CASE REPORT

Fluorescence In Situ Hybridization on Enriched CD138-Positive Cells in Plasma Cell Myeloma

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ABSTRACT

To validate plasma cell enrichment technique for improving the detection of cytogenetic abnormalities in the Plasma cell myeloma (PCM)/multiple myeloma (MM). We compared the abnormality detection rate for overnight unstimulated bone marrow cultures to that for the plasma cell enriched fractions obtained with the use of CD138-coated immunomagnetic beads. Average enrichment factor (EF) was 11. One or more abnormalities were detected in 90% of enriched samples vs. 65% of non-enriched samples, thus resulting in a significantly higher detection rate of total cytogenetic abnormalities in enriched plasma cells (p=0.0038). Additional findings of RB1 deletion, TP53-, 1p-, 1q+ and IGH@ rearrangement seen in the 25% of enriched samples could contribute to the altered risk in the patient. One of the three cases with plasma cells as low as 1% by morphology was positive for a residual disease marker in the enriched sample and negative in the non-enriched sample. The plasma cell enrichment technique increased the detection rate of diagnostic and prognostic markers and is a very sensitive method for detecting minimal residual disease.

Keywords: Myeloma, plasma cell, cytogenetic, enrichment, Fluorescence In Situ Hybridization (FISH).

INTRODUCTION

Plasma Cell Myeloma/multiple myeloma (PCM/MM), is a bone marrow based multifocal plasma cell neoplasm associated with an M protein in serum or urine and disseminated marrow involvement.[1] Myeloma is the second most common hematopoietic malignancy with 22,350 new cases and 10,710 deaths in US in 2013 according to data of National Cancer Institute[2]. Myeloma spans a clinical spectrum from asymptomatic to highly aggressive disease. Staging for myeloma does not use the tumor size, lymph node, metastasis (TNM) system. The International Myeloma Working Group diagnostic criteria is commonly used.[3] The criteria for Symptomatic myeloma include: M-protein ≥ 30 g/L and/or bone marrow clonal cells ≥ 10% and must have evidence of end-organ damage that can be attributed to the plasma cell proliferative process; manifested by CRAB (calcium, renal failure, anemia, and bone lesions).[3] The immunophenotype for myeloma (malignant) cells are $CD38^+$, $CD138^+$, $CD56^+$and $CD19^-$, $CD45^-$ or $CD45^{lo}$, which are different from healthy (benign) bone marrow plasma cells with $CD38^+$, $CD138^+$, $CD19^+$, $CD45^+$, $CD56^-$.[4] Despite recent therapeutic advances, myeloma remains as an incurable tumor with median survival of 6 years.[5] Cytogenetic studies have revealed various chromosome abnormalities which contribute to stratify myeloma into standard and high-risk for drug resistance.[6] There are several diagnostic challenges in identifying cytogenetic abnormalities by interphase FISH, including small sample size and low proportion of disease PCs in the hemodilute BM that can lead to false negative results. In accordance with the International Myeloma Working group (IMWG) recommendations, FISH should be carried out on nuclei from purified plasma cells.[7] There are two available methods to isolate cells: fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS) using surface cell markers. Four studies have been published to use isolation technique enriching plasma cells with improved detection of genetic abnormalities in myeloma bone marrow samples.[8-11]

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Among them, one study[8] is based on FACS, the latest 3 studies have switched to MACS instead.[9-11] Compared to FACS, MACS is a convenient, high-throughput, time-saving, and less costly method for sample enrichment.[12]

In this study we investigated to validate an immunomagnetic positive cell selection protocol for the isolation of CD138 positive myeloma plasma cells to enhance diagnostic sensitivity of interphase FISH analysis by concentrating plasma cells in the sample.

