A Practical Approach to the Detection of Prognostically Significant Genomic Aberrations in Multiple Myeloma

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Multiple myeloma (MM) is a malignancy of differentiated B lymphocytes and has remained an incurable disease. Chromosomal abnormalities are among the most important prognostic parameters for MM. Cytoplasm immunoglobulin-enhanced interphase fluorescent *in situ* hybridization (FISH) has been a standard cell-targeting method for identifying genomic aberrations in MM. We have developed another cell-targeting approach by using CD138 magnetic microbeads to sort plasma cells for FISH analysis. The FISH panel consisted of four probes targeting RB-1, D13S319, immunoglobulin H, and p53 loci. We reviewed the FISH and conventional cytogenetic results of 60 patients with MM. The present cell-targeting approach in conjunction with the FISH probe panel was more sensitive than FISH performed on untargeted cells in detecting prognostically significant genomic aberrations (72 versus 24%, $P = 0.0016$). The frequencies of genomic abnormalities identified were similar to previously reported data obtained with the standard cell-targeting method. Therefore, our cell-targeting approach and FISH panel reliably detect prognostically important genomic abnormalities in MM and are potentially suitable for widespread use. (*J Mol Diagn* 2005, 7:560–565)

Multiple myeloma (MM) is the prototypic monoclonal B-cell neoplasm that is derived from the autonomous proliferation of plasma cells and associated with paraprotein production and osteolytic bone lesions. MM primarily affects middle-aged to elderly patients. Blacks and males are affected more often than whites and females.¹ MM has remained an incurable disease, and effective thera-

peutic approaches are urgently required for patients with MM at different risk groups. Standard prognostic factors include serum β_2 -microglobulin, C-reactive protein, bone marrow plasma cell morphology, and plasma cell proliferation (plasma cell labeling index).^{1–3} These factors are independently associated with prognosis of patients with MM. Recently, there is considerable interest in characterizing genomic markers to establish prognostic models that allow a better estimation of an individual patient's prognosis.

Chromosomal abnormalities are among the most important prognostic parameters for patients with MM. Of particular note, deletions of 13q remain independent adverse prognostic factors.^{4–6} However, conventional karyotyping has been hampered by the slow growth of MM cells in cell cultures, and chromosomal abnormalities are often missed by this technique. Of the cases studied, 50 to 70% showed normal karyotypes originating from the myeloid elements.^{7–11} Therefore, cell-targeting methods are essential for the analysis of genomic aberrations in MM.

Fluorescent *in situ* hybridization (FISH) allows detection of chromosomal aberrations in both actively dividing cells and interphase nuclei. Recently, the cytoplasm immunoglobulin (Ig) enhanced interphase FISH has been used to detect the most common genomic abnormalities in 351 patients with MM, including deletions of 13q14 and 17p13.1 and 14q32 translocations [t(4;14)(p16;q32), t(14;16)(q32;q23), and t(11;14)(q13;q32)].¹² Genomic aberrations have been detected in 54.2% of the tested population for 13q14 deletions, 33.1% for 14q32 translocations, and 10.7% for the 17p13.1 deletion.¹² Importantly, three distinct prognostic groups have been identified, including those with a median survival time of 24.7 months [the t(4;14) and/or t(14;16), and/or 17p13.1 deletion], 42.3 months [13q14 deletions without the t(4;14), t(14;16), or 17p13.1 deletion], and 50.5 months [only the

Supported in part by the Huntsman Cancer Institute Cancer Center support grant P30CA42014, by Affiliated Regional University Pathology Institute for Clinical and Experimental Pathology, by Chinese National Key Program for Basic Research grant 973, 001CB510103 (to S.H.), and by the Outstanding Youth grant 30225038 (to S.H.).

Accepted for publication June 30, 2005.

