의학박사 학위논문

Minimal Residual Disease Detection Using Next-Generation Flow Analysis And Next-Generation Sequencing In Multiple Myeloma Patients: Comparative analysis with IMW treatment response and verification of peripheral blood applicability

다발골수종 환자의 차세대 유세포 분석법과 차세대 염기서열 분석법을 이용한 미세 잔존 질환 검색 : IMW 치료 반응과 비교 분석 및 말초혈액의 사용 가능성 확인

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의과대학 협동과정 중양생물학과

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A thesis for the PhD Degree

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Minimal Residual Disease Detection Using Next-Generation Flow Analysis And Next-Generation Sequencing In Multiple Myeloma Patients: Comparative analysis with IMW treatment response and verification of peripheral blood applicability

by

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ABSTRACT

Introduction: With the advent of 8 color panel which suggested by Euroflow and next generation flow (NGF), a new epoch is marked that malignant PCs clone can be detected with high sensitivity. We are supposed to compare how the MRD results conducted by NGF correspond with IMWG treatment response. In general, MRD employed with BM is gold standard. However, it is invasive method to acquire BM specimen. If we use PB instead of BM, we would have advantage. Other method which operates MRD is identified of *IGH* gene rearrangement. Recently, a method using next generation sequencing (NGS) has emerged. We are supposed to understand differences between NGF MRD and NGS MRD with a some of patients.

Methods: A total of 28 BM and paired PB (27 MM patients at follow-up) was enrolled. We performed NGF using 8-color panel using Navios flow cytometer and Infinicyt. We performed IgH rearrangement NGS using Immunoseq assay (Adaptive Biotechnologies, USA) with 4 patients.

Results: 71.4% (5/7) of patients achieved CR or sCR showed MRD negativity. Nineteen patients showed BM MRD positive. (67.9%; 19/28) CD27 was significantly depressed in PB compared to BM ($p<0.05$). Four patients showed the discrepancy between BM MRD result and response criteria. The response criteria of these 4 patients were VGPR, PR, MR,

and SD, but all the patients were MRD negative by NGF. We found dominants clones in all 4 patients by NGS MRD. One patient had same dominant clone both initial diagnosis BM (87.13%; proportion of clone) and follow-up BM (19.38%). The other 3 patients had newly appeared clones in follow-up BM which clones were not detected in initial diagnosis BM. Clones of 0.7% or more were analyzed as dominant clones.

Conclusions: The results between BM MRD and PB MRD show strong correlation. The expression of CD27 was low in patients with nPC found in peripheral blood, confirming the possibility of using CD27 as a prognostic marker. Malignant clone was detected from all 4 patients who identified NGF MRD negative by BM, after conducting *IGH* gene rearrangement NGS. It means that malignant cells which be missed by NGF can be found by using other approach. Therefore, complementary use of MRD tests using NGF and MRD tests using NGS will allow patients with MRD negative to be identified with higher sensitivity. This will be of great help in determining the patient's treatment or prognosis.

Key words: Multiple Myeloma, Minimal Residual Diseases, Next Generation Flowcytometry, Next Generation Sequencing, IgH Rearrangement

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List of abbreviations

MM : multiple myeloma

ASCT : autologous hematopoietic stem cell transplantation

BCRs : B cell receptors

MGUS : monoclonal gammopathy of undetermined significance

SMM : smoldering multiple myeloma

IMWG : International Myeloma Working Group

sFLC : serum free ligh chain

MRD : minimal residual disease

ASO PCR : allele-specific oligonucleotide PCR

ASO RQ PCR : allele-specific real-time quantitative PCR

NGS : next generation sequencing

MFC : multicolor flow cytometry

NGF : next generation flow cytometry

BM : bone marrow

PC : plasma cell

PEP : protein electrophoresis

IFE : immunofixation test

nPCs : neoplastic plasma cells

ctDNA : circulating tumor DNA

cPCs : circulation plasma cells

CR : complete response

VGPR : very good partial response

PR : partial response

MR : minimal response

SD : stable disease

PD : progressive disease

APS : automatic parameter separation

LOD : limit of detection

LLOQ : lower limit of quantification

CD : cluster of differentiation

NDMM : newly diagnosed multiple myeloma

BMB : bone marrow biopsy

BMA : bone marrow aspiration

FcRn : neonatal Fc receptors

SHMs : somatic hypermutations

IgH-CDR : Ig heavy chain complementary determinant regions

F/U : follow-up

sCR : stringent complete response

NE : not evaluable

Introduction

Multiple myeloma is a malignant tumor that develops after terminal differentiation into plasma cells and is the second most common hematologic malignancy after non-Hodgkin lymphoma. Malignant plasma cells reside mainly in the bone marrow but can also be seen in the peripheral blood and other extramedullary sites such as soft tissues and organs, especially late in the course of the disease. In most patients, multiple myeloma is characterized by the secretion of M proteins (also called monoclonal proteins or monoclonal immunoglobulin proteins) produced by abnormal plasma cells. However, in 15~20% of patients, multiple myeloma cells secrete only monoclonal free light chains, and in less than 3% of patients, these cells do not secrete monoclonal proteins (Drayson et al., 2001; Kyle et al., 2003). Clinical signs of disease are caused by monoclonal proteins, malignant cells or cytokines secreted by malignant cells and include signs of end-organ damage such as hypercalcemia, renal failure, anemia and/or bone disease with lytic lesions. That is, a lesion or pathological fracture due to a disease process, collectively known as a CRAB hallmark (Rajkumar et al., 2014).

Globally, the incidence of multiple myeloma varies and is highest in developed countries such as the United States,

Western Europe, and Australia. The higher incidence in developed countries is probably due to greater clinical awareness of the disease and the availability of better diagnostic techniques. Looking at racial differences, the incidence of multiple myeloma is 2–3 times higher among blacks than whites, but lower among Asians and Hispanics (Waxman et al., 2010; Huang et al., 2007). The prevalence of multiple myeloma has increased due to better diagnostic techniques and improved patient survival due to the widespread use of autologous hematopoietic stem cell transplantation (ASCT) and the development of new therapeutics (Kumar et al., 2014).

Knowledge of B cell development and plasma cell biology is essential to understanding multiple myeloma. Plasma cells develop from hematopoietic stem cells and undergo multiple differentiations in the bone marrow and secondary lymphoid organs into B cells and finally plasma cells. Immature B cells undergo V(D)J rearrangement, a process that generates a diverse primary immunoglobulin repertoire in the bone marrow. B cells with B cell receptors (BCRs), such as IgH–IgL complexes on the cell surface, migrate to secondary lymphoid organs such as lymph nodes or spleen. In these secondary lymphoid organs, B cells undergo affinity maturation, somatic hypermutation, and class–switch recombination to produce

antibodies with high affinity for specific antigens and a variety of immunoglobulins. Double stranded DNA breaks at immunoglobulin loci are required for class switch recombination and somatic hypermutation. However, these DNA breaks can fuse with other breaks that occur elsewhere in the genome, resulting in an abnormal fusion of DNA and chromosomal translocations. Most of these chromosomal translocations are insignificant because these cells do not produce progeny, and this is most likely the result of the lack of growth advantage conferred by the translocation. However, translocations involving specific oncogenes can provide growth advantages to cells, which can lead to the development of pathological conditions such as monoclonal gammopathy of undetermined significance (MGUS), smoldering multiple myeloma (SMM), and eventually multiple myeloma. Thus, chromosomal translocation itself is the starting point that can give rise to a subset of multiple myeloma cases.

The diagnostic criteria used by the majority of clinicians are the updated International Myeloma Working Group (IMWG) criteria. Therefore, in addition to novel biomarkers and CRAB characteristics, diagnosis should be made according to these criteria, which are based on the bone marrow infiltration of clonal plasma cells, and monoclonal protein levels. After diagnosis, some of these tests can be used to monitor

treatment response. The new biomarkers are based on serum free light chain (sFLC) levels or ratio, bone marrow plasma cell infiltration levels, and the presence of two or more focal lesions on MRI, which identify patients with SMM at imminent risk of progression to active disease (Rajkumar et al., 2014). The presence of events that define myeloma is a key feature that distinguishes multiple myeloma from other plasma cell disorders such as MGUS or SMM. Further evaluation may be necessary to confirm that the CRAB feature is due to multiple myeloma and not due to other comorbidities or concomitant disease.

Much research and efforts to understand the biological and clinical aspects of multiple myeloma have led to the development of new therapies, and therapeutic approaches that currently offer benefits to patients. Treatment efficacy is reflected by a large increase in the number of patients achieving complete remission, followed by a prolonged progression-free period. Nevertheless, multiple myeloma remains an uncontrollable disease and an incurable disease that can recur and even lead to death.

It is of clinical importance to be able to predict eventual recurrence by evaluating the effectiveness-depth of the selected treatment, guided by the basic principle that the deeper the remission, the better the disease can be controlled.

The presence of minimal residual disease (MRD), that is, the presence of traces of myeloma cells that may remain in a patient's bone marrow after treatment, has proven important for monitoring remission status and is considered a major cause of recurrence (Davies et al., 2001; Rawstron et al., 2002; Bakkus et al., 2004; Fenk et al., 2004). Current technology enables the detection of MRD at levels as low as one myeloma cell in a million whole tested cells, opening up whole new opportunities for clinicians and patients alike.

Although the importance of MRD in the clinical setting of multiple myeloma has been reported, its clear effect has been widely recognized with the development of more sensitive techniques. Traditional molecular methods, namely allele-specific oligonucleotide PCR (ASO PCR) or real-time quantitative PCR (ASO RQ PCR) (Putkonen et al., 2010; Puig et al., 2014; Silvennoinen et al., 2014), are being replaced by the advent of next-generation sequencing (NGS), 4, 6, or 8-color multicolor flow cytometry (MFC) approaches are being replaced by next-generation flow cytometry (NGF) or other similar high-sensitivity multicolor panels (Flores-Montero et al., 2017; Roshal et al., 2017).

Presently, several different technologies are utilized for MRD monitoring in myeloma, including multi-parameter flow cytometry and next generation sequencing of IgH or light chain

(Paiva et al., 2015). They have different sensitivities and even among multi-parameter flow cytometry technologies, each have different sensitivities among laboratories, depending on the number of fluorochromes, number of acquired cells, or interpreters of flow histogram (Mailankody et al., 2015). Consequently, the definition of MRD negativity varies among laboratories. Together, the practical barriers to the clinical use of flow cytometry MRD monitoring, including the sensitivity of techniques, sample processing, sample quality control, and standardization across laboratories, have not yet been accomplished.

The sensitivity of NGS and NGF can be used for detection of rare residual myeloma BM cells at levels 10^{-5} and higher. There are few reports comparing the frequency of MRD positivity when using both techniques.

CHAPTER 1

Significance of analyzing circulating plasma cells in multiple myeloma – differences from measuring minimal residual disease in bone marrow

Introduction

Plasma cell myeloma is a neoplastic proliferation of the plasma cells. In bone marrow (BM), plasma cell percentage (PC%) is counted for follow-up during treatment, and the clonality of plasma cells is defined by immunochemistry, immunofluorescence, or flow cytometric analysis (Swerdlow et al., 2016). To monitor the quantitative changes in clonal plasma cells indirectly, we measured the serum light chains by protein electrophoresis (PEP) or immunofixation test (IFE), which are the resulting products of neoplastic plasma cells (nPCs). Diagnostic criteria and treatment response adopt these tests, and recently, a deeper treatment response of up to 10^{-5} sensitivity has been achieved, including minimal residual disease (MRD) flow negativity and MRD next generation sequencing (NGS) negativity (Flores-Montero et al, 2017; Ladetto et al, 2014). Plasma cell count percentage, MRD flow (MRD^{flow}), and MRD NGS were performed using BM aspirates.

How can we determine the clonality of cells? We assessed the clonal plasma cells directly by flow cytometry through the light chain, in which plasma cells are expressed in the cytoplasm or cell surface. When BM aspirates are diluted by peripheral blood or clotted during the BM procedure, the

assessment of clonal plasma cells is significantly disrupted (Batinic et al., 2014; Ladetto et al., 2014). If the BM aspirate is diluted 5-fold using peripheral blood, the resulting residual cells will be reduced to 20% of the actual residual cells. Assessment of residual plasma cells in peripheral blood will escape this problem, but nPC cells usually adhere to BM stromal cells and in part (Brooimans et al., 2009), escape from BM and circulate in peripheral blood (PB). It is well reported that the higher the number of circulating plasma cells (cPCs) in PB, the poorer the survival (Dingli et al., 2006).

Some studies seek the option of detecting MRDs in PB. Because of the potential to overcome invasive BM biopsy procedures and detect myeloma plasma cells in special situations such as extramedullary localization. Another reason for searching for MRDs in PB is to avoid false negative results due to the distribution of myeloma cells in the bone marrow. Analysis of circulating tumor DNA (ctDNA) in liquid biopsy, NGS, or NGF, in combination with imaging methods for PB circulating plasma cells monitoring, may indicate a valid strategy. (Sanoja-Flores et al. 2019) However, all of these efforts have so far shown no clear advantage in PB analysis with respect to BM. In fact, a recent NGS MRD study found that in 137 MM patients, after treatment 55 patients still had

detectable MRDs by NGF in the BM, while MRDs were not detected in PB. (Oberle et al. 2017)

In the present study, we investigated the utility of cPC and determined the advantages of cPC relative to other monitoring tests, such as the detection of residual cells, which would be missed by other tests. We performed MRD^{flow} using BM aspirates and PB, comparing the results with the treatment response of the IMW group.

MATERIAL AND METHODS

1. Patients

Among the patients diagnosed with multiple myeloma from November 2000 to December 2016, 28 patients whose bone marrow was collected for follow-up study at Seoul National University Hospital from November 2016 to May 2017 were selected for this study. This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB No. 1612-042-813). All study subjects provided their informed consent to participate in the study. During the period, BM was collected twice from one of a total of 27 patients, and a total of 28 BM aspiration samples were obtained. PB was collected at the same time as BM was collected, and a total of 28 pairs of BM and PB samples were studied. (Table 1-1) For each patient, laboratory tests at the time of BM examination, including CBC, admission panel for chemistry assay, M protein, serum free light chain (sFLC), PEP, IFE, and reports of BM examinations were retrospectively investigated through electrical medical record. The manual counting of PC% in BM aspiration and biopsy was evaluated by two independent medical doctors who had majored in hematology.