METHODS

Twenty heparinized bone marrow aspirates (500 ul to 2 ml) were obtained. The bone marrow samples were divided into 2 aliquotes. One aliquote (nonenriched) was used to set up the culture for 48 hours with IL-4 stimulation. The other aliquote (enriched) was used for plasma cell enrichment. FISH for myeloma markers was performed on both non-enriched and enriched specimens within 48 hours of collection time. CD138-coated immunomagnetic beads (Miltenyi plasma cell isolation kit II, Miltenyi Biotec Inc. CA. USA) were used to enrich the plasma cells. A single cell suspension was prepared after lysing blood in bone marrow specimens. The suspended cells were centrifuged at 300×g for 10 minutes. After aspirating supernatant completely, the cell pellet was resuspended in preparing buffer (10^8 cells per 400 μL). Then 100 μL of Non-Plasma Cell Biotin-Antibody Cocktail was added and incubated for 10 minutes at 4°C. With addition of 200 μL of buffer, 200 μL of Non-Plasma Cell MicroBead Cocktail (CD2, CD3, CD10, CD13, CD15, CD22, CD34, CD123, and CD235a), and 100 μL of CD56 MicroBeads, the cells were further incubated for 10 minutes at 4°C. The cells were washed by adding 5–10 mL of buffer and centrifuged at 300×g for 10 minutes. By using magnetic separation with the autoMACS® Separator, the negative fraction from outlet port was collected and centrifuged at 300×g for 10 minutes. After removing the supernatant, 50 μL of buffer was added to resuspend the cells. The cells were incubated with 50 μL of CD138 MicroBeads for 15 minutes at 4°C. By adding 5–10 mL of buffer the cells were washed and centrifuged at 300×g for 10 minutes. The supernatant was completely aspirated. Up to 10^8 cells were resuspended in 500 μL of buffer. The cells were applied on to a pre-rinsed MS Column in the magnetic field of MACS Separator. The column was washed with 3×500 μL of buffer. After removing the column from the magnetic field, the magnetically retained CD138+ plasma cells were fixed in fixative and slides were made for MUM1 staining as well as FISH testing. Standard special stain for plasma cells by methyl green pyronin (MGP) was later performed to compare the efficacy of both staining techniques. Samples were analyzed using routine FISH tests that were performed with a myeloma panel of FISH probes to assess copy number changes for hyperdiploidy (1,7,9,11) and non-hyperdiploidy (IGH rearrangement including translocations t(11;14), t(4;14), t(14;16), t(8;14), RB1 and TP53 deletion, and chromosome 1 abnormalities). Cut-off value for CEP7 is 4%, CEP9 (6%), CEP11 (4%), CCND1/IGH (0.6%), t(11;14) (0.6%), t(4;14) (0.6%), t(14;16) (0.6%), IGH@ (7%), RB1(8%), TP53 (8%), 1p del, 1q gain (2%), t(8;14) (0.6%), t(6;14) (0.6%), t(14;20) (0.6%). Anti-MUM1 antibodies for staining nuclei of plasma cells were received from Ventana Medical Systems (Tucson, AZ). 200 nuclei were counted for routine FISH and 100 nuclei were counted for enriched samples. The hybridization protocol and the image analysis were performed as previously described.[13] Hybridization was performed with the aforementioned probes overnight at 37°C in a humidified chamber. All probes were purchased from Vysis (Downers Grove, IL), except for 1p32 (CDKN2C)/1q21 (CKS1B) which were obtained from Empire Genomics (Buffalo, NY). Slides were washed with saline-sodium citrate buffer and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for interpretation using a fluorescent microscope. Scoring was performed by recording the total number of green signals and orange signals for each cell, with a minimum of 100 cells evaluated per sample.

Statistical Methods

Comparisons of frequencies in the different groups were analyzed with the χ^2 test or Fisher's exact test. For continuous measurements the Mann-Whitney U or paired t test was used. Linear correlation coefficient (r) was calculated to evaluate the correlation. P values <0.05 (two-sided) are considered statistically significant.

RESULTS

The clinicopathological status of 20 patients with plasma cell neoplasm are summarized in Table 1. There were 9 men and 11 women, with ages from 47 to 88 years. The initial diagnosis was plasma cell myeloma in 19 cases, Monoclonal gammopathy of undetermined significance (MGUS) in 1 case. Among them, 16 cases were new diagnosis; 3 cases were persistent disease, and 1 case of status post stem cell transplant. Bone lesion was present in 2 cases. Serum or urine M protein was detected in 15 cases.