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t(11;14) or none of the abnormalities tested] in the patients treated with conventional chemotherapy.¹²

The FISH analysis reported by Fonseca et al¹² used a triple-color interphase FISH with immunofluorescence detection of the cytoplasmic Ig light chain and has been considered a standard cell-targeting method for the detection of chromosomal abnormalities in MM. Our group has developed an alternative processing protocol targeting both normal and abnormal plasma cells together with a "MM FISH panel" designed to detect the genomic aberrations with proven clinical significance. Our approach used four commercially available probes for FISH analysis on CD138⁺ cells and reliably detected the prognostically significant genomic aberrations, thus allowing clinicians to assess the biological risk of disease progression in individual patients with MM.

Materials and Methods

Patients

Only cytogenetically normal patients were enrolled in the analysis. Sixty patients at diagnosis were included in the present study that had cytogenetics and FISH analysis performed on their bone marrow (BM) samples for evaluation of MM. The clinical diagnosis of MM was based on BM cell characteristics of morphology, cytochemical staining, and immunophenotype. The median age of our patients was 65.4 years. There were 38 men and 22 women in our series. Our series of 60 patients were divided into two groups representing two different phases for analysis: 1) group A (collected between 2002 and 2003) composed of 42 cases was evaluated by FISH performed only on unsorted BM cells from routine chromosomal preparations; and 2) group B (collected within 2004) consisting of 18 cases was assessed by FISH performed on CD138⁺-sorted BM cells.

Chromosome Analysis

Karyotypic analysis was performed on all patients. BM cells were cultured in RPMI 1640 supplemented with 15% of fetal calf serum. Two cultures were left unstimulated for 24 hours, and one was stimulated with phytohemagglutinin/interleukin-2 for 72 hours.¹³ Chromosomes were characterized by a trypsin G-banding method.

Magnetic Cell Sorting of CD138⁺ Cells

CD138 microbeads are developed for isolation of human plasma cells (Miltenyi Biotec, Auburn, CA). CD138 is expressed on normal and malignant plasma cells but not on circulating B cells, T cells, and monocytes.^{14–16} Cell isolation was performed according to the manufacturer's protocol. In principle, at least 1 ml of BM was required to obtain sufficient plasma cells for analysis. BM cells were magnetically labeled with CD138 microbeads and separated on a column that was placed in the magnetic field of a magnetic cell sorting separator. The magnetically labeled CD138⁺ cells were retained in the column while

the unlabeled cells ran through. After removal of the column from the magnetic field, the retained CD138⁺ cells were collected as positively selected cell fraction. Purity of more than 90% was confirmed morphologically by May-Giemsa-Gruenwald staining. Subsequently, the CD138⁺ cells were incubated with hypotonic solution (0.068 mol/L KCL) for 10 minutes at 37°C and then fixed once with Carnoy's fixative for 10 minutes at room temperature before being dropped on slides for FISH analysis.

MM FISH Panel Analysis

Unsorted BM cells from routine chromosomal preparations of unstimulated cultures or CD138⁺-sorted BM cells were analyzed by FISH using a set of commercially available FISH probes specific for RB-1 (13q14), D13S319 (13q14.3), IgH (14q32), and p53 (17p13.1) loci (Vysis, Downers Grove, IL). These four probes constituted the MM FISH panel. All four probes were set up separately for each patient. For FISH pretreatment, slides were aged for 30 minutes at 56°C and then incubated in fresh RNase solution (100 µg/ml) for 1 hour at 37°C. Subsequently, the slides were rinsed three times in 2× standard saline citrate at room temperature (2 minutes each); dehydrated in 70, 85, and 100% ethanol at –20°C (2 minutes each); and air dried before denaturing. Probe hybridization and detection of hybridization signals were performed according to the manufacturer's protocols. At least two technologists scored the same case. Damaged and overlapping nuclei as well as areas of the slide where hybridization was absent or suboptimal were avoided for scoring. For each probe, 200 nuclei were evaluated. BM samples from 20 individuals without apparent hematological diseases and with normal karyotypes were used as controls. Means and standard deviations (SD) of the percentages of nuclei with one, two, and three or breakapart hybridization signals were calculated. Results were considered abnormal if the percentage of nuclei with the abnormal hybridization signals was greater than 3 SD from the mean. Therefore, the following cutoff values were used: 9.9% for the RB-1 deletion, 6.3% for the D13S319 deletion, 9.9% for the deletion of p53, and 2.6% for IgH rearrangements.

