogeneity of myeloma PCs from individual patients. Using operator-independent PCA-based tools, we showed that clonal diversity is observed in around one third of a large series of 116 newly-diagnosed MM patients. Because we are looking at the (surface) protein level, clonal heterogeneity by MFC was less frequently detected than previously reported by genomic techniques; conversely, the sensitivity of MFC to monitor and detect MRD is an advantage to perform on individual patients longitudinal comparisons between clonal diversity at diagnosis vs. MRD. Accordingly, in the present study we showed for the first time that therapeutic pressure over ≥2 diagnostic phenotypic subclones frequently leads to *in vivo* selection of primary chemoresistant myeloma PCs (i.e.: MRD). Because of the limited number of patients, it was not possible to fully address whether such therapeutic pressure was more evident with sequential vs. alterning chemotherapy; further investigations to fully address this question are now warranted. Other important aspect of MFC is the possibility to sort distinct clonal PC subsets according to patient-specific iPEPs, and perform additional

studies on such highly-purified myeloma PC subpopulations. Using the latter applicability, we showed that distinct phenotypic subclones display different clonogenic potential. However, it should be noted that, despite quantitative differences, in many patients the clonogenic potential was not restricted to a single phenotypic subclone, supporting the concept that different tumor cell populations could harbor the capacity to self-renew.(29-31). Moreover, the colony efficacy observed herein (4/10 patients) is in line with previously reported data on primary MM cells (32, 33), and suggests that truly clonogenic cells in MM would constitute a minor population within the tumor bulk.

The different functional behavior of coexisting phenotypic subclones prompted us to investigate if such subclones could also harbor different genetic alterations. Interestingly, we found that in approximately half of the patients coexisting myeloma PC subsets defined by the presence vs. absence of specific antigens may also show different cytogenetic profiles. Importantly, some of the cytogenetic alterations that were unequally distributed among distinct phenotypic myeloma PC subclones define patients with high-risk disease [e.g.: del(17p13)], and depending whether one looks to overall populations of CD138+ sorted PCs or to FACS-sorted myeloma PC subclones, a given patient may have different percentages of PCs carrying a specific cytogenetic abnormality or most importantly, be classified as negative vs. positive depending on the relative frequency of the coexisting myeloma PC subclones (Figure 4B). Furthermore, one could hypothesize that similarly to CLL, also in MM small subclones with TP53 deletion identified before treatment may become the predominant population at relapse (34) and hence influence the prognosis (although their initial low frequency).(35) Whether or not these small subclones are also primary chemoresistant and predominant in between disease diagnosis and relapse (i.e.: MRD) remains to be determined; however, if this happens to occur, it could perhaps change our perception on how to monitor patients' residual disease after initial chemotherapy. Thus, these observations suggest that monitoring of MM patients could benefit from a more

comprehensive approach that on top of the quantification of the minor MRD clones, also informs on their cytogenetic background based on the information collected at diagnosis. Of note, the most informative markers to dissect PC intraclonal heterogeneity were CD56, CD27 and CD81, all of which are consensually used to screen for PC clonality at baseline, as well as at the MRD stage. Consequently, the methodology reported here could be implemented in routine practice without additional efforts.

In summary, in this study we demonstrate the phenotypic subclonal diversity within tumor PCs from individual MM patients, such subclones also carrying frequently different cytogenetic and functional profiles. Since the balance between the different phenotypic subclones may fluctuate during the course of the disease (e.g.: in the setting of persistent MRD), the same could also be true to the predominance vs. disappearance of specific genetic alterations [e.g.: del(17p13)] and the patients' risk of relapse-free survival. Thus, cytogenetic characterization of myeloma PC phenotypic subclones easily recognizable at diagnosis and their subsequent monitoring after therapy may contribute to better tailor treatment and overcome chemoresistance in MM. Larger cooperative studies are now warranted to confirm such hypothesis.