The percentage of plasma cells in the original BM samples was determined by flow cytometry and morphologic analysis. In this study, the plasma cell percentage in nonenriched marrow was less than 6% by flow cytometry whereas it varied from 1% to 85% by morphologic analysis as flow cytometric analysis underestimated the number of plasma cells. For morphologic analysis of the bone marrow samples, immunohistochemistry for CD138 and in situ hybridization studies for kappa and lambda immunoglobulin light chain were performed in conjunction

Table 1: Clinicopathologic status of 20 plasma cell neoplasm patients at plasma cell enrichment study

Case no.	Age; sex	Clinical status	Pathology diagnosis	Bone lesion	M protein
1	78/M	Newly diagnosed	Plasma cell myeloma	No	IgG κ
2	87/M	Newly diagnosed	Plasma cell myeloma	No	No
3	47/M	Newly diagnosed	Plasma cell myeloma	No	No
4	75/F	Newly diagnosed	Plasma cell myeloma	No	IgG κ
5	75/F	Newly diagnosed	Plasma cell myeloma	yes	IgG κ
6	67/F	Newly diagnosed	Plasma cell myeloma	No	IgA κ
7	64/F	Newly diagnosed	Plasma cell myeloma	No	IgA λ
8	60/M	Persistent disease	Plasma cell myeloma	No	IgG λ
9	54/M	Newly diagnosed	Plasma cell myeloma	No	IgA λ
10	80/M	Newly diagnosed	Plasma cell myeloma	No	Ig κ
11	56/F	Newly diagnosed	Plasma cell myeloma	No	IgG κ
12	52/F	Newly diagnosed	Plasma cell myeloma	No	No
13	88/F	Newly diagnosed	Plasma cell myeloma	No	No
14	83/M	Newly diagnosed	Plasma cell myeloma	No	IgA κ
15	72/F	Newly diagnosed	Plasma cell myeloma	No	No
16	66/F	s; P SCT	Plasma cell myeloma	yes	IgG κ
17	77/F	Persistent disease	MGUS	No	IgA λ
18	67/F	Persistent disease	Plasma cell myeloma	No	IgG κ
19	51/M	Newly diagnosed	Plasma cell myeloma	No	IgA λ
20	80/M	Newly diagnosed	Plasma cell myeloma	No	Free λ

Abbreviations: M: Male; F: Female; s; p: Status post; SCT: Stem cell transplant; MGUS: Monoclonal gammopathy of undetermined significance

with H&E to further characterize the distribution, percentage, and intracytoplasmic immunoglobulin light chain expression in the plasma cells. Plasma cell count in enriched sample ranged from 2 x 10^5-2 x 10^6. Standard special stain for plasma cells by methyl green pyronin (MGP)[14] was performed in comparison with MUM1 staining. The enriched plasma cells from case 5 demonstrated MUM1 nuclear and MGP cytoplasmic staining, respectively (Figure 1A and B). By comparing 3 enriched samples, the linear correlation coefficiency between MUM1 and MGP staining was confirmed (Figure1C). Although both staining methods are available, we found the MUM1 stained well after treatment of the fixative provided by the enrichment kit, whereas MGP had better quality before the step of using the fixative. Either one can be used depending on the work flow. In this study, we used MUM1 nuclear staining throughout the study to identify plasma cells.

The plasma cell enrichment factor (EF) was calculated as the percentage of MUM1 positive plasma cells in the enriched sample divided by the percentage of plasma cells in the nonenriched sample by H&E morphologic analysis. The enrichment significantly increased plasma cell concentrations with average EF of 11 (Figure 1D).

Plasma cell enrichment improved detection of cytogenetic abnormality with interphase FISH. Representative pictures are displayed in Figure 2 are from case 7. Nonenriched samples showed normal signal pattern of 2 orange and 2 green signals (1p/1q, RB1/Cep11, and TP53/Cep17), and 2 fusions in IGH tests (Figure 2 A, C, E, and G). By enrichment method, there were 4 orange (1q) signals and 2 green (1p) signals (Fig 2B), indicating a 1q gain status. Deletion of 13q (RB1) and 17 (P53) was also detected by enriched method (Figure 2D and 2H). In Figure 2E, the non-enriched sample showed two orange/green (yellow) fusion (2F) signal pattern with IGH@ break-apart probe suggesting a normal signal pattern. By enrichment (Figure 2F), one orange, one green, and one orange/green fusion signal pattern is observed (1O1G1F). This signal pattern indicates that the genomic targets for the IGH flanking probes have been physically separated as a result of the translocation.