Table 1-1. Baseline characteristics of 28 enrolled patients

Characteristics	Total (N = 28)
Age, median (range)	62.5 (57.75-64)
<65 years, n (%)	6 (21.4)
≥65 years, n (%)	22 (78.6)
Sex, n (%)	
Male	18 (64.3)
Female	10 (35.7)
Hemoglobin (g/dL), median (range)	11.7 (9.55-12.525)
Platelets (10^9/L), median (range)	169.5 (106-245.25)
Calcium (mg/dL), median (range)	8.95 (8.7-9.3)
Creatinine (mg/dL), median (range)	0.74 (0.65-0.9625)
Albumin (mg/dL), median (range)	3.9 (3.35-4.15)
IMWG response, n (%)	
Complete response (CR)	7 (25)
Very good partial response (VGPR)	3 (10.7)
Partial response (PR)	5 (17.9)
Minimal response (MR)	2 (7.1)
Stable disease (SD)	5 (17.9)
Progressive disease (PD)	3 (10.7)
Relapse	3 (10.7)
BM plasma cells (%), median (range)	
Aspirates	0.9 (0.4-3.65)
Biopsy	5 (5-5)

Values are presented as median (Interquartile Range)

Abbreviations: IMWG, International Myeloma Working Group

2. Detection of multiple myeloma minimal residual disease using next-generation flow cytometry (NGF)

2.1 Reagents and antibodies used for detection of MRD

MM MRD was performed in the same way as the MM MRD panel presented by EuroFlow. CYT-MM-MRD-CE-IVD (Cytognos, Salamanca, Spain) kit was used. A total of 10 antibodies are required for the MM MRD panel. Eight of these antibodies (CD38-FITC, CD56-PE, CD45-PerCP-Cyanine 5.5, CD19-PE-Cyanine7, CD117-APC, CD81-APC-C750, CyIg κ -APC, CyIg λ -APC-C750) were included in the kit. The other two antibodies, CD27-BV510 (BioLegend, San Diego, CA, USA) and CD138-BV421 (BD Bioscience, NJ, USA) were purchased separately and used. (Table 1-2)

Table 1-2. Characteristics and list of FACS antibodies for MM-MRD NGF

Tube	BV421	BV510	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-C750
1	CD138	CD27	CD38	CD56	CD45	CD19	CD117	CD81
2	CD138	CD27	CD38	CD56	CD45	CD19	CyIg κ	CyIg λ

Marker	Fluorochrome	Clone	Source	Catalogue number	Application in EuroFlow panel	uL/ test
CD19	PE-Cy7	J3-119	Beckman Coulter	IM3628	MM-MRD, ALOT, LST, SST, BCP-ALL, PCD, B-CLPD, BCP-ALL MRD	5
CD27	BV510	O323	BioLegend	302835	MM-MRD	10
CD38	FITC	Multi-epitope	Cytognos	CYT-38F2	MM-MRD	6
CD45	PerCP-Cy5.5	HI30	BioLegend	304028	MM-MRD	10
CD56	PE	C5.9	Cytognos	CYT-56PE	MM-MRD, PCD, AML	2
CD81	APC-C750	M38	Cytognos	CYT-81AC750	MM-MRD	6
CD117	APC	104D2	BD Biosciences	333233	MM-MRD, PCD, BCP-ALL	5
CD138	BV421	MI15	BD Biosciences	562935	MM-MRD	2
CyIg κ	APC	Polyclonal	Dako	C0222	MM-MRD, PCD	5
CyIg λ	APC-C750	Polyclonal	Cytognos	CYT-LAC750	MM-MRD, PCD, BCP-ALL	3

2.2 Sample preparation

Bone marrow aspirate or peripheral blood is obtained in EDTA tubes (BD Bioscience, USA). After determining how many cells are in the blood per uL, proceed with a blood volume containing at least 10 x 10^6 cells. Red blood cell lysis was carried out by filling up to 2 mL of blood in a 50 mL conical tube and filling up to 50 mL of BulkLysis^{TM}(Cytognos, Spain).

When the number of cells per uL was small and more than 2 mL of blood was required, several 50 mL tubes were used.

After mixing well, incubate the tube at room temperature for 15 minutes. Centrifuge the tube at 800 g for 10 minutes and remove the supernatant using a Pasteur pipette. To washing the cells, resuspend the cell pellet with 2 mL of PBS mixed with 0.5% BSA, 0.09% NaN_3. After that, fill up to 50 mL with the PBS mixed with 0.5% BSA, 0.09% NaN_3 and mix well. After centrifugation of the tube at 800 g for 5 minutes, the supernatant is removed. After resuspending in the same way as above, transfer the resuspend to a 5 mL FACS tube. Centrifuge the FACS tube at 540 g for 5 minutes, and then the supernatant is poured and decanted. After resuspending the cell pellet with 180 uL of PBS, it is used for the subsequent steps.

2.3 Staining process and acquisition

To staining surface antigens with FACS antibodies, add 120 uL of distilled water to the surface staining reagent included in the MM-MRD kit. Mix the reagents well and leave it for at least 30 minutes at room temperature before use. Because of CD27 and CD137 antibodies were not included in MM-MRD kit, staining was carried out separately. First, add CD27 and CD138 antibodies to 20 uL of surface staining mixture and put them into two FACS tubes. Divide the resuspension prepared in step 2.2 into two tubes and put them all. Mix the tube well

and leave for 30 minutes in the dark at room temperature for staining. Thereafter, 2 mL of PBS was added, mixed the tube well, and centrifuged at 540 g for 5 minutes. Remove the supernatant, leaving only about 100 uL in each tube. Since the staining for cell surface antigen staining has been completed, only one of the two tubes for intracellular staining proceeds with the following process. The other tube is stored at room temperature in a dark place.

To staining the intracellular antigens, add 100 uL of Reagent A (Fix&Perm, Nordic-MUBio BV, The Netherlands) to the tube prepared above, and mix the tube thoroughly. To fix the cells, store the tube for 15 minutes in the dark at room temperature. To washing the cell, add 2 mL of PBS, mix, and centrifuge the tube at 540 g for 5 minutes. Remove the supernatant, leaving only about 100 uL. For the next step, resuspend the cell pellet. To permeabilize the cell, add 100 uL of Reagent B (Fix&Perm, Nordic-MUBio BV, The Netherlands) to the tube and mix well. To use intracellular FACS antibodies, add 70 uL of distilled water to the cytoplasmic staining reagent in the MM-MRD kit and mix, and 10 uL of this is added to the tube containing 200 uL mixture of Reagent B. Mix the tube well and to staining the antibodies store the tube in the dark at room temperature for 15 minutes. Centrifuge the tube at 540 g for 5 minutes and remove the supernatant after leaving only about

100 uL. The staining of both tubes has now been completed for FACS analysis.

Before inserting FACS tubes into analyzer, 500 uL of PBS was added to both tubes to resuspend all cell precipitates. A analysis was performed using Navios (Beckman Coulter Inc., Miami, FL, USA). The analysis was performed immediately after the staining was completed. In case of the previous staining procedure was delayed or the cell acquisition was delayed, the analysis was performed after storing the tube at 4℃. The flow cytometer' s acquisition speed is set to an intermediate level for analysis.

2.4 MM-MRD results analysis using Infinicyt program and gating strategy

Infinicyt (Cytognos, Spain) was used to analyze the results. The gating strategy followed the consensus guidelines and methods recommended by EuroFlow (Stetler-Stevenson et al., 2016).

First, the population with bright positive CD38 expression is broadly gated. Then, by gating the CD138 positive population, dying cells and non-plasma cells such as NK cells are excluded. After that, the plasma cell was refined by performing CD38 bright gating once more on the CD38-CD45 dot plot. Then, the remaining non-plasma cells are excluded using the

Automatic Parameter Separation (APS) diagram provided by Infinicyt software. The APS diagram is a diagram that clusters cell populations according to the expression patterns of CD45, CD138, CD38, CD19, CD56, and CD28, which are commonly included in intracellular and surface staining tubes. After gating each cluster analyzed in the APS diagram, non-plasma cells can be ruled out by checking the antigen expression pattern. (Figure 1) Finally, when defining Neoplastic plasma cells, CyIg κ/CyIg λ plasma cell ratio information is helpful. This is because neoplastic plasma cells have an extreme CyIg κ/CyIg λ plasma cell ratio.

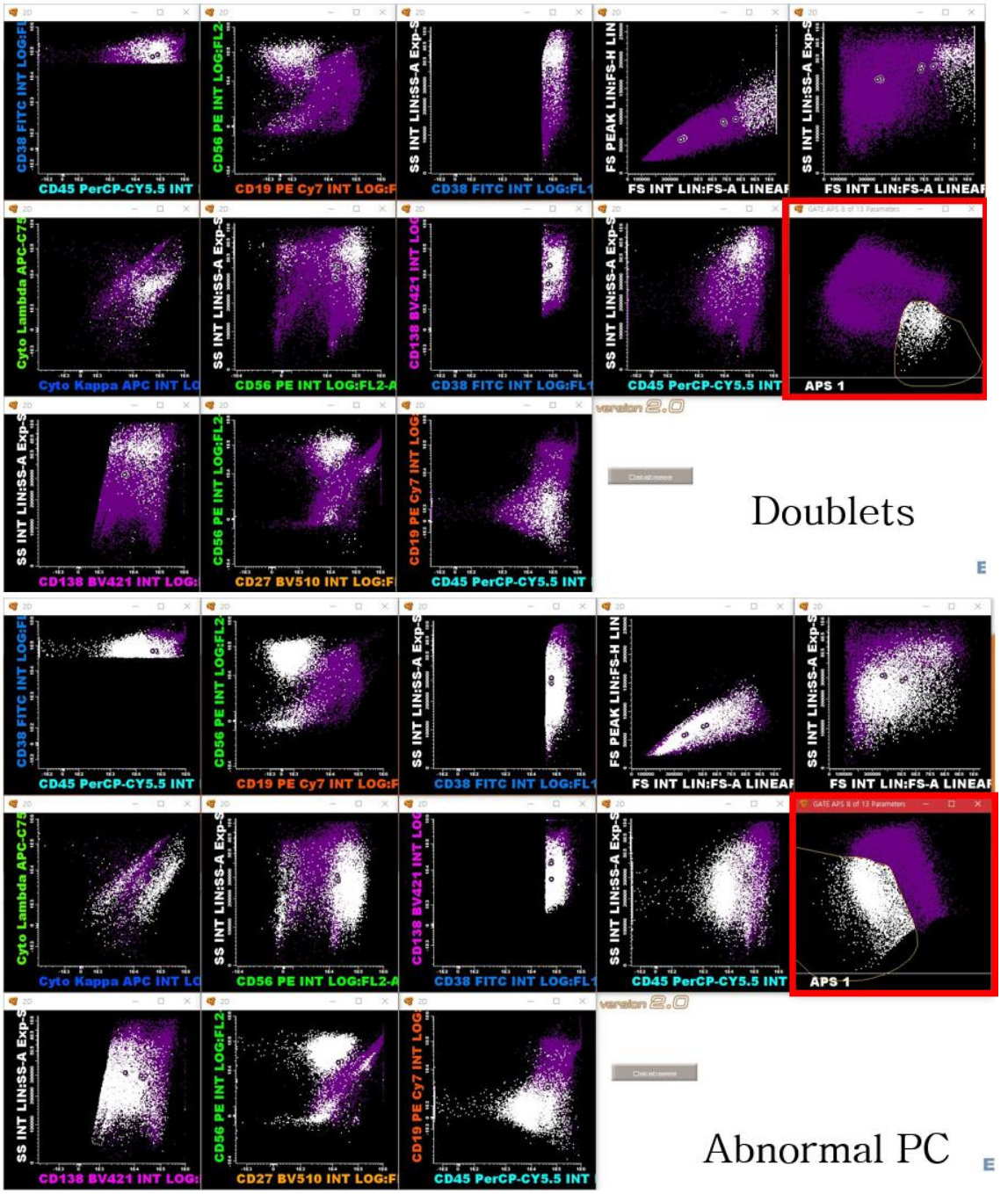


Figure 1-1. Illustration of cell separation via APS diagram in Infinicyt software

2.5 Assessment of minimal residual diseases by NGF

To evaluate the appropriateness of the MRD by NGF, the number of acquired cells was counted, and the viability of the acquired cells was calculated. Samples with a minimum number of 5,000,000 or more acquired cells and a viability of 85% or more were determined to be appropriate for the evaluation. The detection sensitivity of the acquired cells was calculated as: (total number of cells/limit of detection (LOD))/ (total number of acquired cells). In addition, the LOD (<20) and lower limit of quantification (LLOQ) (>50) of each specimen were assessed, and patients with LOD ≥20 with BM and/or PB were defined as positive for BM MRD and/or cPC, respectively.

2.6 Immunophenotypic expression features of plasma cell by flow cytometry

The expression patterns of six immunophenotyping molecules (CD56, CD27, CD19, CD45, CD117, and CD81) of neoplastic clonal PC and normal PC in PB and BM samples were analyzed. The expression patterns of cluster of differentiation (CD) molecules were divided into five categories. Positive was divided into three categories: dim, moderate, and bright; the other two categories were negative and heterogeneous.

Heterogeneity refers to a pattern with both negative and positive expression.

3. Linearity test of NGF

The linearity of flow cytometry was evaluated using plasma cell lines (U266, ATCC-TIB196, ATCC) and BM aspirates from patients with newly diagnosed multiple myeloma (NDMM). The plasma cell line U266, and BM aspirates from patients with NDMM were diluted with normal nucleated cells in BM to 1%, 5%, and 10%, and 0.01%, 1%, and 50%, respectively, to verify linearity. BM aspirates from staging patient with malignant lymphoma without BM invasion was used to normal BM nucleated cells.

4. Immunoturbidimetric assay (Measurement of serum free light chain level)

The serum free light chain level was measured by the immunoturbidi-metric assay. The reagents used were Optilite Freelite Kappa kit and Optilite Freelite Lambda kit (The Binding Site, Birmingham, UK). Assay was all conducted using an automated device (TBS Optilite), and the results were also analyzed in the device.

5. Statistics

Pearson's correlation assay was used to analyze the correlation between PB/BM and each laboratory test. The Mann-Whitney U test was also applied to compare the clinical features and results of various laboratory tests according to polyclonality. For comparison of the immunophenotypic expression patterns between PB and BM, and between normal and nPC, Chi-square test and Fisher's exact test were performed. All statistical analyses were performed using PASW statistics version 18 (SPSS Inc., Chicago, USA) and MedCalc version 12.0 (MedCalc Software, Mariakerke, Belgium).

RESULTS

Evaluation results of flow cytometry: validation and linearity

The linearity of the NGF was verified using multiple myeloma cell lines and primary myeloma cells from multiple myeloma patient. Myeloma cell line U266 (ATCC-TIB-196, ATCC) was used. Since U266 cell line has high expression of CD138, population of cell line was confirmed by gating the CD138 bright area in FACS analysis. The cell lines were diluted to 1%, 5%, and 10% with normal bone marrow nucleated cells to confirm linearity. For the normal bone marrow nucleated cells, the bone marrow of a patient without bone marrow invasion was used among the bone marrow aspirate samples performed to stage malignant lymphoma. The results of the detected percentage of U266 cell line by using NGF and the dilution ratio of U266 cell line showed a strong correlation. (R^2=0.9994, p<0.001)

Primary myeloma cells were diluted to 0.01%, 1%, and 50% with normal blood marrow nucleated cells to verify linearity. Same as above, for the normal bone marrow nucleated cells, the bone marrow of a patient without bone marrow invasion was used among the bone marrow aspirate samples performed to stage malignant lymphoma. The proportion of bone marrow plasma cells at the time of initial diagnosis of the patient was

18.2% by bone marrow differential count of bone marrow aspiration. The analysis of the results was based on the assumption that 18.2% plasma cells were 100% and diluted with normal bone marrow nucleated cells. For example, in the case of 50% dilution, it was predicted that there would be 9.1% plasma cells, which is 50% of 18.2%. The percentage of diluted primary myeloma cells and the percentage of primary myeloma cells detected using NGF showed a strong correlation. (R^2=0.9999, p<0.001) (Figure 1-2)

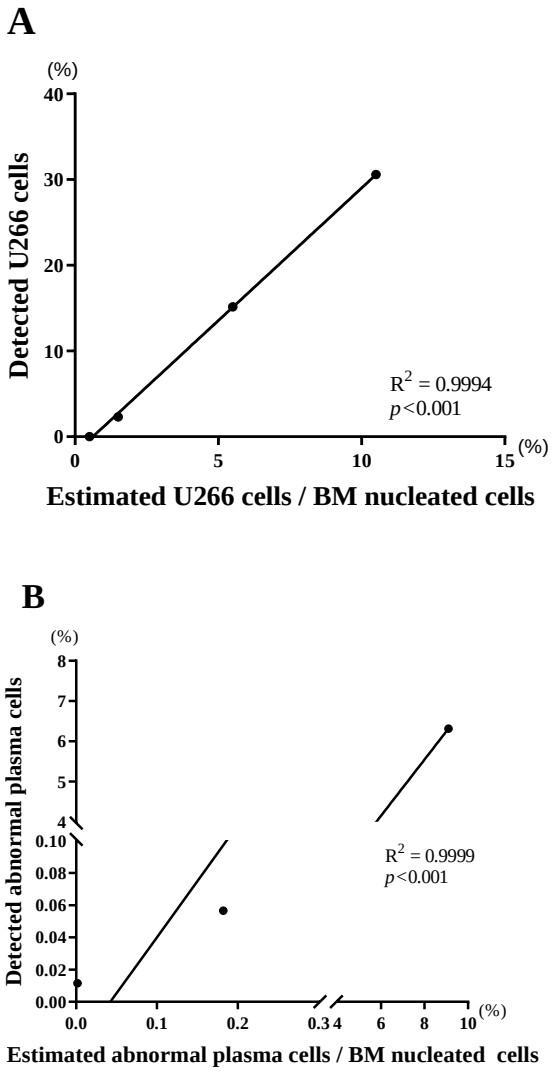


Figure 1-2. Validation of linearity of next generation flow cytometry analysis using U266 myeloma cell line and primary myeloma cells of myeloma patient at initial diagnosis. (A) The results of U266 cells by the next generation flowcytometry after mixing the myeloma cell line U266 with normal bone marrow specimen in the ratio of 1%, 5%, and 10% (R^2=0.9994, p<0.001) (B) The results of the neoplastic plasma cells after

mixing of primary myeloma cells from patients with normal bone marrow mononuclear cells (the ratio of 0.01%, 1%, and 50%) (R^2=0.9999, p<0.001)

IMWG Treatment response of patients

Twenty-eight pairs of bone marrow and peripheral blood were tested for MRD and circulating abnormal plasma cell by NGF. As a result, bone marrow MRD were positive in 19 patients (19/28, 67.9%), and circulating abnormal plasma cells in peripheral blood were positive in 11 patients (11/28, 39.2%). The circulating abnormal plasma cells in peripheral blood were detected in 11 of 19 patients (57.9%) who were positive for bone marrow MRD. All 11 patients with peripheral blood circulating abnormal plasma cells were positive for MRD in the bone marrow. MRD was positive in 40.0% (4/10) of CR+VGPR patients, and MRD was positive in 83.3% (15/18) of patients with a PR, MR, SD, PD, and relapse.