Supplementary information is available at Leukemia's website

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Figure Legends

Figure 1. Intratumoral phenotypic heterogeneity in MM. The immunophenotypic expression profile (iPEP) of newly-diagnosed MM patients was determined by principal component analysis (PCA) based on simultaneous assessment of 23-markers on single bone marrow (BM) clonal plasma cells (PCs) by multidimensional flow cytometry. Presence of >1 phenotypically distinct subclones was determined operator-independently through the automated population separator (APS) density-based view of flow data (A), and confirmed by typical bivariate (B) and single-parameter histogram (C) diagrams. Three additional representative examples are shown in (D). Also, those markers significantly contributing to identification of such subclones are ranked from these being less (-) to more (+) frequently informative (E).

Figure 2. Therapeutic selection of distinct phenotypic myeloma PC subclones at the MRD stage. The iPEP of diagnostic (baseline) myeloma PCs (represented by lines corresponding to one and two SD) was merged with that of chemoresistant myeloma PCs (MRD) after 9 induction cycles (black dots). The most informative markers for the identification of the distinct baseline phenotypic subclones from individual MM patients were chosen for the bivariate (PC1 vs. PC2) merged representations of patients without clonal selection (A-E) and with clonal selection (F-J) after chemotherapy.

Figure 3. Distinct phenotypic myeloma PC subclones show different clonogenic potential. The clonogenic potential of FACS-sorted phenotypic subclones of myeloma PCs (CD45+ and CD45-) from patient 105321 (A) and patient 107234 (B) was analyzed. Images for each population at day +14 were taken with a Hamamatsu digital camera (Hamamatsu Photonics, Japan) coupled to a Zeiss microscope (Carl Zeiss Iberia, S.L., Madrid, Spain) using the software Openlab 4.0.3. Magnification of the lens: 10x for pictures from patient 105321 and 20x for pictures from patient 107234.

Figure 4. Distinct phenotypic myeloma PC subclones show different cytogenetic profiles. (A) FACS-sorted β7+ and β7- subclones from patient 100172 were hybridized with probes for t(14q32) (red-green spots; left panel), 1q25 and 1p36 (green and red spots, respectively; middle panel), as well as 13q14 and 17p13 (green and red spots, respectively; right panel). The percentage of altered nuclei is indicated for each probe and myeloma PC subclone; probes with a diploid pattern were classified as 2N. (B) FACS-sorted $CD56^{+}$ and $CD56^{-}$ subclones from patient 380486 were hybridized with probes for t(14q32) (red-green spots; left panel) chromosomal regions, 13q14 (red spots; middle panel), and 17p13 (red spots; right panel). The percentage of altered nuclei is indicated for each probe and myeloma PC subclone; probes with a diploid pattern were classified as 2N.

Table 1. Clonogenic potential of distinct phenotypic FACS-sorted subclones of myeloma PCs. Frequency of colonies and clusters scored at day +14 after co-culture of the same number of distinct clonal PCs antigenic subsets with the human mesenchymal stem cell line hMSC-TERT.

Patient	Subclone	N. of clonal PCs plated	N. of colonies	N. of clusters
105321	CD45+	18000	208	NS
	CD45-	18000	0	0
106344	CD45+	38000	0	6
	CD45-	38000	0	3
106774	CD56+	10000	0	0
	CD56-	10000	0	0
107234	CD45+	43000	0	17
	CD45-	43000	0	58
109027	CD56+	200000	1	2
	CD56-	200000	5	20
106234	CD19+	30000	0	0
	CD19-	30000	0	0

110803	CD81+	10000	1	2
	CD81-	10000	1	4
112061	CD117+	18000	0	1
	CD117-	18000	0	4
114053	CD81+	23000	0	5
	CD81-	23000	0	1
115085	CD117+	500000	36	NS
	CD117-	500000	35	NS