Statistical Analysis

Statistical analysis was performed using Stata software (Release 8.2; StataCorp, College Station, TX) and SPSS (Release 12.0; SPSS, Inc., Chicago, IL). All reported significance levels are two-sided. Differences between proportions of abnormalities as detected by FISH on CD138⁺-sorted cells and on unsorted cells were tested using χ^2 test for independent samples.

Results

Only cytogenetically normal patients were enrolled in the present study. Our series of 60 patients were divided into

Table 1. Clinical Features and FISH Analyses (Listed in Percentage of Nuclei for Each Probe) for Each Patient in Group A (Unsorted Cells) with Abnormal FISH Results

Case	Age*	Sex	RB-1 probe [†]	D13S319 probe [†]	IgH probe [†]	p53 probe [†]
4	76	F	0	2.5	<u>23.5</u>	2.5
15	74	M	1.5	1	<u>7.6</u>	0
17	48	F	1.5	0.5	<u>13.5</u>	0
20	75	M	2.5	0.5	<u>4.4</u>	2
26	59	M	5	1.5	<u>19.5</u>	2.5
30	64	M	<u>19</u>	<u>13</u>	<u>14</u>	3.5
35	63	F	5	<u>9.6</u>	2	1
37	53	F	<u>56</u>	<u>44.5</u>	<u>35</u>	<u>30.5</u>
40	79	M	1.5	3.5	<u>17</u>	2.5
42	72	M	<u>20</u>	<u>31</u>	2.5	4

M, male; F, female.

*Age in years.

[†]Underline indicates abnormal result.

two groups representing two different phases for analysis: 1) group A (collected between 2002 and 2003) composed of 42 cases was evaluated by FISH performed only on unsorted BM cells from routine chromosomal preparations; and 2) group B (collected within 2004) consisting of 18 cases was assessed by FISH performed on CD138⁺-sorted BM cells. Overall, 10 of 42 (24%) patients were found to be abnormal in group A, and 13 of 18 (72%) patients were found to be abnormal in group B (Tables 1 and 2). Importantly, in group B, FISH was also performed subsequently on unsorted BM cells with probes specific for the abnormalities as identified on CD138⁺-sorted cells in nine abnormal cases; only two of them showed the same aberrations as detected in sorted cells. Therefore, although we were not able to perform FISH simultaneously on both sorted and unsorted BM cells in all of the 60 patients, the present results still demonstrated clearly that the MM FISH panel on CD138⁺-sorted BM cells is significantly more sensitive

than FISH on unsorted BM cells in detecting chromosomal abnormalities in MM cells ($P = 0.0016$).

Among the abnormalities identified in group A, 1 case (2.4%) had abnormalities for RB-1/D13S319/IgH/p53; 1 case (2.4%), abnormalities for RB-1/D13S319/IgH; 1 case (2.4%), deletions in both RB-1 and D13S319; 1 case (2.4%), a deletion in D13S319; and 6 cases (14%), IgH rearrangements. Overall, IgH rearrangements were detected in 8 of 42 (19%) of the patients, 13q14 deletions in 4 of 42 (9.5%), and the p53 deletion in 1 of 42 (2.4%) patients. The percentage of cells positive for each probe was in the range of 4.4 to 35% for IgH rearrangements, and 19 to 56% and 9.6 to 44.5%, respectively, for deletions of RB-1 and D13S319. For the p53 deletion, 30.5% was detected.