Of the 19 MRD positive samples, more than 50 abnormal plasma cells, which are lower limit of quantification (LLOQ), were detected in 18 positive samples excluding 1 sample that was a PR. Two of the 7 Complete Response specimens (28.6%) showed MRD positive, and 71.4% of CR patients showed MRD negative by NGF. Both Progressive Disease (n=3) and Relapse (n=3) patients were positive for MRD. In 80% of Partial

Response (n=5) and Stable Disease (n=5) patients, bone marrow MRD was positive. On the other hand, 20% of PR and SD patients were MRD negative. 66.7% of patients with Very Good Partial Response (n=3) and 50% of patients with Minimal Response (n=2) were MRD positive. Of a total of 28 bone marrow samples, 19 samples (67.9%) were MRD positive by NGF. (Table 1-3)

Table 1-3. Treatment response of patients who were subjected to next generation flow cytometry

Treatment Response According to IMW criteria	Number of Specimens (n=28)	Number of patients showing BM MRD positivity (%)	Number of Patients showing circulating abnormal plasma cells in peripheral blood †
Complete Response	7	2/7 (28.6%)	1/7 (14.3%)
Very Good Partial Response	3	2/3 (66.7%)	0/3 (0%)
Partial Response	5	4/5 (80%)	3/5 (60%)
Minimal Response	2	1/2 (50%)	1/2 (50%)
Stable Disease	5	4/5 (80%)	1/5 (20%)
Progressive Disease	3	3/3 (100%)	3/3 (100%)
Relapse	3	3/3 (100%)	2/3 (66.7%)
Total	**28**	**19/28 (67.9%)**	**11/28 (39.3%)**

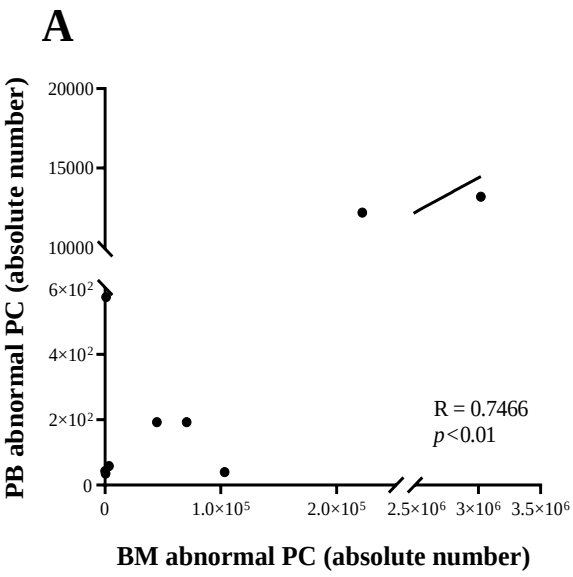
† All of patients showing circulating abnormal plasma cell in peripheral blood showed MRD positivity in bone marrow

Comparison with the fraction and absolute number of neoplastic plasma cell in BM and PB

The number of peripheral blood circulating plasma cells and the corresponding number of MRD cells in the bone marrow showed a clear positive correlation. (R=0.7466, p<0.01) (Figure 1-3A) However, the number of peripheral blood circulating plasma cells was significantly lower than MRD cells of the bone marrow. (p<0.01) The treatment response of patients with positive peripheral circulating plasma cells were CR, PR, MR, SD, PD, and Relapse. Surprisingly, abnormal plasma cells were also found in the peripheral blood of patients who showed CR. (1/9, 14.3%) Peripheral circulating plasma cells were detected in all three patients of PD, in 66.7% (2/3) of relapsed patients and in 60% (3/5) of PR patients. In the case of negative MRD in bone marrow, no abnormal plasma cells were found in paired peripheral blood.

Peripheral circulating abnormal plasma cells were detected in 11 of 19 patients (57.9%) with positive bone marrow MRD. In 19 samples in which MRD cells were detected in the bone marrow, the correlation between the fraction of bone marrow MRD cells and the fraction of peripheral circulating abnormal plasma cells was analyzed. (Figure 1-3B) Compared to the MRD fraction detected in bone marrow, the fraction of abnormal plasma cells found in peripheral blood was

significantly smaller. However, the patient with more malignant clonal plasma cells found in the bone marrow, the higher the number of abnormal plasma cells detected in the peripheral blood, which showed a strong positive correlation. ($R=0.8726$, $p<0.001$) That is, as the number of peripheral circulating abnormal plasma cells increased, the number of malignant clonal cells in the bone marrow increased.



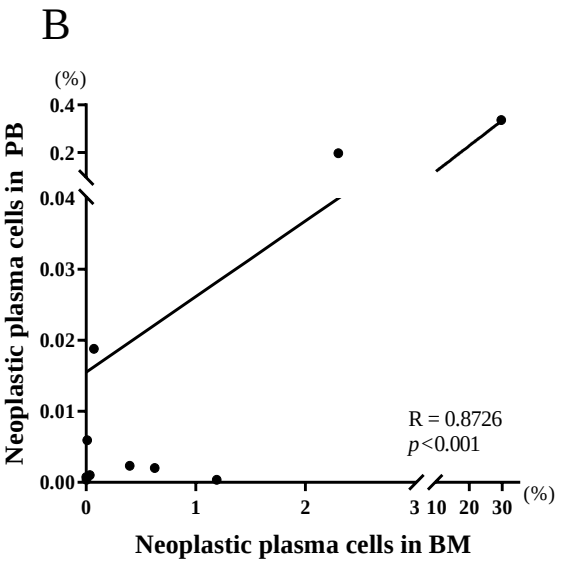


Figure 1-3. (A) Correlation between absolute number of minimal residual cells in bone marrow and in peripheral blood by next generation flow cytometry (B) Correlation between BM minimal residual cells (%) and PB circulating abnormal

plasma cells (%) by NGF (A) showed a strong correlation (R=0.7466, p<0.01, n=11) (B) showed a very strong correlation (R=0.8726, p<0.001, n=11)

Quantitative data of NGF MRD in BM and PB

At least 5,000,000 bone marrow cells must be acquired, and the viability of the analyzed cells must be 85% or more for the determination of the MRD by NGF in a bone marrow sample. All 28 bone marrow samples performed in this study satisfied the above two conditions. (Table 1-4)

The median number of acquired bone marrow cells was 11,211,380, and the detection sensitivity (limit of detection / total number of acquired cells) was 1.78×10^{-6}. The median value of a acquired peripheral blood cells was a 10,098,128 cells, and a sensitivity of 1.98×10^{-6}, which was similar to that of the bone marrow sample. Among the bone marrow samples, the median number of detected malignant clonal plasma cells (MRD cells) in 18 samples in which 50 or more cells were found, which is the lower limit of quantification that can be used statistically, was 4,769.

In contrast, the median number of abnormal plasma cells detected in peripheral blood was 193, which was significantly smaller in the number of neoplastic plasma cells found compared to bone marrow samples. The number of MRD

observed in one patient was above the LOD and below the LLOQ. In this patient, the number of MRD cells found was not used for statistical analysis because the MRD was positive but not quantifiable. There were patients with less than 20 MRD cells, but this was MRD negative because it corresponds to less than LOD, and 9 patients correspond to this. Among the peripheral blood samples, 11 patients detected cPC above the LOD, and no patients found cPC below the LOD.

Table 1-4. Quantitative results of MRD^{NGF} in bone marrow and peripheral blood

	Bone Marrow	Peripheral Blood	MRD positive Bone Marrow	Circulating abnormal PC positive Peripheral Blood
Number of samples	28	28	19	11
Total acquisition cell counts, median (range)	11,211,380 (7,174,946-12,000,000)	10,098,128 (2,715,395-12,000,000)	11,146,239 (7,174,946-12,000,000)	10,086,287 (4,184,845-12,000,000)
No. of samples meeting total acquisition cell counts >5,000,000	28	23	19	10
No. of samples which are >85% of viability	28	27	19	11
No. of samples which showed abnormal cells, <20 (LOD)	9	17	0	0
No. of samples which showed abnormal cells, >20 and <50	1	3	1	3
No. of samples which showed abnormal cells, >50 (LLOQ)	18	8	18	8
Median of Absolute number of MRD which are >50 (LLOQ), (range) †	4,769 (52-3,018,627)	193 (35-13,199)	4,769 (52-3,018,627)	193 (35-13,199)

PC; plasma cell

† In the case of peripheral blood, >0 not >50

Comparison with the conventional BM analysis and NGF

In 28 bone marrow samples, the correlation between the fraction of plasma cells on the differential calculation of bone marrow leukocytes and the fraction of plasma cells detected

through NGF was analyzed. (Figure 1-4) The two values showed a strong positive correlation with an R value of 0.985. However, the fraction of plasma cells calculated through differential count tended to be more than twice as large as the fraction of plasma cells detected through NGF. (p<0.0001) For example, in one patient, 70.4% of bone marrow cells were confirmed to be plasma cells through differential count, but only 29.7% were confirmed to plasma cells by NGF. In the other patient, 32.2% were plasma cells by differential count of bone marrow, but 10.1% were detected as plasma cells by NGF. This is presumed to be since the sample for flow cytometry was significantly diluted by peripheral blood.

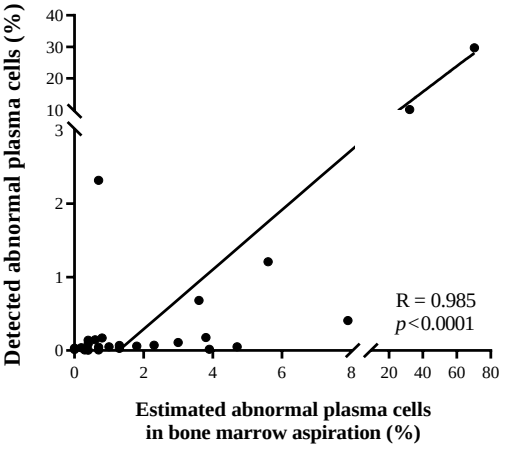


Figure 1-4. Correlation between differential count of plasma cells in bone marrow aspiration and minimal residual cells by

next generation flowcytometry Showed a very strong correlation (R=0.985, p<0.0001, n=28)

In addition, the correlation between the fraction of plasma cells calculated from bone marrow biopsy and the fraction of plasma cells detected through NGF was analyzed. (Figure 1-5) The correlation coefficient R value was 0.7417 (p<0.0001), showing a positive correlation. The reason for showing relatively weak correlation compared to the correlation between the fraction of plasma cells calculated from bone marrow aspiration and the fraction of plasma cells detected from NGF is that the plasma cells detected through NGF were very small in the two samples compared to the fraction of plasma cells calculated in the bone marrow biopsy. Plasma cells (%) by bone marrow biopsy and MRD^{NGF} cells (%) by NGF of these two patients were 90% and 2.3%, respectively, and 40.0% and 0.4%. This significant difference in numerical values is presumed to be due to the dilution of the bone marrow sample by peripheral blood.

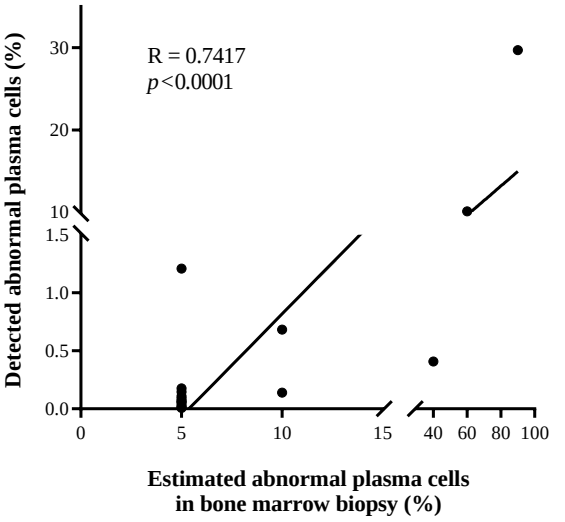


Figure 1-5. Correlation between $CD138^+$ plasma cells in bone marrow section and minimal residual cells by NGF showed a strong correlation (R=0.7417, p<0.0001, n=28)

Comparison with the Free Light Chain level and NGF MRD

The correlation between the percentage of malignant clonal plasma cells found by NGF and the serum free light chain measured in patients was analyzed. (Figure 1-6) The R value was -0.0992, showing a very weak negative correlation (p=0.6154)

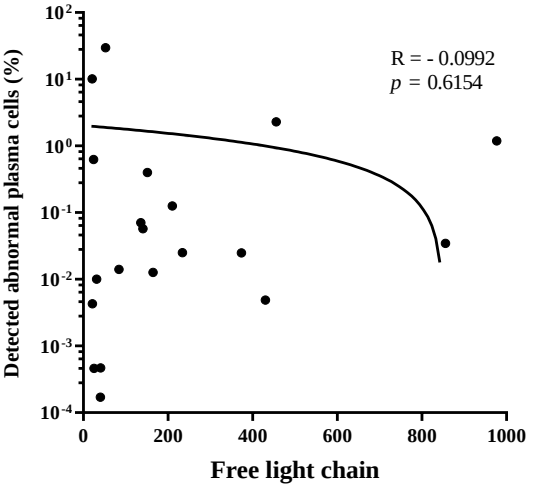


Figure 1-6. Correlation between MRD by NGF and serum free light chain showed a very weak negative correlation (R= -0.0992, p=0.6154, n=28)

The correlation between the number of malignant plasma cells and serum free light chain as well as the fraction of malignant plasma cells found in BM was also analyzed. (Figure 1-7) The correlation showed a very weak negative correlation like the fraction. (p= 0.6152) However, in both results, the absolute value of the R value was less than 0.1, showing no significant correlation, and the p value was also greater than 0.05, which was not statistically significant.