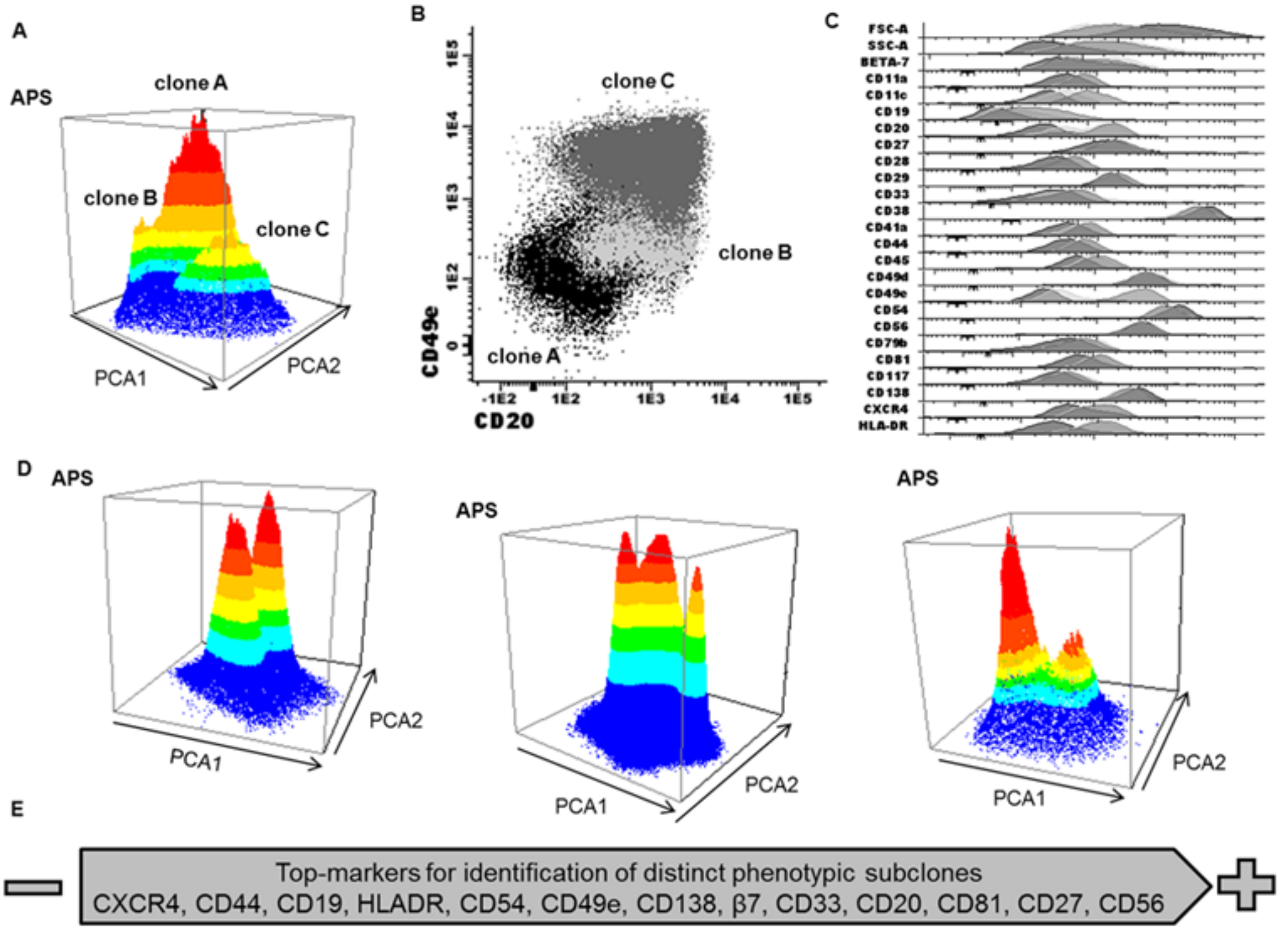
Abbreviations: NS=not scored

Table 2. Cytogenetic profile of distinct phenotypic FACS-sorted subclones of myeloma PCs.