The use of enriched plasma cells detected more cases with cytogenetic abnormality than that of nonenriched samples (18 cases vs 13 cases in Table 2). Five cases (case 1, 7, 10, 11, 19) with IgH gene rearrangement but unknown partners were further investigated with additional probes t(6;14), t(8;14), t(14;20) (Table 3). We evaluated abnormal events or in other words frequency of positive test results for individual abnormalities (Table 4). Comparison of total number of events between enriched and non-enriched samples produced significant results with a *P* value of 0.0038 (Figure 3). Gain of chromosomes 7, 9, or 11 was the most commonly detected events in this study accounting for 40% of the total abnormalities detected. IGH rearrangement was the second most common abnormality detected (24%). Some rare abnormal cytogenetic events were only reported in plasma cell-enriched samples including 1 case for each abnormality of t(6;14), t(14;20), and del(TP53/CEP17). High risk abnormalities including del13q (RB1) and 1q gain were detected more in plasma cell-enriched samples (7 vs 2 cases

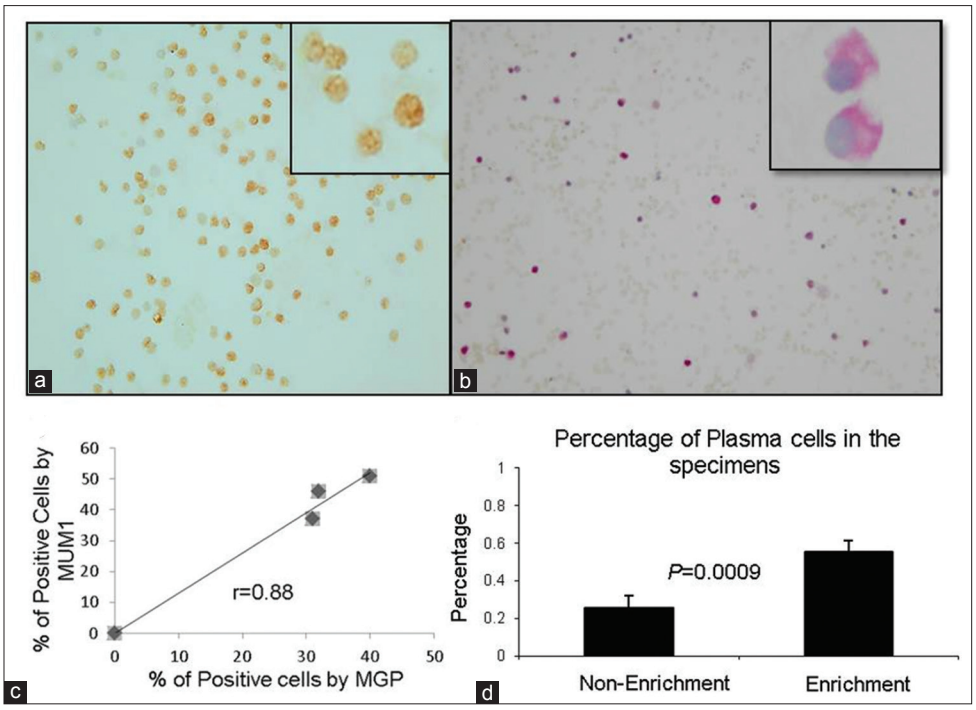

Figure 1: Plasma cells in enriched bone marrow samples. A, Enriched bone marrow aspirate from case 5 showing 51% of MUM1+ plasma cells with an insert showing nuclear staining by higher magnification. (original magnification x400). B, MGP staining from the same sample with an insert showing cytoplasmic staining by higher magnification. (original magnification x400). C, Linear correlation coefficient between MUM1 and MGP staining. D, Comparison of MUM1+ plasma cells in enriched bone marrow samples with those in nonenriched samples analyzed by morphology. MGP, methyl green pyronin. MUM1, multiple myeloma oncogene 1.