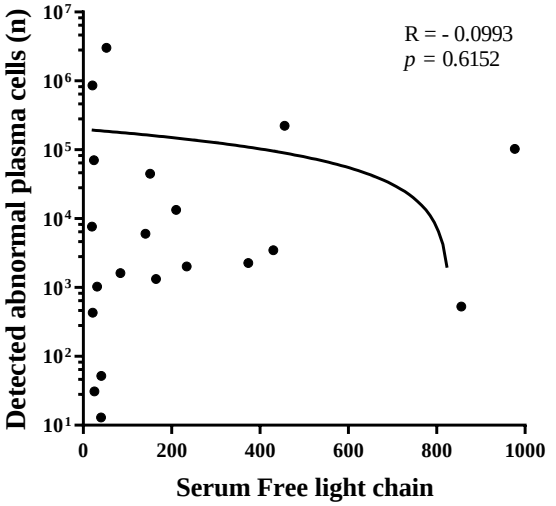


Figure 1-7. Correlation between absolute number of neoplastic plasma cells in BM and serum FLC showed a very weak negative correlation (R= −0.0993, p=0.6152, n=28)

In addition to comparison of bone marrow MRD cells and serum FLC, the fraction of circulating neoplastic plasma cells found in PB and serum FLC were compared. (Figure 1-8A) As a result, it showed a very weak negative correlation similar to results of comparison between malignant plasma cells found in bone marrow and FLC. (R= −0.008, p=0.9676) The FLC correlation analysis was also performed with the absolute number of circulating nPCs in the PB (Figure 1-8B), which showed a very weak positive correlation. (R=0.0059, p=0.9762) Same as the bone marrow MRD compare, in both results, the absolute value of the R value was less than 0.1,

showing no significant correlation, and the p value was also greater than 0.05, which was not statistically significant.

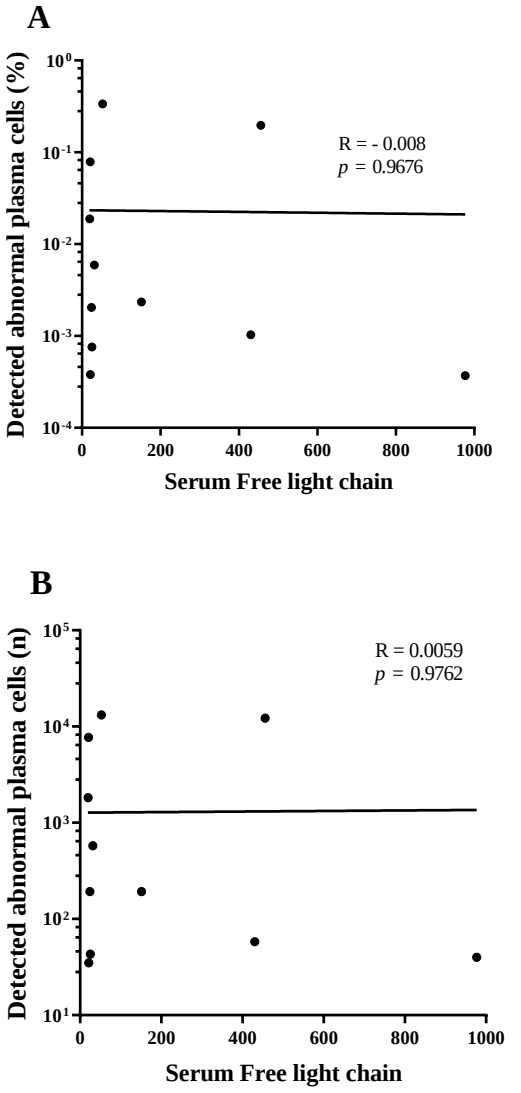


Figure 1-8. (A) Correlation between circulating nPC in PB and serum free light chain (B) Correlation between absolute number of nPC in PB and serum free light chain (A) showed a

very weak negative correlation (R= −0.008, p=0.9676, n=11) (B) showed a very weak positive correlation (R= 0.0059, p=0.9762, n=11)

Comparison with the FLC level/ ratio

The average values of the absolute number of FLC of nPCs measured by flow cytometry in BM and PB were 71,519.4 and 488.8, respectively. Moderate correlations were observed (R=0.606, p=0.0006). The average value of the sFLC level measured by automated immunoassay (Optilite, The Binding Site, Birmingham, UK) was 157.9 (95% CI: 64.1−251.7). When serum FLC was compared to the FLC level of BM/PB nPC using flow cytometry (FLC^{flow}), no statistically significant correlation was found. When rank correlation analysis was applied, there was a moderate correlation between BM FLC^{flow} and sFLC levels (R = 0.472, p=0.0112). Serum FLC levels also showed a moderate correlation with BM MRD% (R = 0.493, p=0.0077).

For the FLC ratio (rFLC), the median value of rFLC of nPC in BM (BM $rFLC^{flow}$) and PB (cPC $rFLC^{flow}$) was 435.0 (95% CI: 2.5 – 2089.3) and 0 (95% CI: 0.0−22.3), respectively. Serum rFLC had a median value of 2.71 (95% CI: 1.62 −8.79). Based on an rFLC ≥ 8, patients were classified as either

involved or uninvolved in rFLC abnormality. Five patients had an rFLC $\geq$ 8 in all three specimens, and eight patients had an rFLC < 8 in all three specimens. Six patients were uninvolved only in serum rFLC, and two patients were only involved in cPC $rFLC^{flow}$. Five patients had rFLC abnormality only in BM PC $rFLC^{flow}$ and no patient was only uninvolved in BM $rFLC^{flow}$. In a total of 28 patients, 15 patients showed discrepancies (Table 1-5)

Table 1-5. Patients had discrepancy in the FLC ratio in serum, BM minimal residual cells, and circulating plasma cells.

Patient number	IMWG response	FLC ratio*			Initial type	PC% (manual counting)		Absolute No. of PC†		M protein (g/dL)
		Serum	BM MRD	cPC		BMA	BMB	BM MRD	cPC	
Pt 17	PD	7.76	9065.74	337.5	Lambda	70.4	90	3018627	13199	5.9
Pt 30	PR	2.18	4569.14	3681000	Kappa	32.2	60	860141	7719	2.44
Pt 37	Relapse	1.34	2984.091	58000	Kappa	3.6	10	70481	193	0.73
Pt 36	CR	0.78	373	347000	Lambda	1	5	1030	576	0
Pt 13	SD	1.53	163000	10000	Kappa	0.2	5	429	35	0.47
Pt 6	PR	1.96	7000	23000	Kappa	0.7	5	31	43	0.59
Pt 12	PR	7.11	4513	NA	Lambda	0.4	10	13423	0	NA
Pt 19	CR	4.28	680.25	NA	Lambda	3	5	6055	0	0
Pt 35	SD	1.92	673000	NA	Lambda	0	5	1613	0	0.7
Pt 31	VGPR	7.99	99.4	NA	Lambda	0.4	5	1333	0	0
Pt 1	PR	2.72	7000	NA	Kappa	0.6	5	13	0	NA
Pt 8	Relapse	27.04	290	NA	Kappa	3.8	5	2267	0	0
Pt 10	SD	13.39	55.0833	NA	Kappa	1.3	5	2017	0	NA (suspected)
Pt 28	SD	9.94	5.1613	NA	Lambda	2.3	5	529	0	0.99
Pt 26	SD	11.36	NA	NA	Kappa	0.8	5	0	0	NA

*FLC ratio was calculated by automated immunoassay for serum and by flow cytometry for BM aspirates (BM MRD) and peripheral blood (cPC) specimens.

†Values represent the absolute number of BM MRDs and circulating plasma cells in PB as measured by flow cytometry.

Abbreviations: FLC, free light chain; BMB, bone marrow biopsy; BMA, bone marrow aspiration; IMWG, International Myeloma Working Group; MRD, minimal residual disease; cPC, circulating plasma cells

Comparison with the M-protein percent and level

BM MRD% and cPC% measured by flow cytometry showed significant correlations with both the quantitative value and percentage of the M protein in serum. For BM MRD%, there was a strong correlation with the quantitative value of the M protein ($R = 0.9000$, $p<0.001$) and a moderate correlation with the M protein% ($R^2 = 0.755$, $p<0.001$). In addition, cPC% showed moderate or higher correlation with both the quantitative M protein values ($R^2 = 0.735$, $p<0.001$) and M protein% ($R^2 = 0.582$, $p<0.735$).

The laboratory test results, including conventional tests for routine BM diagnosis and subsequent workup, and absolute number of nPCs in BM and PB performed by flow cytometry are presented. (Table 1-6) Four patients (Pt17, Pt20, Pt37, and Pt25) were diagnosed with PD based on manual counting of PCs in BM aspiration and/or BM biopsy. These patients were positive for BM MRD% and cPCs, as determined by flow cytometry. For Pt 18 and Pt 8, there was no significant increase in PC in both BM aspiration and biopsy; however, they were diagnosed as PD due to a marked change in the quantitative values of the M protein in serum and urine, respectively. Both patients were positive for BM MRD%, and Pt18 was also cPC positive.

Table 1-6. Demographics and characteristics of patients with progressive disease or relapse.

Patient number	Sex	Age	BMA PC%	BMB PC%	M-protein (s, g/dL)	M-protein (u,%)	IFE (Ig)	IFE (type)	Serum rFLC	BM MRD*	cPC*	BM $rFLC^{flow}$	cPC $rFLC^{flow}$
Pt 17	F	61	70.4	90.0	5.9	3.4	IgG	lambda	7.76	3018627	13199	9065.7400	337.5
Pt 20	M	75	0.7	90.0	0	65.2	NA	NA	59.72	222210	12196	178.1779	525.2
Pt 18	M	62	5.6	5.0	2.58	59.9	IgG	Kappa	41.8	103262	40	1525.5000	21000.00
Pt 37	M	49	3.6	10	0.73	0	NA	NA	1.34	70481	193	2984.0909	58000.00
Pt 25	F	64	7.9	40.0	0.73	NA	NA	NA	16.35	44859	193	2494.8000	47000
Pt 8	M	60	3.8	5.0	0	7.9	NA	NA	27.04	2267	0	290.0000	NA

*Values represent the absolute number of BM MRD and circulating plasma cells in PB as measured by flow cytometry.

Abbreviations: BMA, bone marrow aspiration; BMB, bone marrow biopsy; rFLC, free light chain ratio; BM, bone marrow; IFE, immunofixation; MRD, minimal residual disease; cPC, circulating plasma cells

Difference expression pattern between normal plasma cells and abnormal plasma cells

The expression patterns of the immunophenotype between malignant clonal plasma cells and normal plasma cells found by NGF were compared and analyzed in patients with positive bone marrow residual disease. (Table 1-7) The expression patterns of the cluster of differentiation (CD) molecules were divided into five categories. Positive is expressed as Dim, Moderate, and Bright according to the intensity of expression, and the other two are negative and heterogeneous. Negative means that the corresponding immunophenotype is not expressed, and heterogeneous has both negative and positive expression patterns. (Figure 1-9) A total of 6 CD molecules were analyzed (CD56, CD27, CD19, CD45, CD117, CD81).

Among them, there was no difference in expression pattern of CD81 between malignant clonal plasma cells and normal plasma cells ($p = 0.053$). All of the other CD marker molecules showed significant differences with p values of 0.05 or less. CD56 was positive in most malignant clonal plasma cells (16/19, 84.2%), but negative in 3 patients. On the other hand, the normal plasma cells showed CD56 negative in 94.7% (18/19). Unlike CD56, CD27 showed bright positive in all normal plasma cells, but in malignant clonal plasma cells, 31.6% (6/19) of samples were negative, 47.4% (9/19) were dim,

moderate positive. Compared to normal plasma cells, the expression level of CD27 was relatively weak in malignant plasma cells. Similar with CD27, CD19 showed bright positive in 94.7% (18/19) of samples in normal plasma cells, but negative in 78.9% (15/19) of samples in malignant clonal plasma cells. CD45 was positive in both normal plasma cells and malignant clonal plasma cells, but the degree of expression was different. In the case of normal plasma cells, moderate and bright positives were 100% (19/19), but in the case of malignant clonal plasma cells, dim positive was 68.4% (13/19). Malignant clonal plasma cells showed a tendency to lower CD45 expression than normal plasma cells. ($p < 0.05$) CD117 was all negative in normal plasma cells. Malignant clonal plasma cells were positive in 52.6% (10/19), and all of the 10 positive samples were dim and moderate positive. CD81 showed a stronger expression in normal plasma cells. CD81 was positive in 89.5% (17/19), whereas only 47.4% (9/19) of malignant clonal plasma cells were positive. However, the difference in positive expression between these two groups was not significant, and both showed only dim positive, moderate positive, and heterogeneous patterns. ($p = 0.053$). Taken together, it was found that the six types of CD molecules used in MRD analysis were suitable for

distinguishing malignant clonal plasma cells from normal plasma cells.

Table 1-7. Comparison of immunophenotype expression between neoplastic and normal plasma cells in bone marrow, detected by next generation flowcytometry

	Plasma cell type	Negative	Positive				*p*-value
			Dim	Moderate	Bright	Hetero†	
CD56	Abnormal	3	-	-	14	2	$p<0.05$
	Normal	18	-	-	-	1	
CD27	Abnormal	6	6	3	-	4	$p<0.05$
	Normal	-	-	-	19	-	
CD19	Abnormal	15	3	-	-	1	$p<0.05$
	Normal	-	-	1	18	-	
CD45	Abnormal	-	13	2	2	2	$p<0.05$
	Normal	-	-	13	6	-	
CD117	Abnormal	9	7	2	-	1	$p<0.05$
	Normal	19	-	-	-	-	
CD81	Abnormal	10	4	1	-	4	$p=0.05$
	Normal	2	7	7	-	3	3

†Hetrerogeneous; Abnormal plasma cell clone showed negative to positive expression

Abbreviations: BM, bone marrow; MRD, minimal residual diseases

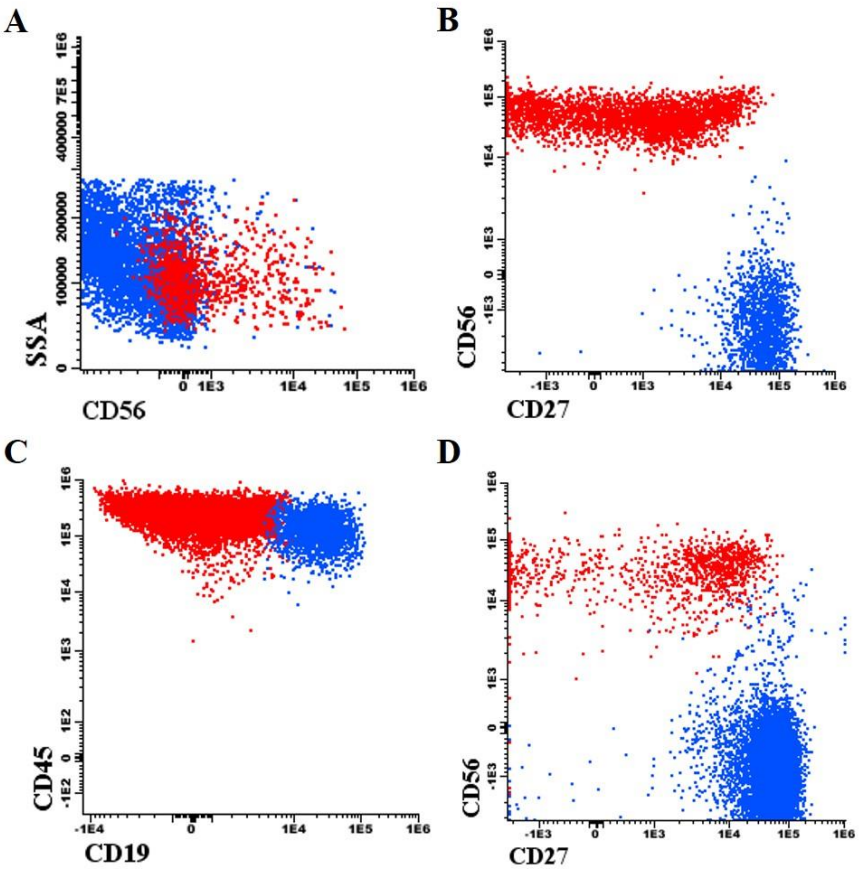


Figure 1-9. Profiles of heterogeneous immunophenotype expression of residual cells in BM; red dot, abnormal plasma cells; blue dot, normal plasma cells. (A) CD56 heterogeneous (B) CD27 heterogeneous (C) CD19 heterogeneous (D) CD27 heterogeneous

Difference expression pattern between abnormal plasma cell in BM and peripheral circulating plasma cells

Immunophenotype expression patterns of peripheral blood circulating plasma cells and malignant plasma cells of bone marrow were compared; CD56, CD27, CD19, CD45, CD117, CD81 (Table 1-8). Except for the CD27, the expression patterns of abnormal plasma cells in peripheral blood and malignant clonal plasma cells in bone marrow were not significantly different in all other CD molecules. In the case of CD27, there was a significant difference with $p < 0.05$. In bone marrow malignant plasma cells, CD27 was negative in 31.6% (6/19) samples, whereas peripheral blood circulating plasma cells were negative in 90.9% (10/11) samples. CD81 had a similar tendency. The expression of CD81 was negative in 52.6% (10/19) of malignant plasma cells of the bone marrow, but 90.9% (10/11) of abnormal plasma cells of peripheral blood were negative. (p = 0.085). The CD marker showing the most similar expression pattern was CD45, which was dim positive in 68.4% (13/19) of bone marrow malignant plasma cells and dim positive in 63.6% (7/11) of peripheral blood circulating plasma cells. The greatest difference in immunophenotype expression pattern was CD27, followed by CD81, CD19, CD56=CD117, and CD45. (Figure 1-10) Bivariate dot plots

showing the immunophenotype expression of all 11 patients with cPC found in PB are illustrated in Figure 1-11.