Patient	Subclones	1p	1q	t(14q32)	t(4;14)	t(11;14)	t(14;16)	*RB1* (13q14)	*TP53* (17p13)
100644	$CD81^+$	2N	2N	neg	NT	NT	NT	2N	2N
	$CD81^-$	2N	2N	neg	NT	NT	NT	2N	14% del
100172	$B7^+$	2N	46% +1q	80%	neg	neg	neg	2N	2N
	$B7^-$	2N	77% +1q	91%	neg	neg	neg	78% del	11% del
381155	$CD45^+$	2N	2N	neg	NT	NT	NT	2N	2N
	$CD45^-$	2N	2N	neg	NT	NT	NT	66% del	2N
100643	$CD56^-$, $CD81^-$	2N	2N	61%	neg	pos	neg	2N	2N
	$CD56^+$, $CD81^+$	NT	NT	56%	neg	pos	neg	2N	2N
381111	$CD56^+$	11% -1p	2N	neg	NT	NT	NT	2N	2N
	$CD56^-$	53% -1p	2N	neg	NT	NT	NT	2N	2N
380486	$CD56^+$	50% +1p	50% +1q	67%*	NT	NT	NT	70% del	60% del
	$CD56^-$	50% +1p	50% +1q	15% *	NT	NT	NT	30% del	2N
113790	$CD19^+$	2N	2N	neg	NT	NT	NT	2N	NT
	$CD19^-$	2N	2N	neg	NT	NT	NT	2N	NT
113919	$CD38^+$,SSChigh	NT	NT	26%	neg	NT	NT	2N	2N
	$CD38^{low}$SSClow	2N	2N	84%*	neg	NT	NT	87% del	87% del
114053	$CD81^-$	29%+1p	29%+1p	neg	NT	NT	NT	2N	2N
	$CD81^+$	35%+1p	35%+1p	neg	NT	NT	NT	2N	2N
114661	$CD56^+$	NT	NT	24%	neg	NT	neg	2N	2N
	$CD56^-$	NT	NT	neg	neg	NT	neg	15% del	2N
114681	$CD56^+$	NT	NT	neg	NT	NT	NT	100% del	100% del
	$CD56^-$	NT	NT	neg	NT	NT	NT	100% del	100% del

Abbreviations: *del(14q32); NT= not tested; 2N= diploid; neg= negative; pos=positive