In group B, 1 case (5.6%) had anomalies for RB-1/D13S319/IgH/p53; 1 case (5.6%), anomalies for RB-1/D13S319/IgH; 3 cases (16.7%), deletions in both RB-1 and D13S319 (Figure 1); 2 cases (11%); abnormalities for

Table 2. Clinical Features and FISH Analyses (Listed in Percentage of Nuclei for Each Probe) for Each Patient in Group B (CD138⁺ Sorted Cells) with Abnormal FISH Results

Case	Age*	Sex	RB-1 probe [†]	D13S319 probe [†]	IgH probe [†]	p53 probe [†]
1	63	F	<u>28.5</u>	<u>26</u>	<u>97</u>	<u>15</u>
			(1.5)	(5)	(1.5)	(3.5)
2	78	F	<u>31.5</u>	<u>40.5</u>	<u>4</u>	1.5
4	80	M	4	5	<u>8.4</u>	2.5
5	63	M	<u>12.5</u>	4.5	<u>30</u>	5
6	80	F	<u>21</u>	5	<u>19</u>	7
			(16)		(18)	
8	91	M	9	4	<u>6.5</u>	<u>16.5</u>
10	71	M	2.5	3.5	<u>5</u>	3
11	59	F	<u>40.5</u>	<u>45.5</u>	2.5	1.5
			(3.5)	(6)		
13	82	M	0.5	0	2	<u>49</u>
						(9)
14	53	M	<u>25.3</u>	<u>29.9</u>	0	0
			(2)	(4)		
16	84	F	<u>28</u>	<u>46.5</u>	1.5	4
			(4.5)	(6)		
17	66	M	0.5	0.5	<u>4</u>	0
					(0.5)	
18	67	F	3	<u>34.8</u>	0	0
				(33.5)		

M, male; F, female.

*Age in years.

[†]Underline indicates abnormal result; parentheses indicate FISH analysis also performed on unsorted cells.

Figure 1. One cell with two RB-1 orange signals representing a normal cell (A) and two cells each with one orange signal indicating the RB-1 deletion (B and C).

both RB-1 and IgH; 1 case (5.6%), a deletion in D13S319; 3 cases (16.7%), IgH rearrangements (Figure 2); 1 case (5.6%), abnormalities for both IgH and p53; and 1 case (5.6%), a deletion in p53. Overall, 13q14 deletions were observed in 8 of 18 (44%) patients, IgH rearrangements in 8 of 18 (44%) patients, and the p53 deletion in 3 of 18 (16.7%) patients. The positively identified cells for each probe ranged from 12.5 to 40.5% and from 26 to 46.5%, respectively, for deletions of RB-1 and D13S319; from 4 to 97% for IgH rearrangements; and from 15% to 49% for the p53 deletion.

Discussion

As reported previously, traditional cytogenetics only detects 30 to 50% of abnormalities in MM due to the low yield of MM cells in cultures.⁷⁻¹¹ Interphase FISH in conjunction with cell-targeting methods has been considered the best approach for the detection of chromosomal aberrations in MM. There are two types of cell-targeting methods that can be potentially used for FISH analysis of MM, ie, combining interphase FISH with immunofluores-

cent detection of the cytoplasmic Ig light chain^{12,17} or cell sorting by CD138 microbeads. CD138, also known as Syndecan-1, is expressed on normal and malignant plasma cells, but not on B or T cells and monocytes.¹⁴⁻¹⁶ CD138 is also expressed on the basolateral surfaces of endothelial cells, embryonic mesenchymal cells, vascular smooth muscle cells, endothelium, and neural cells.^{18,19} Therefore, cells of BM origin sorted by CD138 microbeads, which are predominantly composed of plasma cells, are suitable for FISH analysis of MM cells. The sorting procedures used were easily performed and were able to yield enough cells for FISH analysis with the MM FISH panel in our series. It has been reported that the detection sensitivity of CD138 on plasma cells ranges from 60 to 100%. This range likely represents technical problems.²⁰ For example, loss of CD138 has been found in samples stored in a cold environment or delayed in processing at the initial stage of setting-up. The most significant loss has been observed in samples separated with Ficoll-Hypaque or lysed with overnight incubation before testing.²⁰ If it is suspected that loss of CD138 has occurred, ie, no or not a significant amount of CD138⁺