Table 1-8. Comparison of immunophenotype expression between BM MRD^{NGF} cells and peripheral circulating plasma cells

			Positive				vs. BM abnormal plasma cells
		Negative	Dim	Moderate	Bright	Hetero†	
CD56	PB	2	-	1	7	1	p=0.672
	BM	3	-	-	14	2	
CD27	PB	10	-	-	-	1	p<0.05
	BM	6	6	3	-	4	
CD19	PB	10	1	-	-	-	p=0.582
	BM	15	3	-	-	1	
CD45	PB	-	7	3	1	-	p=0.966
	BM	-	13	2	2	2	
CD117	PB	4	5	2	-	-	p=0.672
	BM	9	7	2	-	1	
CD81	PB	10	-	1	-	-	p=0.085
	BM	10	4	1	-	4	

†Heterogeneous; Abnormal plasma cell clone showed negative to positive expression

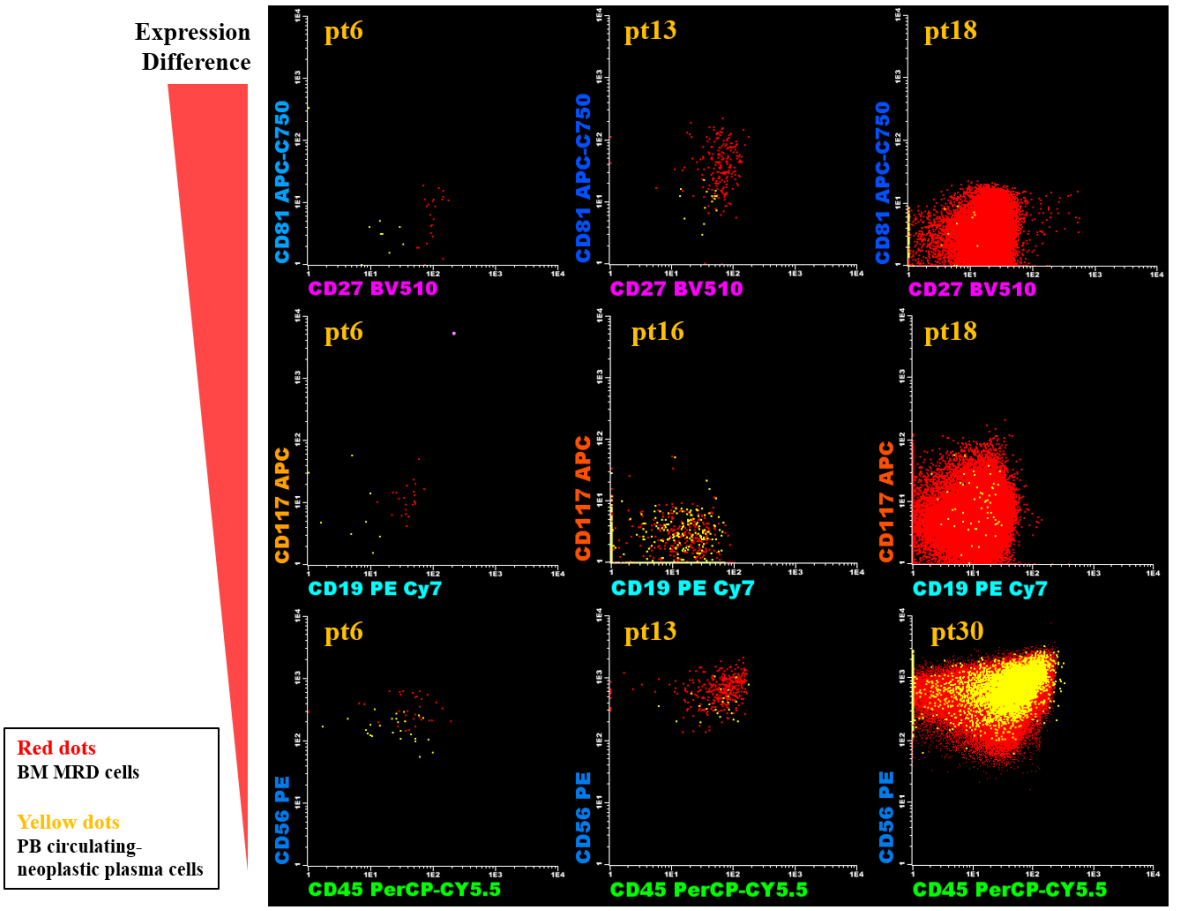
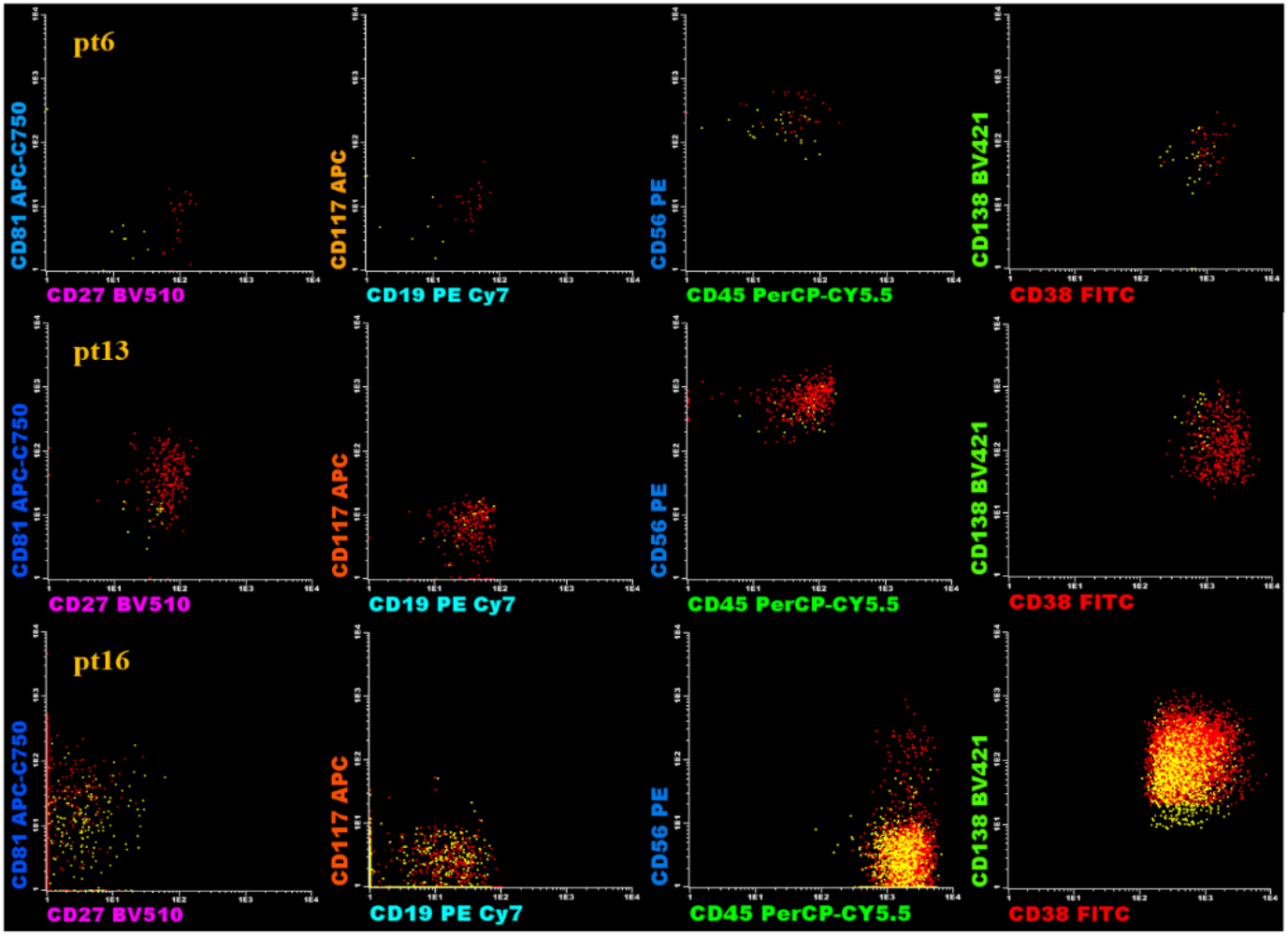


Figure 1-10. Illustrating graphical representations of the difference of the immunophenotype expression between BM MRD cells (red dots) and PB circulating plasma cells (yellow dots). BM MRD cells showed stronger CD27 expression than cPC in PB ($p < 0.001$), and CD45 had little difference in expression between the two cell groups ($p = 0.966$)


pt6
CD81 APC-C750
CD27 BV510
CD117 APC
CD19 PE Cy7
CD56 PE
CD45 PerCP-CY5.5
CD138 BV421
CD38 FITC
pt13
CD81 APC-C750
CD27 BV510
CD117 APC
CD19 PE Cy7
CD56 PE
CD45 PerCP-CY5.5
CD138 BV421
CD38 FITC
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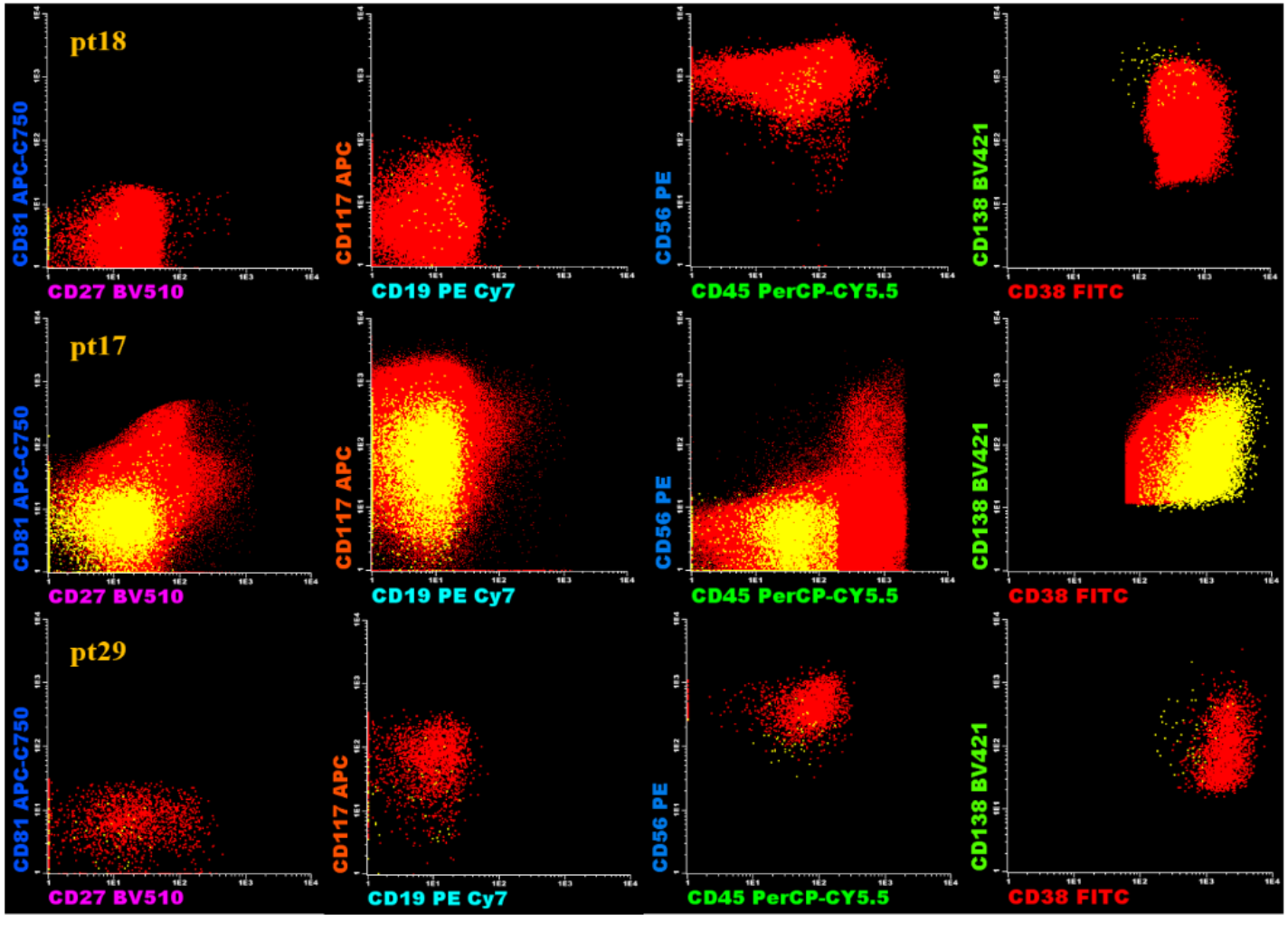
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CD56 PE
CD45 PerCP-CY5.5
CD138 BV421
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CD27 BV510
CD117 APC
CD19 PE Cy7
CD56 PE
CD45 PerCP-CY5.5
CD138 BV421
CD38 FITC
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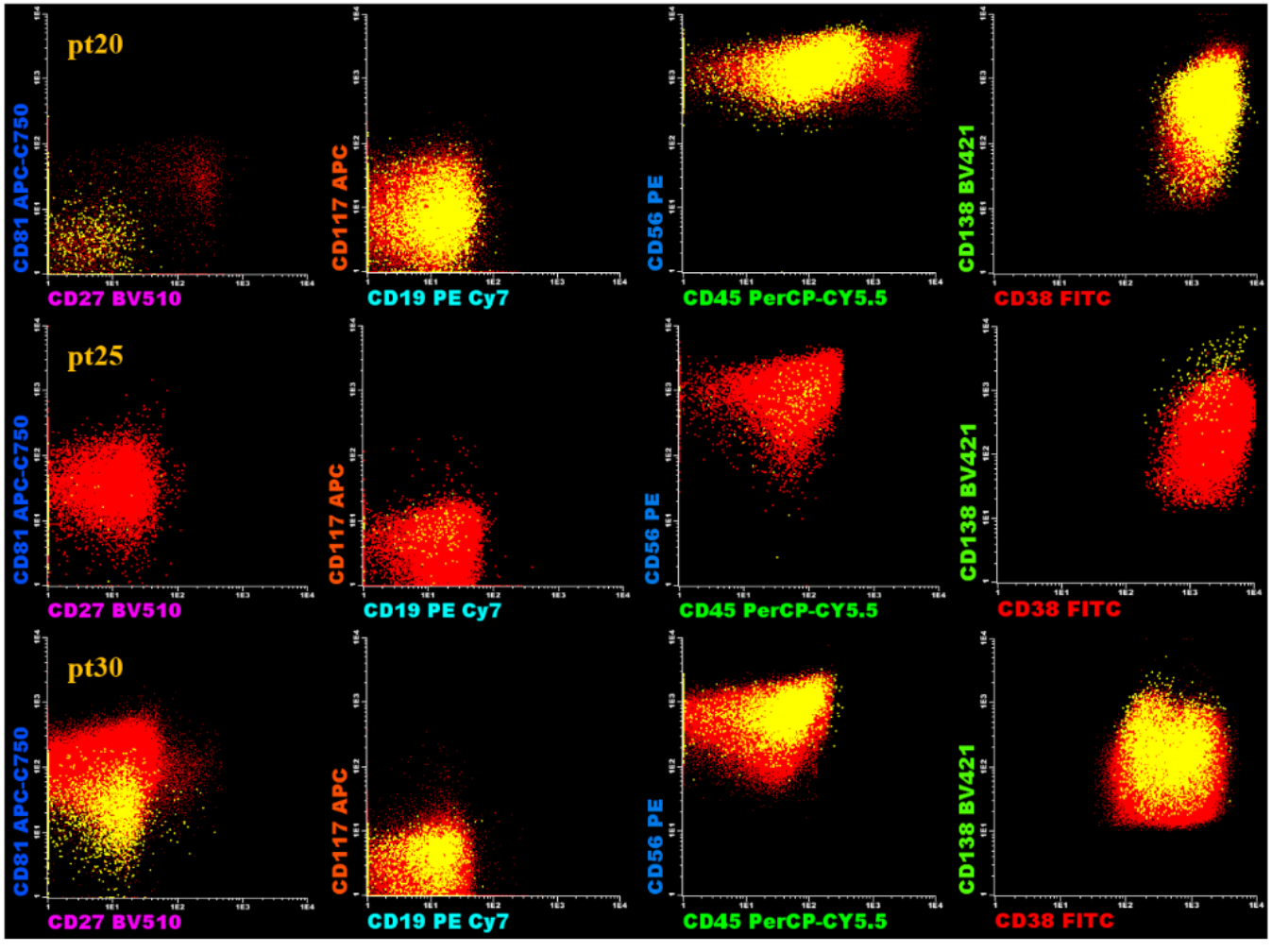
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CD45 PerCP-CY5.5
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CD27 BV510
CD117 APC
CD19 PE Cy7
CD56 PE
CD45 PerCP-CY5.5
CD138 BV421
CD38 FITC
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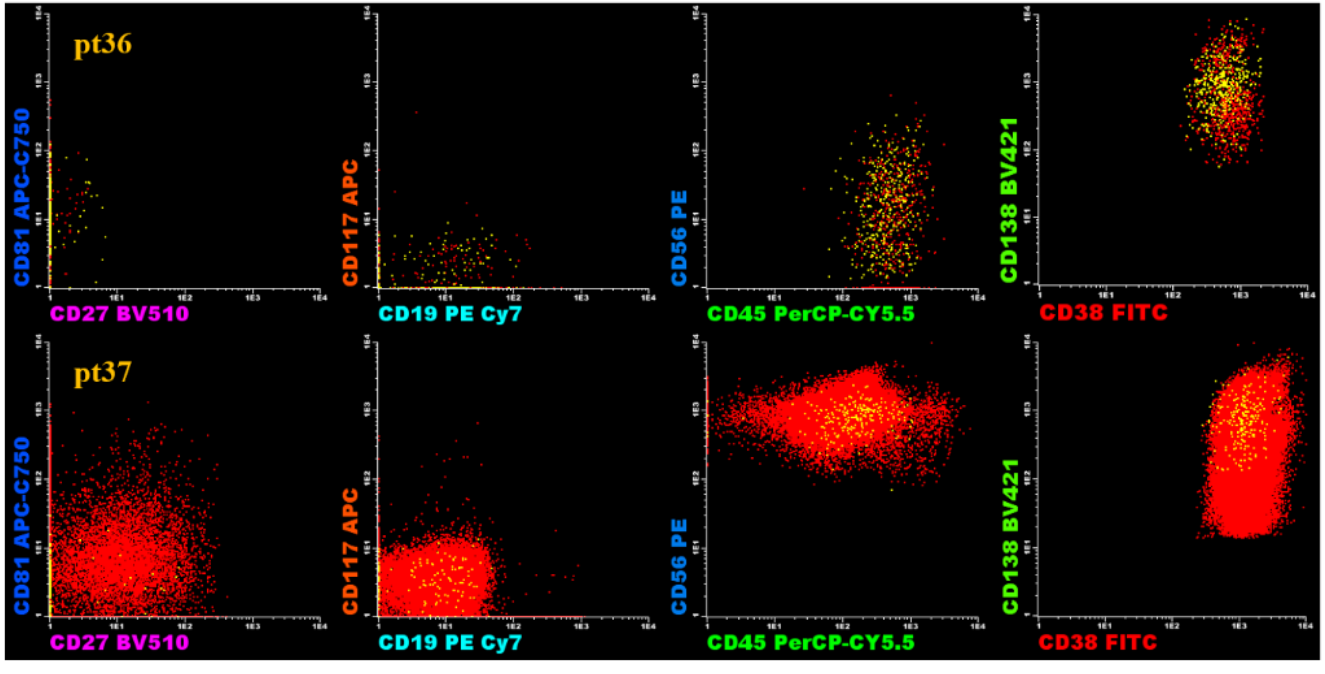