Figure 1.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APS
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clone B
clone C
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PCA2
B
clone C
clone B
clone A
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CD20
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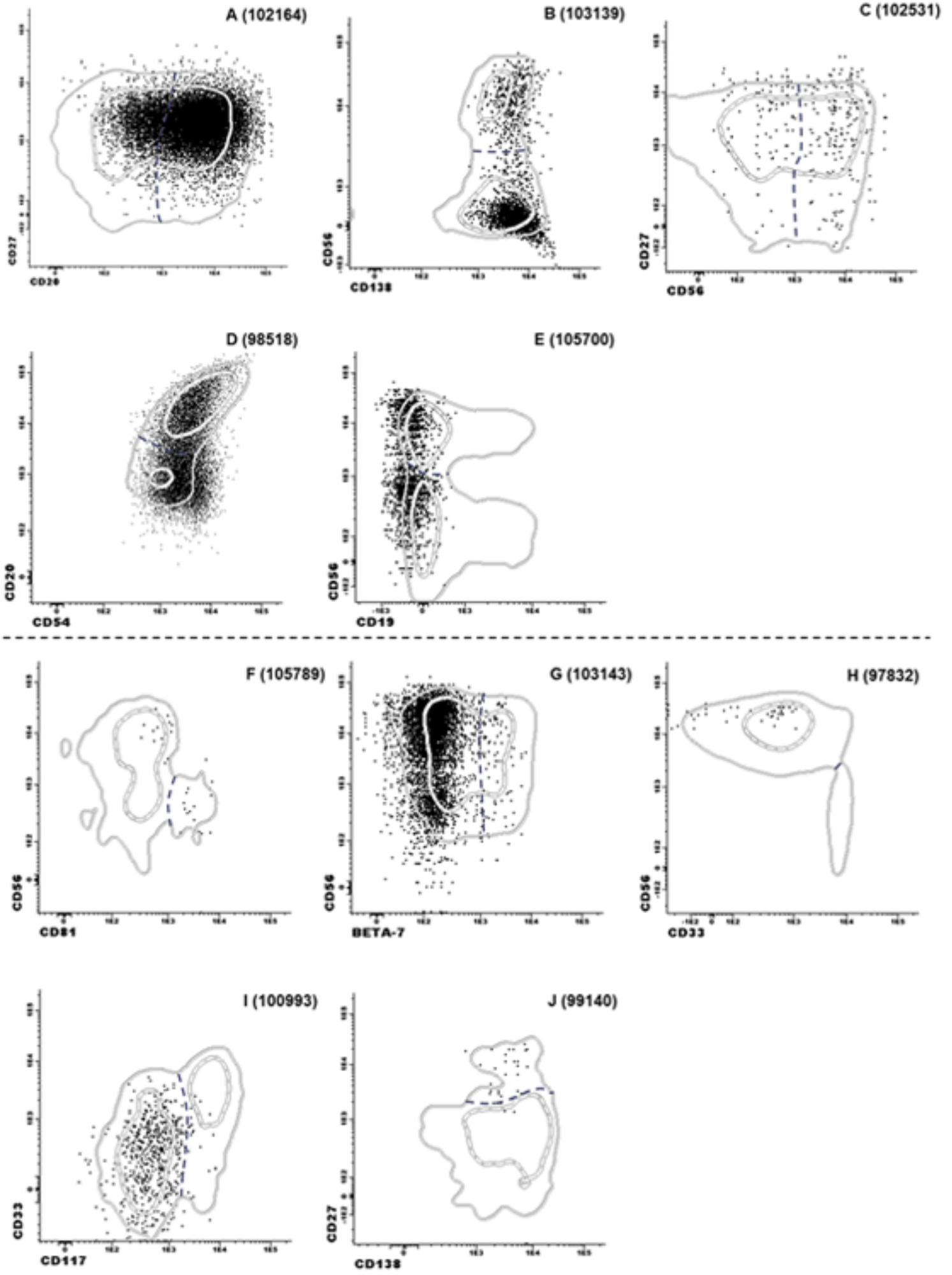
1E4
1E3
1E2
0
-1E2
1E2
1E3
1E4
1E5
C
FSC-A
SSC-A
BETA-7
CD11a
CD11c
CD19
CD20
CD27
CD28
CD29
CD33
CD38
CD41a
CD44
CD45
CD49d
CD49e
CD54
CD56
CD79b
CD81
CD117
CD138
CXCR4
HLA-DR
D
APS
PCA1
PCA2
APS
PCA1
PCA2
APS
PCA1
PCA2
E
Top-markers for identification of distinct phenotypic subclones
CXCR4, CD44, CD19, HLADR, CD54, CD49e, CD138, β7, CD33, CD20, CD81, CD27, CD56