Table 2: Results of FISH analysis in non-enriched and enriched samples[a]

Case no.	Hyperdiploidy						IGH@								(RB1)		TP53/ CEP17		Loss of 1p		Gain of 1q	
	CEP7		CEP9		CEP11		t (11;14)		t (4;14)		t (14;16)		IGH@									
	NE	E	NE	E	NE	E	NE	E	NE	E	NE	E	NE	E	NE	E	NE	E	NE	E	NE	E
1	11	41	9.5	33	10	39	0	0	0	0	0	0	1	9	0	0	1	2	0	0	0.5	0
2	1	0	1	4	3	4	0	0	0	0	0	0	1.5	0	0	0	1	3	0	0	11	0
3	2.5	3	3.5	13	0	14	0	0	0	0	0	0	1	0	0.5	1	6	0	0.5	0	0	1
4	1.5	0	1	0	0.5	0	0	0	39	72	0	0	24	40	39	81	4	5	53	83	53	82
5	3.5	2	7	37	12	68	0	0	0	0	0	0	3	5	3.5	0	5	0	1	3	6	4
6	0	0	0	0	7.5	10	0	0	0	0	0	0	0	7	0	12	0	1	0	6	0	0
7	0	3	0	3	0	1	0	0	0	0	0	0	4	64	6	27	5	35	0	0	5	63
8	0	0	0	0	0	0	0	0	0	0	0	0	3.5	4	2.5	3	3	3	1	0	0	0
9	0.5	9	1.5	7	1	12	0	0	0	0	0	0	1.5	2	0	12	1	0	0.5	0	1	2
10	43	59	43	59	31	56	0	0	0	0	0	0	40	59	7	0	0	0	0	0	30	40
11	0	0	0	0	0	0	0	0	0	0	0	0	2	53	2	46	3	0	0	1	0	0
12	1	0	1	3	0	72	0	29	0	0	0	0	3	0	3	37	5	1	2	4	5	12
13	41	72	26	54	53	80	0	0	0	0	0	0	0	0	0	0	19	0	0	0	0	0
14	77	81	77	83	66	0	0	0	0	0	0	0	30	0	57	92	2	0	1	0	67	91
15	0	0	0	0	0	0	0	0	0	0	0	0	1.5	0	0	0	0	0	0	0	0	0
16	2	0	1.5	0	0	0	0	0	0	0	0	0	3	0	2.5	2	2	0	0	0	0	3
17	0	2	5.5	0	0	0	0	0	0	0	5.5	14	2.5	7	4	5	2	0	2.5	2	0.5	1
18	0	0	0	4	0	3	0	0	0	0	0	0	8	0	3	0	5	0	0.5	0	0.5	1
19	0	0	0	23	15	31	0	0	0	0	0	0	3	28	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	7	7	0	0	0	0	0

Abbreviations: IGH@, IGH rearrangement; [a]The number in the table represent the percentage of interphases positive for the corresponding individual probe in nonenriched (NE) and enriched (E) samples, respectively.

in del13q(RB1)), (8 vs 7 cases in gain of 1q). One of the three cases with plasma cells as low as 1% by morphology was positive for 1q gain (case 16) in the enriched sample and negative in the non-enriched sample, which is from a patient post stem cell transplant, implying the usefulness of enrichment method to monitor residual disease.