Figure 1−11. Bivariate flow cytometry dot plots illustrating immunophenotype (CD27, CD81, CD19, CD117, CD45, CD56, CD38, CD138) expression of BM MRD cells(red dots) and PB cPCs(yellow dots) from 11 patients with BM MRD and PB cPC positive.

Heterogeneity and Polyclonality of malignant plasma cells

Malignant plasma cells were heterogeneous rather than monoclonal in 9 of 19 (47.4%) patients with bone marrow MRD positive. In most cases there was one dominant clone, and an additional small one. In patients with heterogeneous, the small clones had different expression patterns of at least one (up to 3) immunophenotype from the dominant clone. Among the patients who showed heterogeneous by MRD analysis, two cases in which CD45 expression patterns were divided, on case in which CD19 expression patterns were divided, and a total of 3 cases were illustrated in dot plots. (Figure 1-12)

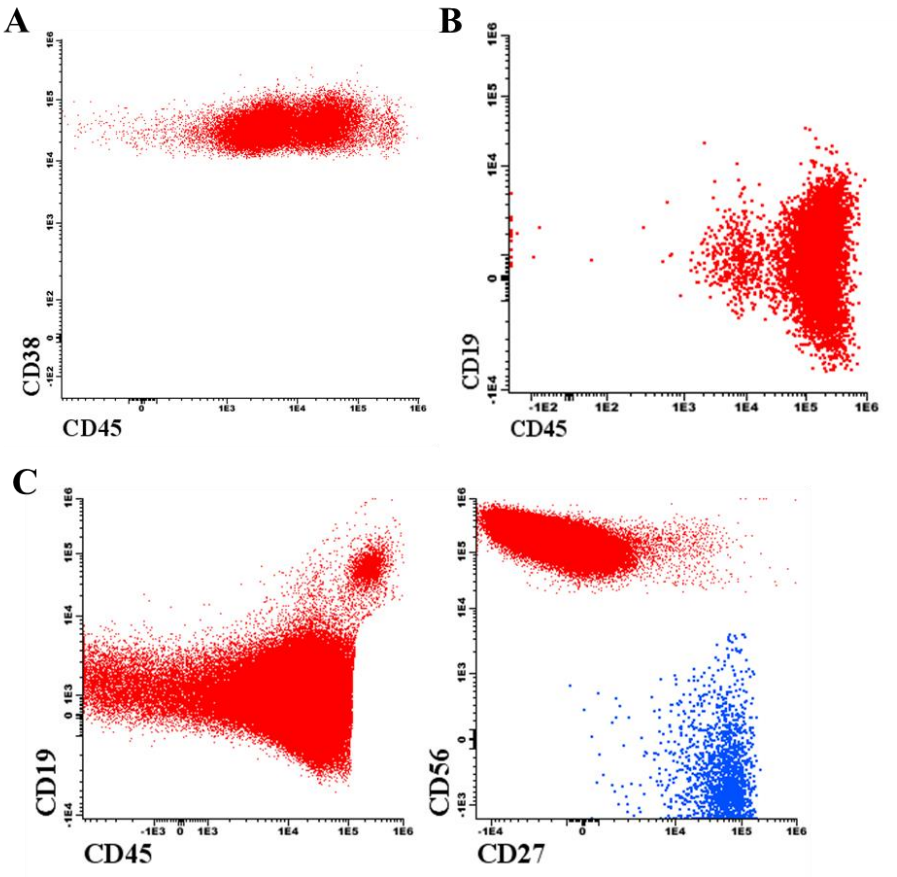

Figure 1-12. Three representative cases of showing heterogenous neoplastic plasma cells in bone marrow; red dot, abnormal plasma cell; blue dot, normal plasma cell (A) CD45 dim/moderate heterogenous cells (B) CD45 moderate/bright heterogenous cells (C) CD19 negative/positive cells. (left) All the plasma cells with CD19 negative/positive in same sample show CD56 positive and CD27 negative (right)

In the IMW response of 9 patients with heterogeneous MRD cells, 3 patients were PD, 3 patients were relapse, and 3

patients were each from CR, PR, SD. Among the subjects of this study, both patient groups with PD and relapse had heterogeneous pattern of malignant plasma cells in the bone marrow. Patients with the bone marrow MRD pattern as heterogeneous had more malignant plasma cells in the bone marrow than those who were monoclonal. (p<0.01, Figure 1-13) The number of circulating nPCs found in PB and heterogeneous patterns were also analyzed. Like the malignant plasma cells of the bone marrow, heterogeneous patients had more nPCs found in PB than monoclonal patients. (p<0.0001, Figure 1-14)

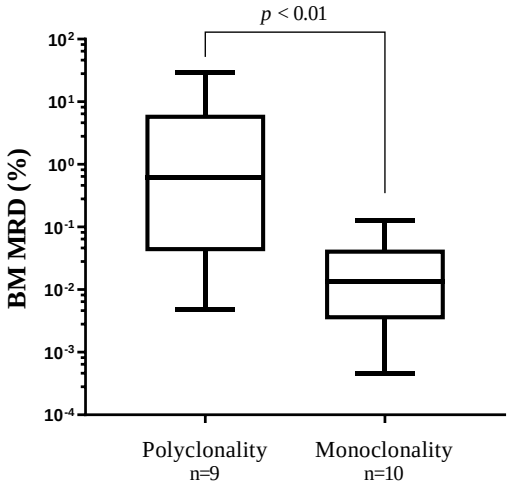

Figure 1-13. BM MRD(%) difference between polyclonality group (n=9) and monoclonality group (n=10) within BM MRD positive patients (n=19)

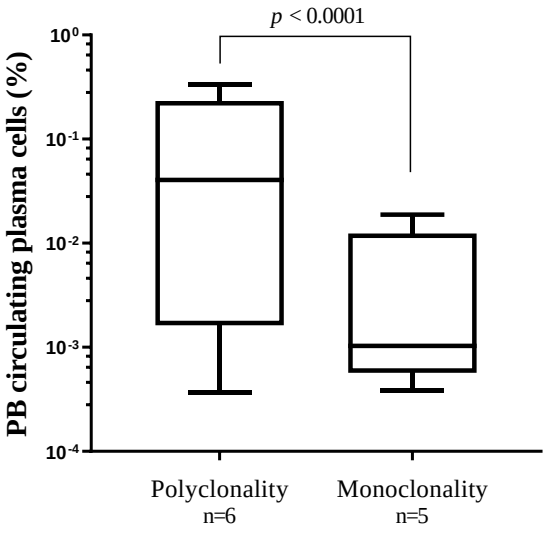

Figure 1-14. Circulating neoplastic plasma cells in PB (%) difference between polyclonality group (n=6) and monoclonality group (n=5)

Overall comparison between clinical features and NGF results

All patients with cPC found in peripheral blood had bone marrow MRD positive. The clinical characteristics of these patients and the immunophenotype expression pattern of BM MRD cells were compared with those of BM MRD positive patients who did not detect cPC in PB. (Figure 1-15) Among the clinical characteristics, the items that showed statistically significant difference were IMW treatment response (p=0.011) and FISH results (p=0.023). Most of the patients with cPC found in PB had PR, Relapse, PD, and MR with IMW treatment

response, and those with positive only BM MRD were VGPR, SD, and CR.

Looking at the FISH (Flourescence *in situ* Hybridization) results, among the patients with cPC found in PB, all 7 patients who underwent FISH were positive, but out of the patients with positive only BM MRD, only 2 out of 5 patients who underwent the FISH were positive. Positive FISH results included RB1 deletion (66.7%; 6/9), IgH rearrangement (55.6%; 5/9), 1q gain (55.6%; 5/9), p53 deletion (11.1%; 1/9), and chromosome 9 gain (33.3%; 3/9). There was no statistically significant difference of the FISH results between the group of patients with cPC and those without cPC in PB.

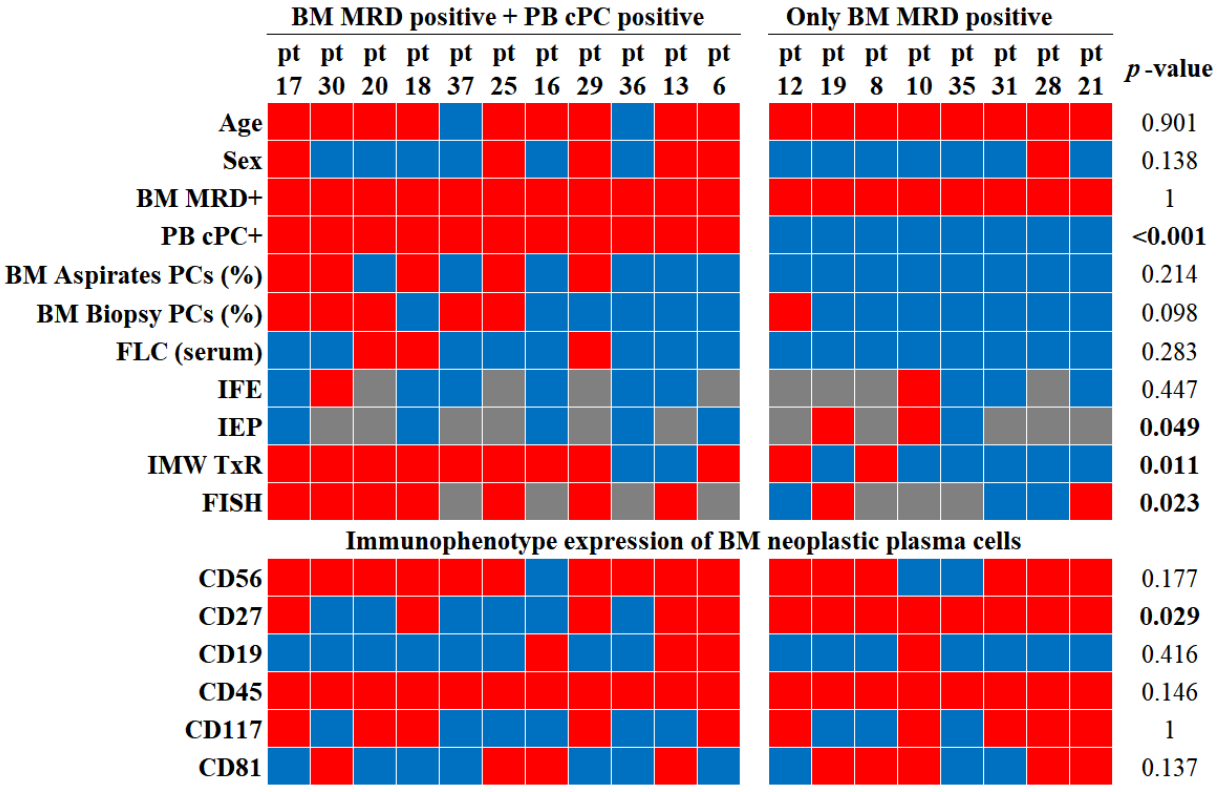

Figure 1−15. Individual profiles of PB circulating plasma cell positive patients and only bone marrow MRD positive patients

(BLUE) Age: 55 or less, Sex: Male, BM MRD+ & PB cPC+: negative, BM Aspirates PCs (%): under 4%, BM Biopsy PCs (%): under 10%, FLC level (serum): under 400, IFE & IEP: IgG, IMW TxR: VGPR SD CR, FISH: normal, Immunophenotype expression: negative (RED) Age: over 55, Sex: Female, BM MRD+ & PB cPC+: positive, BM Aspirates PCs (%): 4% or more, BM Biopsy PCs (%): 10% or more, FLC level (serum): 400 or more, IFE & IEP: IgA, IMW TxR: PR Relapse PD MR, FISH: positive, Immunophenotype expression: dim moderate

bright heterogeneous (negative to positive) (GRAY) Not attempted

Both patient groups were positive for BM MRD. Accordingly, the immunophenotype expression patterns of BM MRD cells in the patient group with cPC found in PB were compared with those in the undetected patient group. Among the 6 antigens (CD45, CD56, CD27, CD19, CD81, CD117), the antigen showing a statistically significant difference was CD27 ($p=0.029$). Among the patients with cPC found in PB, 5 out of 11 patients (45.5%) showed positive (dim, moderate, bright, heterogeneous) CD27 expression in BM MRD cells, but 8 out of 8 patients (100%) with no cPC found in PB showed positive CD27 expression. All of them were positive in CD27 expression.