Figure 2.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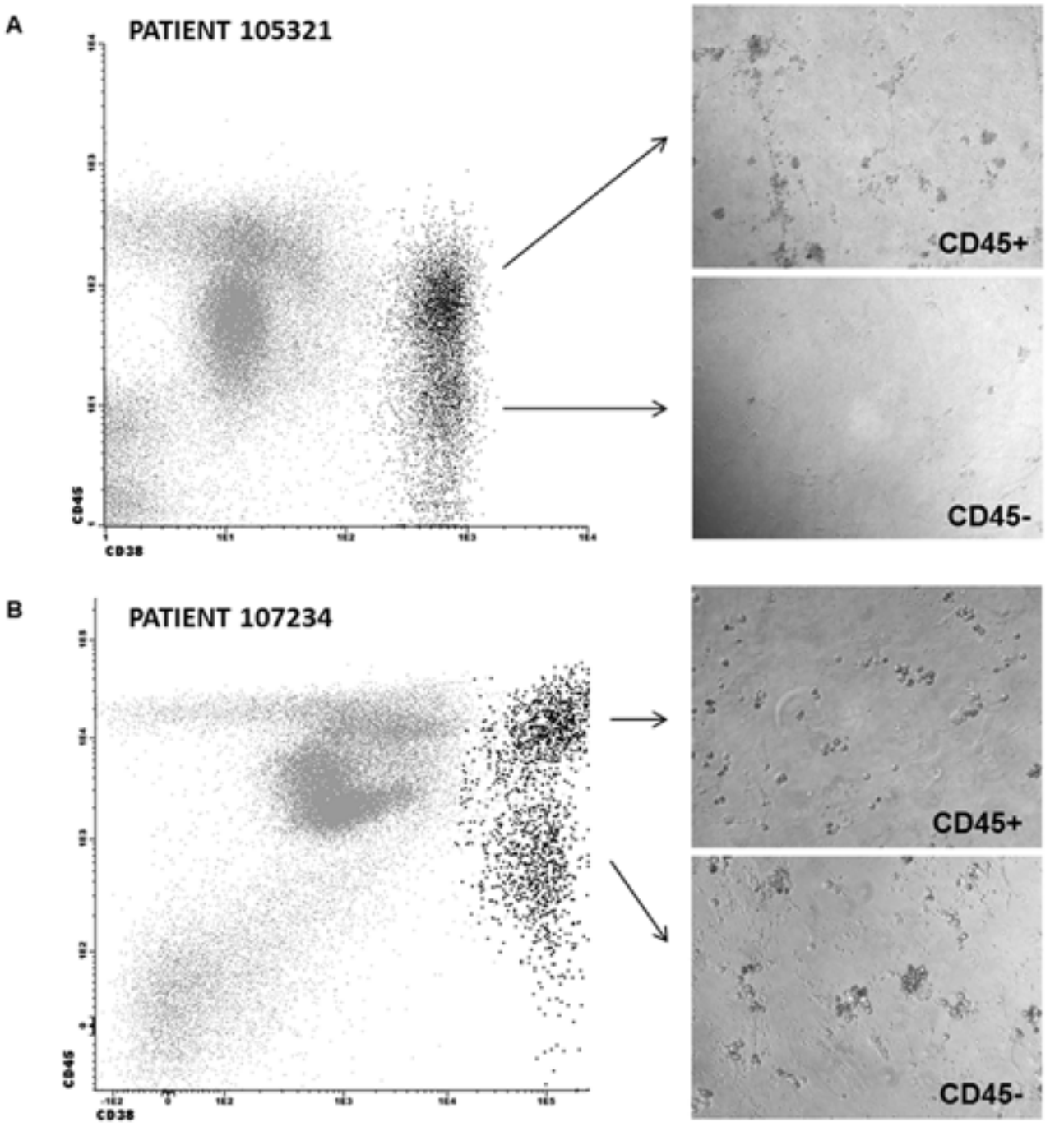
CD20
B (103139)
CD56
CD138
C (102531)
CD27
CD56
D (98518)
CD20
CD54
E (105700)
CD56
CD19
F (105789)
CD56
CD81
G (103143)
CD56
BETA-7
H (97832)
CD56
CD33
I (100993)
CD33
CD117
J (99140)
CD27
CD138