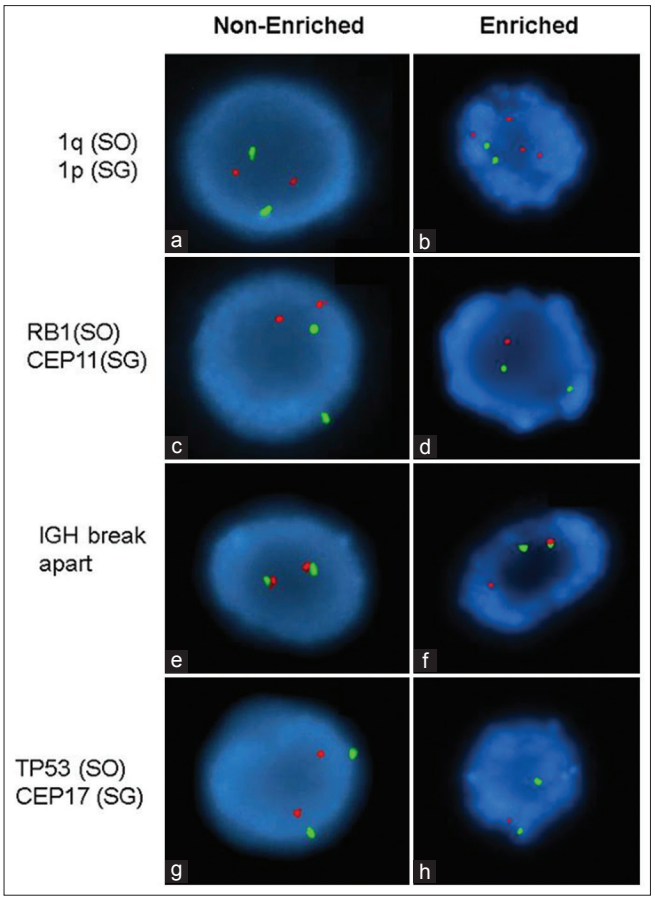

Figure 2: Representative micrographs of interphase FISH of marrow sample from a patient with plasma cell myeloma in case 7. Dual probes conjugated with signal orange (SO) and signal green (SG) were used in A through H. case 7. Normal pattern as of 2 orange and 2 green signals were present in the nonenriched samples (Figure 2 A, C, E, and G). Signal gain or loss of either orange or green is abnormal pattern seen in enrichment group. There were 4 orange (1q) signals and 2 green (1p) signal (Fig 2B), indicatingan 1q gain status. Deletion of 13 (RB1) and 17 (P53) was also detected by enriched method (Figure 2D and 2H). In Figure 2E, the non-enriched sample showed two orange/green (yellow) fusion (2F) signal pattern. By enrichment (figure 2F), one orange, one green, and one orange/green fusion signal pattern is observed (1O1G1F). This signal pattern indicates that the genomic targets for the IGH flanking probes have been physically separated as a result of the translocation. Original magnification x1000.

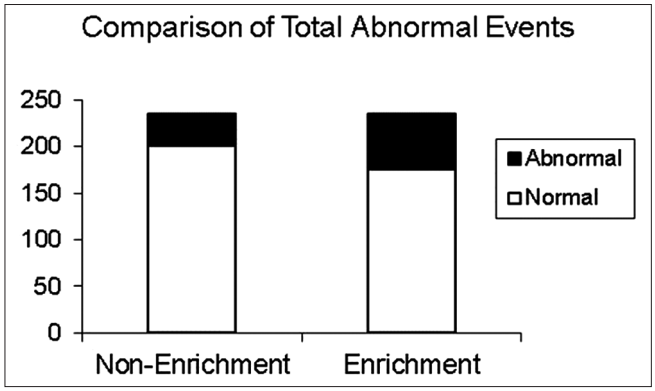

Figure 3: Comparison of Cytogenetic Abnormalities Identified by FISH between Nonenriched and Enriched Samples. Fisher's exact test was performed to obtain the one-tailed P value.

Table 3: Results of FISH analysis in non-enriched and enriched samples[a] with additional probes

Case no.	IgH					
	t (6;14)		t (8;14)		t (14;20)	
	NE	E	NE	E	NE	E
1	0	0	0	4	0	0
7	0	0	0	0	0	0
10	0	0	0	0	0	10
11	0	0	0	0	1	37
19	0	0	0	0	0	0

Abbreviations: IGH@, IGH rearrangement; [a]The numbers in the table represent the percentage of interphases positive for the corresponding individual probe in nonenriched (NE) and enriched (E) samples, respectively

DISCUSSION

In this study, we demonstrated that the plasma cell enrichment technique using CD138 selection kit is a highly effective technique for isolation of plasma cells thereby improving the diagnostic sensitivity of FISH in plasma cell neoplasms.