DISCUSSION

The linearity test conducted with the U266 cell line and the patient's primary myeloma cell was confirmed to be well detected in the FACS analysis according to the dilution rates. (R^2 = 0.9994 & 0.9999, Figure 1-2) When seeing that the result of dilution of the primary cell up to 0.01% was well detected, the detection conducted in this method has a sensitivity of 10^{-5} or more. This corresponds to the sensitivity of next generation flow cytometry, so it can be considered that the results we performed with satisfy the criteria for next generation flow cytometry.

Among the patients with CR, 28.6% (2/7) showed MRD positivity. This is similar to other studies. (Munshi et al., 2017; Lahuerta et al. 2017) (Table 1-3) It was confirmed that MRD may be positive even if the IMWG treatment response criteria is a CR. Each patient who was diagnosed with VGPR, PR, MR, and SD showed MRD negativity. In other studies, there were patients who showed MRD negative despite achieving below VGPR treatment response, but the reason for this phenomenon was not specifically mentioned. We applied the next generation sequencing MRD method to these patients, which well be covered in Chapter 2.

The comparison between nPC of PB and BM in the present study revealed that the number of PB circulating nPCs was lower than that of BM. cPC was detected in 57.9% (11/19) of patients with MRD^{flow} positivity in the BM, and the quantitative value of cPC (mean number 193/ml) was 25-fold lower than that of BM nPC (4,769/ml). (Table 1-4) At the same time, it can be inferred that the nPC in the BM escape to peripheral blood because all patient with cPC were BM MRD positive. (Table 1-2) cPC was also found in patients with CR, which means that even if the IMWG treatment response is good, cPC can be found in peripheral blood. This means that an MRD test using peripheral blood is not impossible.

BM MRD assessment is possible through invasive BMA, and pre-analytical errors that can influence the results of MRD are numerous in BM aspirates. Even in patients who confirmed that 70.4% of bone marrow cells were plasma cells by differential count, only 29.7% of its detected by NGF. (Figure 1-4) The reason why this result occurs may be because of PB contamination. This could be seen by comparing the ratio of plasma cells identified through bone marrow biopsy and the ratio of plasma cells identified through NGF, as well as the differential count using bone marrow aspirates. (Figure 1-5) Contamination of BMA by PB is frequent, which results in a significantly lower MRD level (Ladetto et al., 2014). The

frequency of PB contamination is up to 30% in the BMA procedure (Astle et al., 2017). BMA clotting was also not infrequent. Clotting can cause either falsely high MRD cells or falsely low MRD. The actual incidence of BM clotting in BM is 10% to 25% (Riley et al., 2004).

FACS analysis was conducted for a total of 10 markers. These markers were verified and selected by the EuroFlow Consortium. Among them, CD38 and CD138 were used as common markers to determine plasma cells. Six of surface markers were used, except for the intracellular marker Kappa and Lambda. Of the six, five (CD56, CD27, CD19, CD45, CD117) were statistically significant in distinguishing between normal plasma cells and neoplastic plasma cells. (Table 1-7) This confirms that markers verified by EuroFlow Consortium can also be applied to Korean multiple myeloma patients who are Asian. Although CD81 has a p-value of 0.053 for the difference in expression between normal and neoplastic plasma cells, it is considered meaningful because it is an approximation close to the statistical significance criterion of 0.05.

In our study, CD27 was significantly depressed in PB compared to BM (31.6% [6/19] in BM vs. 90.9% [10/11] in PB). (Table 1-8) Such characteristics are also confirmed in other studies, but the reason is not explanined. (Mack et al.

2022) CD27 antigen is a member of the tumor necrosis factor receptor family, and low expression of CD27 has been reported in previous studies to be associated with poor prognosis (Chu et al., 2020; Guikema et al., 2003). Circulating neoplastic cells are migratory cancer stem cells with metastatic potential (Pantel et al., 2016); usually, myeloma cells reside in the BM and adhere to BM stromal cells. cPCs have a more immature phenotype than nPCs in BM, which differs from actively dividing nPC in BM (Paiva et al., 2013). The difference in the expression of CD27 in cPC suggests its possibility of being utilized as a prognostic factor in the future. Additionally, when patients with BM MRD positive were divided into patients with cPCs found and non-cPCs found, BM neoplastic plasma cells in patients with cPCs found showed a low expression of CD27. (54.5% [6/11]) (Figure 1-15) Conversely, patients with no cPCs detected had no low expression of CD27 in BM neoplastic plasma cells. (0% [0/8]) Patients with low CD27 expression neoplastic plasma cells may have a tendency for some plasma cells to circulate.

The IMW treatment response criteria include test items that can be assessed with PB, including serum light chain quantitation with ratio, IFE, and serum PEP (Hillengass et al., 2019). Although they can be easily assessed, they have limitations in their interpretation. The five primary classes of

immunoglobulins are IgG, IgM, IgA, IgD, and IgE, and their half-lives in vivo are different (Waldmann et al., 1969). As most serum proteins are too large for renal filtration, they are removed by pinocytosis from the plasma. Therefore, the half-life of IgA and IgM is approximately 5-6 days. In contrast, IgG has a concentration-dependent half-life of approximately 21 days due to recycling by neonatal Fc receptors (FcRn) (Kim et al., 2007; Anderson et al., 2006). Such finding suggests that the concentration of IgG in the serum may not reflect the actual status of nPC that produces IgG. Herein, serum FLC did not correlate with PB or BM MRDflow. Theoretically, the amount of monoclonal IgG residing in the serum for a long time coincides with the amount of nPCs.

One of the most important tests for detecting monoclonal proteins is the immunofixation test, and negative IFE is a requisite for stringent CR. Frequent false positive IFE is reported, which is much higher than expected. Although sFLC quantitation provides a rapid indicator of response, the role of the sFLC assay has been questioned because of the presence of discordant abnormal sFLC ratios in a significant proportion of patients displaying CR (Nasir et al., 2011). In addition, atypical oligoclonal bands in IFE are frequently observed, resulting in a vague interpretation, and can lead to false-positive IFE results. Protein EP also shows frequent false

positive results, and almost 30% of cases with PEP monoclonal band proved to be real by IFE test (Huang et al., 2014). When comparing the number and fraction of BM MRD cells with the sFLC level, there was no statistically significant correlation. (Figure 1-6, Figure 1-7) The number and fraction of cPC found in PB also showed no significant correlation with the sFLC level. (Figure 1-8) Through this, it was found that MRD cells and cPCs detected by NGF were not correlated with IFE results.

Regarding the sFLC ratio, we compared the sFLC and clonal PC/normal PC ratios. (Table 1-5) The serum FLC ratio and the FLC ratio confirmed by flow cytometry did not show the same result. Generally, the clonal PC ratio in BM and PB is maximized compared to the sFLC ratio (Martinez-Lopez et al., 2015; Xu et al., 2017). Of note, patients with a borderline sFLC ratio of 8 had a maximized clonal PC ratio.

The M protein level of serum showed a high correlation with BM MRD fractions ($R = 0.9$, $p<0.001$), and a moderate correlation with the fractions of cPCs. ($R^2 = 0.735$, $p<0.001$) There were two patients who achieved PD due to an increase in M protein in serum, although there was no increase in plasma cells in BMA and BMB. Both patients had BM MRD positivity. Through this, it can be considered that when a quantitative increase is observed with the M protein detection, it tends to

be MRD positive. These two patients are "Pt" 8 and "Pt 18". "Pt 18" was diagnosed with PD owing to the presence of the M protein in the absence of BM residual cells; however, that case was positive for BM MRD^{flow} and cPC. "Pt 8" was determined to relapse due to the presence of urine M protein; this case showed BM MRD^{flow} positivity, but cPC was not detected (Table 1-6).

In this study, it was observed that 9 of the MRD positive patients had polyclonal neoplastic plasma cells. (47.4%, Figure 1-13) If the clone of the neoplastic plasma cell is polyclonal, there is a result that the prognosis is poor compared to the patient who is not. (Tschautscher et al. 2019; Das et al. 2022) However, studies on polyclonal of malignant plasma cells are being conducted relatively recently, so further studies on the polyclonality is needed.

A dual check on the BM MRD test should be conducted. If the BM specimen is diluted or clotted, the PC counts for BMA/BMB and BM MRD^{flow} will be influenced. To address this problem, cPC is helpful. Our study showed that the cPC correlated with BM MRD and BMA/BMB PC%. In particular, cPC was highly correlated with BMB PC% compared to BMA PC%. BMB PC% was not influenced by the BM specimen problem. If the BM specimen had a specimen problem, BM MRD

and BMA PC% would both be influenced, while cPC and BMB PC% would not be influenced by the BM aspiration error.

Conclusively, we suggest that the measurement of cPC can complement the weak point of tests using BMA, including MRD^{flow} and PC%, in BM smear. Additionally, cPC could complement laboratory errors, such as serum PEP or IEP. The expression of CD27 was low in patients with nPC found in peripheral blood, confirming the possibility of using CD27 as a prognostic marker. In addition, if the expression of CD27 is low in BM MRD cells, it can be used as a biomarker for judgement such as monitoring MRD using PB when confirming the treatment response. For four patients with discrepancies between the IMW treatment response and the BM NGF MRD results, MRD screening should be performed using other methods such as NGS rather than NGF to check whether they consistently show inconsistencies with the IMW treatment response.

CHAPTER 2

Minimal Residual Disease Negativity by Next-Generation Flow in non-CR Myeloma Patients

Introduction

The International Myeloma Working Group (IMWG) defines treatment responses on the basis of the plasma cell percentage in bone marrow (BM), amount of light chains, and monoclonal antibody titers assessed via immunofixation and/or electrophoresis. In 2016, the IMWG included minimal residual disease (MRD) in the response criteria (Kumar et al., 2016). This addition was driven by the recent introduction of novel, less toxic agents, and consequent improvement in the rates of complete response (CR) and survival in multiple myeloma (MM) (Palumbo et al., 2014; Mateos et al., 2014). MRD negativity has been shown to have prognostic significance surpassing the prognostic value of CR (Landgren et al., 2016; Munshi et al., 2017; Lahuerta et al., 2017). At the same time, relapse rates still remain high despite improvements in the treatments, thus necessitating a more in-depth evaluation of patients with highly sensitive methods for MRD detection (Rollig et al., 2015).

Next-generation flow (NGF) and next-generation sequencing (NGS) have been adopted as additional BM assessment tools for detecting MRD in the IMWG response criteria. While conventional flow cytometry had limitations including lower sensitivity and a lack of standardization, the

NGF technology allows interrogation of several million cells, and the EuroFlow consortium has provided fine-tuned, standardized algorithms for identifying clonal plasma cells.

Clonal immunoglobulin gene rearrangements represent a major molecular target in MM MRD detection. (Lionetti et al. 2017) ASO-PCR was used as the first technique for evaluating MM MRD. However, the applicability is limited by the high proportion of SHMs within the IgH-CDR. With millions of read parallel sequencing, NGS overcomes technical ASO-PCR limitations, enabling MRD measurements with high sensitivity. (Bai et al. 2017) The advantages of this approach are also represented by its ability to identify clonal cells in MM patients with low tumor burden, improving knowledge of MM biology, and providing useful information on treatment choices and disease management. The data obtained must be elaborated through specific bioinformatic tools capable to analyze millions of reads.

Several NGS platforms have been tested for MM MRD detection over the past few years. Two of these, ImmunoSEQ (Adaptive Biotechnologies, Seattle, WA, USA) and LimphoSIGHT (known as ClonoSEQ) (Adaptive Biotechnologies, Seattle, WA, USA); the latter was first licensed by the FDA in 2019 and is currently the most widely adopted. These commercial kits are characterized by two-

stage and single-reaction PCR amplifying IgH VDJ rearrangements, respectively.

NGF has detected MRD in many patients among MM patients who reached and remain at CR. Those results are meaningful in that closer monitoring could benefit CR patients with MRD-positivity. However, when NGF fails to detect MRD in non-CR patients, it is difficult to account for this discrepant phenomenon, and its clinical meanings.

To address this issue, we investigated the correlation between NGF MRD results and the IMWG response criteria, the biological implications of NGF in non-CR patients, paying special attention to patients with discrepant results.

MATERIAL AND METHODS

1. Patients

Among the patients diagnosed with multiple myeloma from November 2000 to December 2016, 34 patients whose bone marrow was collected for follow-up study at Seoul National University Hospital from November 2016 to May 2017 were selected for this study. This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB No. 1612-042-813). All study subjects provided their informed consent to participate in the study.

There were four patients who failed to reach CR, yet showed negative-MRD by NGF. For those four patients, the NGS analysis of IgH rearrangements was conducted with paired BM specimens obtained at diagnosis and follow-up evaluation. (Figure 2-1)

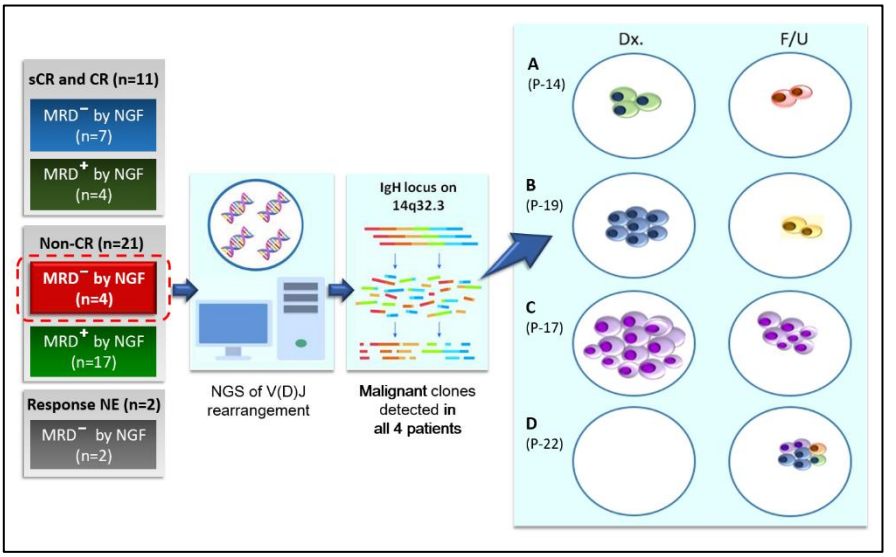

Figure 2-1. NGS of IgH Rearrangements in the 4 non-CR patients with negative MRD by NGF. (A) and (B) Acquisition of new dominant clones in 2 patients (P-14 and P-19). (C) Persistence of the residual clone in 1 patient (P-17). (D) Acquisition of new heterogeneous clones in 1 patient (P-22)

Abbreviation: CR complete response, F/U follow-up, MM, multiple myeloma, MRD minimal residual disease, NE not evaluable, NGF next generation flow, NGS next generation sequencing, sCR stringent complete response

2. Next Generation Sequencing

2.1 Genomic DNA extraction

Genomic DNA was prepared from BM aspirates using the QIAamp Blood Mini Kit (Qiagen, CA, USA). First, put 20 uL of proteinase K into the bottom of a 1.5 mL microcentrifuge tube.