Figure 3.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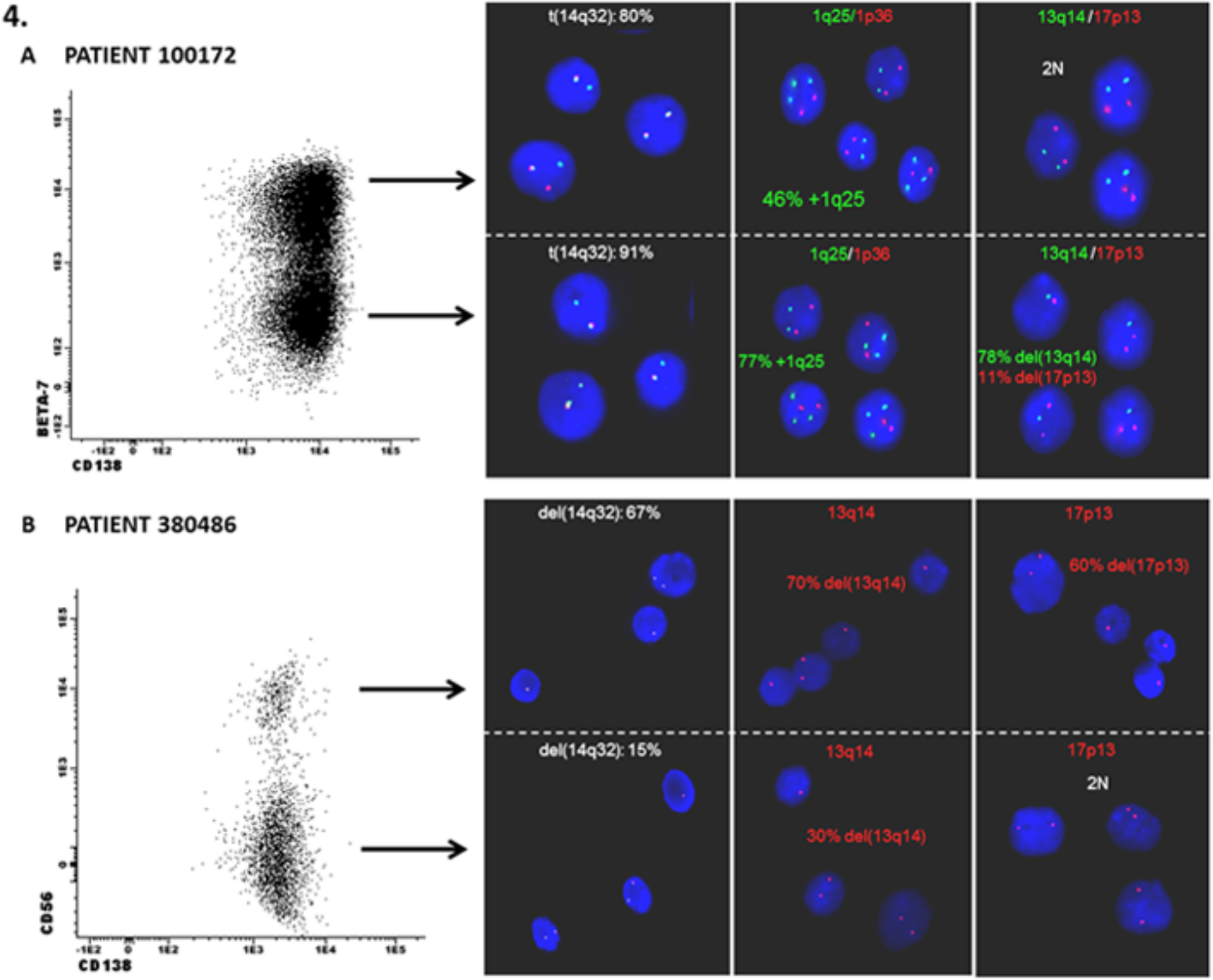
CD38
CD45+
CD45-
B
PATIENT 107234
CD45
CD38
CD45+
CD45-

Figure 4.


A PATIENT 100172
BETA-7
CD138
t(14q32): 80%
1q25/1p36
46% +1q25
13q14/17p13
2N
t(14q32): 91%
1q25/1p36
77% +1q25
13q14/17p13
78% del(13q14)
11% del(17p13)
B PATIENT 380486
CD56
CD138
del(14q32): 67%
13q14
70% del(13q14)
17p13
60% del(17p13)
del(14q32): 15%
13q14
30% del(13q14)
17p13
2N