Bone marrow specimens were all processed within 48 hours after collection as the plasma cell number may significantly decrease with aging samples.[11] Both staining techniques MGP staining before fixation and MUM1 staining after fixation worked efficiently in our study. Since fixation can impair CD138 staining due to cytoplasm loss, MUM1 nuclear staining helps to assess the enrichment after fixation. MUM1 is found mainly in B-cell lymphoma and is useful and specific in identification of plasma cell differentiation.[15,16] To investigate the cut off percentage of plasma cells indicated for enrichment protocol, the plasma cell concentration in the original BM sample were evaluated by both flow cytometry and morphologic analysis. Polyclonal plasma cells constitute up to 2% of the bone marrow.[17] Monoclonal plasma cells may represent low level early disease or after treatment. Previous study[9] has suggested that 10% or less percentage of BM plasma cells be a threshold required for the plasma cell enrichment. We selected cases with marrow plasma cells less than 6% as determined by flow cytometry to make the comparison more significant. However, the plasma cell concentration by morphologic analysis (H&E, immunohistochemistry for CD138 and in situ hybridization studies for kappa and lambda immunoglobulin light chain) in the selected bone marrow samples were much variable from 1%-85%. This is consistent with previous study[18] that flow cytometric analysis underestimated the number of plasma cells partly due to loss of immunostaining during the process. Compared to the original BM sample, enrichment increased the plasma cell concentration to average 11 folds. Detection of the total abnormal events was significantly improved after enrichment. Hyperdiploidy of 7, 9, or 11 was the most common abnormality detected followed by

Table 4: The summary of cytogenitic abnormalities identified by FISH in non-enriched and enriched samples[a]

Sample type	Hyperdiploidy			IgH translocation partners							Del (RB1)	TP53	1p	1q
	CEP7	CEP9	CEP11	11	4	16	6	8	20	IGH@				
Non-Enriched	20	25	35	5	5	5	0	0	0	20	10	0	10	35
Enriched	25	40	50	5	5	5	0	5	5	50	35	5	25	40

Abbreviations: IGH@, IGH rearrangement;. [a]The percentage refers to total number of abnormal (positive) events for each individual probe divided by total 20 cases studied

IGH@ gene rearrangement in this study.

FISH is an important technique to detect cytogenetic abnormalities,[19] by which a risk-stratification model has been established to determine prognosis of myeloma. Short survival and shorter duration of response to therapy have been reported with t(4;14)(p16;q32), t(14; 16)(q32;q23), cytogenetic deletion of 13q-14, and deletion of 17p13 (p53 locus).[20-22] Chromosome 1 abnormalities have been associated with the transition from MGUS/SMM to MM. [20]

Based on these studies,[22] patients with 17p deletion, del (13q)(RB1), hypodiploidy, 1q gain, t(14;16), and t(14;20) are considered to have high-risk myeloma. Patients with t(4;14) translocation are considered an intermediate-risk. All others are considered as standard-risk. In our study, 1 case of each abnormality for t(6;14), t(14;20), and del(TP53/CEP17) were only detected in enriched samples. High risk abnormalities including del13q(RB1) and 1q gain were detected in enriched samples more than in nonenriched group. For 3 cases with plasma cells less than 1% by morphology, nonenriched method failed to detect any abnormality, whereas one case was positive for a high risk abnormality (case 16 with 1q gain) in the enriched sample.

In conclusion, the plasma cell MACS enrichment method is easy and a relatively quick procedure enhancing the abnormality detection rate and disease stratification. Percentage of plasma cells from enriched specimens is at least ten times greater than nonenriched samples, thereby increasing the percent of abnormal cells proportionately. Performing FISH in unsorted samples carries a relatively risk of low sensitivity for detection of chromosome abnormalities by 25% based on our study. Enriched FISH studies can detect abnormalities with as low as 1% plasma cells as analyzed by morphologic evaluation.

It is also noteworthy that cell enrichment may miss co-existing diseases in other cell lineages of the bone marrow. Therefore, careful investigation of patient's history and morphological examination are needed prior to the final interpretation of the results.

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