Add 200 uL BM aspirates to the microcentrifuge tube. 200 uL of AL buffer is added to the tube containing the BM aspirates to lyse the lymphocytes. To ensure efficient lysis, it is essential that the sample and buffer AL are mixed thoroughly to yield a homogeneous solution. Incubate the tube at 56℃ for 10 minutes. Briefly centrifuge the tube to remove drops from the inside of the lid. Add 200 uL ethanol (96−100%) to the tube and mix again by vortexing for 15 seconds. After mixing, briefly centrifuge the tube. Carefully apply the mixture to the QIAamp Mini spin column in a 2 mL collection tube without wetting the rim. Close the cap, and centrifuge at 6000g for 1 minute. Place the spin column in a clean 2 mL collection tube and discard the tube containing the filtrate. Carefully open the spin column and add 500 uL buffer AW1 without wetting rim. Close the cap and centrifuge at 6000g for 1 minute. Place the spin column in a clean 2 mL collection tube and discard the collection tube containing the filtrate. Open the spin column and add 500 uL buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed for 3 minutes. After centrifugation, place the spin column in a new 2 mL collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 minute. This step helps to eliminate the chance of possible buffer AW2 carryover. Place the spin column in a clean 1.5 mL microcentrifuge tube and discard the collection

tube containing the filtrate. Carefully open the spin column and add 20 uL buffer AE. Incubate the tube at room temperature for 1 minute and then centrifuge at 6000g for 1 minute. DNA were quantified using the Dropsense96.

2.2 Generation of IgH rearrangement sequencing data and analysis

Sample data were generated using the immunoSEQ assay (Adaptive Biotechnologies, WA, USA). CDR3 region was amplified from gDNA using an amplification bias-controlled multiplex PCR approach (Robins et al., 2009; Carlson et al., 2013). CDR3 libraries were sequenced on an Illumina instrument according to the manufacturer's instructions. The data were filtered and clustered using both the relative frequency ratio between similar clones and a modified nearest-neighbor algorithm. V, D and J gene definitions were annotated in accordance with the international immunogenetics database. CDR3 sequences were normalized to correct for residual amplification bias and quantified against a set of synthetic CDR3 sequence analogues (Carlson et al., 2013). Data were analyzed using the immunoSEQ Analyzer toolset.

RESULTS

IgH rearrangement sequencing quality and overall characteristics

As a result of sequencing of 8 bone marrow samples, the average number of total clones was 1,014,334.5, and the average number of unique clones was 3,103.4. The number of detected gene arrangements ranged from a minimum of 1,126 to a maximum of 18,189, and the clonality ranged from a minimum of 0.049 to a maximum of 0.927. (Table 2-1)

Table 2-1. Overall characteristics of IgH rearrangement NGS results

Patient ID		Total Clones	Unique Clones	Gene Rearrangements	Clonality
P-14	Dx	1,066,057	5,701	6,119	0.097
	Follow-up	715,578	3,806	4,038	0.062
P-17	Dx	3,440,049	4,189	18,189	0.927
	Follow-up	191,453	944	1,126	0.384
P-19	Dx	1,335,816	3,430	4,921	0.049
	Follow-up	389,141	1,677	1,781	0.074
P-22	Dx	512,532	3,200	3,280	0.055
	Follow-up	464,050	1,880	2,704	0.176

IgH rearrangement analysis of 4 patients by NGS

The NGS study of IgH rearrangements on those four patients revealed residual abnormal PCs, which were not detected by NGF. The patient with PR (P-17) harbored the same dominant clone in both the diagnostic BM (87.13%; proportion of clone) and the follow-up BM (19.38%) samples. Two patients (P-14 and P-19) acquired new dominant clones after treatment, while the dominant IgH rearrangement clone detected in the diagnostic BM disappeared in each patient. The newly acquired dominant clone in P-19 carried a non-productive DJ rearrangement, whereas dominant clones found in the other three patients (P-14, P-17, and P-22) harbored productive VDJ rearrangements. The patient with SD (P-22) displayed heterogeneous clones in the follow-up BM (5.24%, 4.72%, 3.11%, 2.09%), while no IgH rearrangements were detected in the diagnostic 6 BM. The results of NGS of IgH rearrangements are summarized in Table 2-2.

Table 2-2 Results of IgH rearrangement NGS in the Non-CR patients with Negative MRD by NGF

Case No.	IMWG treatment response	NGF MRD (%)	IgH rearrangement NGS (%)		M-protein serum (g/dL)	sFLC κ/λ ratio
			Dx	Follow-up		
P-14	VGPR	0	4.19	0.75†	Not detected	1.61 (normal)
P-17	PR	0.00017*	87.13	19.38	0.23	1.98 (abnormal)
P-19	MR	0	50.80	1.49†	0.13	2.34 (abnormal)
P-22	SD	0	Not detected	1) 5.24† 2) 4.72† 3) 3.11† 4) 2.09†	2.08	11.36 (abnormal)

*Detected under limit of detection

†Acquisition of new dominant clones with disappearance of initial dominant clones at Dx.

Abbreviation: *IFE* immunofixation, *IMWG* International Myeloma Working Group, *MR* minimal response, *NGF* next generation flow, *NGS* next generation sequencing, *PR* partial response, *SD* stable disease, *sFLC* serum free light chain, *VGPR* very good partial response

Characteristics of dominant clone

A total of 10 dominant clones were discovered by IgH rearrangement sequencing by NGS. Among them, amino acid sequences were confirmed in 3 clones. There are 4 known V genes, 7 D genes, and 8 J genes. The V gene was all IGHV03, and the D gene was different in all 7 clones. (IGHD03-10, IGHD06-19*01, IGHD06-13*01, IGHD06-25*01, IGHD03-10*01, IGHD04-23*01, IGHD01-26*01) The J gene was identified in 8 out of 10 clones, but 4 types were identified. (IGHJ06-01*02, IGHJ05-01*02, IGHJ04-01*02, IGHJ02-01*01) There was 1 VDDJ rearrangement, 3 VDJ rearrangements, and 6 DJ rearrangements. Among the dominant clones, the smallest fraction was 0.75% and the largest was 87.13%. Except for the follow-up BM specimen of P-22 patient, the dominant clone was detected more than twice as much as the other clones in all remaining samples. (Table 2-3)

Table 2-3. Genetic information on 10 dominant clones identified by NGS of the *IGH* gene

Patient	Sample	Amino acid sequence	V gene	D gene	J gene	Rearrangement	Clone fraction
P-14	Dx	CARNYYGPGSYYPYYGMDVW	IGHV03	IGHD03-10	IGHJ06-01*02	VDDJ	4.19%
P-14	Follow-up	Unresolved	-	IGHD06-19*01	-	DJ	0.75%
P-17	Dx	CVRESLAWSIAAANWFDPW	IGHV03	-	IGHJ05-01*02	VDJ	87.13%
P-17	Follow-up	CVRESLAWSIAAANWFDPW	IGHV03	-	IGHJ05-01*02	VDJ	19.38%
P-19	Dx	Unresolved	IGHV03	-	-	VDJ	50.80%
P-19	Follow-up	Unresolved	-	IGHD06-13*01	IGHJ04-01*02	DJ	1.49%
P-22	Follow-up	Unresolved	-	IGHD06-25*01	IGHJ05-01*02	DJ	5.24%
P-22	Follow-up	Unresolved	-	IGHD03-10*01	IGHJ02-01*01	DJ	4.72%
P-22	Follow-up	Unresolved	-	IGHD04-23*01	IGHJ02-01*01	DJ	3.11%
P-22	Follow-up	Unresolved	-	IGHD01-26*01	IGHJ06-01*02	DJ	2.09%

DISCUSSION

The clinical impact of MRD negativity cannot be exaggerated in MM. Changes in the immunophenotype after treatment are not infrequent in MM, thus potentially yielding false negative MRD results on flow cytometry. The current EuroFlow NGF method, however, provides meticulous, sequential steps for yielding the highest resolution between normal and abnormal PCs, even if the immunophenotype is altered (Flores-Montero et al., 2017). Some studies have accounted for MRD-negativity in non-CR patients by the nature of M protein, which is more inert and has a longer half-life; hence, M-protein levels may not decrease promptly in response to treatment (Oberle et al., 2017). Normally, most serum proteins that are too large for renal filtration are cleared away through pinocytosis, which occurs in almost all nucleated cells. IgG has a concentration-dependent half-life of approximately 3 weeks because of the recycling process via FcRn receptors (Waldmann et al., 1969; Kim et al., 2007; Anderson et al., 2006). Furthermore, the IMWG criteria of treatment response primarily depend on the M-protein and light chain concentrations; however, they do not consider the BM PC%, except that sCR and CR require a BM PC% <5. In other words,

residual M-proteins, which are cleared slowly, can lead to misclassification of virtual CR as non-CR.

Antigenic drift of IgH rearrangements is frequent in B lymphoid malignancies, but NGS-MRD measurement in myeloma has overcome these variations. In patients receiving treatment, we observe clonal antigenic evolution during the persistence of the residual clone and the emergence of new dominant and heterogeneous clones. However, an interpretation for dominant clones is not yet standardized for NGS. The criteria for defining dominant sequences are rather arbitrary. A commercial NGS service provider, Clonoseq, has defined a dominant sequence as those comprising at least 3% of all similar sequences in sequences among IgH, IgK, and IgL for diagnostic purposes. Some studies adopted 0.3 to 0.5% as the threshold for a dominant clone (Kriangkum et al., 2015; Tak et al., 2017). In our study, the threshold for dominant sequences was set at 0.7%, and NGS of IgH rearrangement revealed dominant neoplastic sequences in all four non-CR patients with negative MRD on NGF. This suggests that the BM samples were neither diluted nor inadequate for evaluation. The percentage of mast cells in BM samples further indicated that peripheral blood contamination was an unlikely explanation for MRD negativity on NGF.

The present study shows that NGS can be used to detect residual clones in patients who test MRD-negative by NGF. We suggest that NGF and NGS should be performed in a complementary manner to determine the MRD status. We also suggest that once one method (NGF or NGS) yields negative results, the other be applied to assess the validity of the negative finding. Therefore, NGF and NGS can compensate for the deficits in the IMWG treatment response criteria, which are mainly based on M-protein levels. The IMWG suggests that MRD tests be initiated only at suspected CR. However, since the prognostic value of MRD negativity has been proven to surpass CR in studies, it is necessary for all patients to be screened for the MRD status regardless of their response criteria (Landgren et al., 2016; Lahuerta et al., 2017). In addition, whether non-CR patients with MRD negativity on NGF have better outcomes than MRD-positive patients within the same response groups warrants further investigation.

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국문 요약

서론: EuroFlow 가 제안한 8 가지 종류의 형광을 이용한 패널과 차세대 유세포 분석법의 등장으로 악성 형질세포 클론을 높은 민감도로 검출할 수 있게 되었다. 차세대 유세포 분석법으로 진행한 미세 잔존 질환 결과가 기존에 사용되고 있는 IMWG 치료 반응과 어떻게 일치하는 지 비교하려 한다. 일반적으로, 미세 잔존 질환은 골수를 이용해서 결과를 얻는 것이 최선의 방법이다. 하지만 골수를 얻는 방법은 환자에게 있어 침습적인 방법이기 때문에, 만약 말초혈액을 골수혈액 대신 이용할 수 있다면 그 자체로 강점을 가질 수 있다. 미세 잔존 질환을 확인하는 다른 방법은 면역글로불린 중쇄 재배열 서열을 분석하는 것이다. 최근에 차세대 염기서열 분석 기술을 이용하여 면역글로불린 중쇄 재배열 서열을 분석하는 것이 대두되고 있다. 본 연구에서는 차세대 유세포 분석법과 차세대 염기서열 분석법, 두 가지 분석법으로 얻는 미세 잔존 질환 결과를 비교 분석하여 두 방법의 차이점을 논의하고자 한다.

연구방법: 서울대병원을 내원한 27 명의 다발성 골수종 환자의 28 개 골수 혈액과 말초 혈액을 이용하였다. Navios 유세포 분석기를 사용하여 8 가지 종류의 형광을 이용한 패널을 통해 유세포 분석을 하였고, 그 결과를 Infinicyt 프로그램을 사용하여 분석하였다. 그 중 4 명의 환자를 대상으로 ImmunoSeq 을 사용하여 *IGH* 유전자 재배열 서열 분석을 진행하였다

결과: 완전 관해 판정을 받은 환자의 71.4% (5/7)가 미세 잔존 질환 음성을 보였다. 19 명의 환자는 최소 검출 한계 이상의 악성 형질세포가 관찰되었다. (67.9%; 19/28) CD27 은 골수 혈액에서 발견된 악성 형질세포보다 말초혈액에서 발견된 악성 형질세포에서 유의미하게 저하되었다. (p<0.05) 4 명의 환자는 미세 잔존 질환 검사 결과와 치료

반응과 일치하지 않는 결과를 얻었다. 4 명의 치료 반응은 매우 좋은 부분 관해, 부분 관해, 최소 관해, 안정 병변이었지만 4 명의 환자 모두에서 차세대 유세포 분석법으로 미세 잔존 질환이 관찰되지 않았다. 이 환자들을 대상으로 차세대 염기서열 분석 기술을 이용하여 면역글로불린 중쇄 재배열 서열 분석을 한 결과, 1 명의 환자는 초기 진단 당시에 87.13%였던 클론이 동일하게 추적 관찰 당시에 19.38%로 남아있었다. 다른 세 명의 환자는 추적 관찰 당시 골수 혈액에서 초기 진단 당시 골수 혈액에서 발견되지 않았던 새로운 클론이 나타났다. 0.7%가 넘는 클론을 우세 클론으로 분석하였다.

결론: 골수 혈액을 이용한 미세 잔존 질환 검색과 말초 혈액을 이용한 검색은 강한 상관관계를 보였다. 골수에서 발견된 미세 잔존 질환 세포에 비해 말초 혈액에서 발견된 악성 형질세포에서 CD27 의 발현이 억제되어 있었고, 이는 CD27 을 하나의 예후에 대한 마커로 사용할 수 있다는 가능성을 확인한 것이다. 골수 혈액에서 차세대 유세포 분석법으로 미세 잔존 질환이 음성으로 확인된 4 명의 환자를 대상으로 IGH 유전자 재배열 서열 분석을 차세대 염기서열 분석법으로 시행하였을 때, 모든 환자에서 우세한 클론을 검출할 수 있었다. 이는 차세대 유세포 분석법으로 확인할 수 없는 악성 형질세포를 차세대 염기서열 분석법으로 찾을 수 있음을 뜻한다. 따라서 차세대 유세포 분석법을 이용한 미세 잔존 질환 검색과 차세대 염기서열 분석법을 이용한 미세 잔존 질환 검색을 상호 보완적으로 사용하면, 더 높은 민감도로 미세 잔존 질환을 검색할 수 있다. 이것은 환자의 치료와 예후에 대한 판단을 내리는 데 큰 도움이 될 것이다.

주요어: 다발성 골수종, 미세 잔존 질환, 차세대 유세포 분석법, 차세대 염기서열 분석법, IGH 유전자 재배열

학 번: 2016-22017