Figure 2. One cell with two IgH orange/green (yellow) fusion signals representing a normal cell (A) and two cells each with an one orange, one green, and one orange/green (yellow) fusion signal pattern indicating the presence of an IgH rearrangement (B and C).

cells were collected after magnetic cell sorting, as a back-up, FISH may be performed on CD138⁺ cells collected or on unsorted cells to evaluate genomic aberrations. In our series, we did not encounter such situations. Avet-Loiseau et al^{21,22} and Fiserova et al²³ also reported using similar techniques to study MM in the literature. However, they did not perform the FISH analyses by using probe panels. In their studies, only 14q32 rearrangements and/or 13q14 deletions were investigated; the p53 locus was not analyzed. Therefore, we have developed a practical cell-targeting approach to evaluate the efficacy of a FISH panel in screening prognostically significant chromosomal aberrations in MM.

Even though studies with the cytoplasm Ig-enhanced interphase FISH have indicated that almost all MM cells are chromosomally abnormal,^{24–26} not all of the abnormalities identified are prognostically significant. Fonseca et al¹² used the same approach to analyze a group of MM patients and reported that FISH probes targeting at 13q14, 17p13, and IgH loci can serve as genetic markers stratifying MM patients into three distinct prognostic categories. Based on their findings, we performed FISH analysis in a group of MM patients with the probes targeting at the similar loci as in Fonseca's study, and we were able to detect chromosomal abnormalities in CD138⁺-sorted cells in 72% of the tested MM patients compared with 24% by FISH evaluation performed on unsorted BM cells ($P = 0.0016$). FISH on CD138⁺-sorted cells revealed the frequencies of individual genomic abnormalities in our study population similar to the frequencies observed in prior studies, such as 13q14 deletions in 44% compared with 30 to 54%,^{4,6,12,27,28} the p53 deletion in 16.7% compared with 10 to 11%,^{12,29} and IgH rearrangements in 44% compared with 50 to 60% in the literature.^{30–32} Of note, FISH on unsorted BM cells identified that the frequencies of individual genomic aberrations in our study series were all lower than those reported in the literature, further supporting FISH on untargeted cells as an inefficient approach for the identification of chromosomal abnormalities in MM.

In our series (Table 2), several patients showed a relatively low percentage of cells detected with IgH rearrangements compared with the percentages reported.^{12,21} Our results are believable and were generated on the basis of high-quality imaging analysis by experienced technologists (Figure 2) and solid statistical evaluation. In theory, IgH rearrangements normally play a primary role in the pathogenesis of MM. In such conditions, IgH rearrangements likely serve as an initial pathogenic factor affecting stemlines of MM. However, although less commonly, IgH rearrangements can also appear as a secondary anomaly involved in the pathogenesis of MM. As a secondary change, IgH rearrangements likely only affect sidelines of a tumor cell population, which may explain the low-level positivity of IgH rearrangements in some of our patients. In addition, unknown technical issues resulting in contamination of the enriched cell populations by CD138⁺ cells may serve as another explanation. Additional studies are warranted to further investigate this observation.

It is worth mentioning that conventional cytogenetics and/or FISH have identified some chromosomal abnormalities in MM, such as trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19, and 21; monosomies of chromosomes 14, 16, and 22; and deletions of 13q at breakpoints other than 13q14^{7,8,10,11,26,33} that were not included in our FISH panel for detection. Although it has been shown that patients with complex karyotypes may have poorer survival, the specific prognostic significance of each of these chromosomal aberrations in MM is not known.⁷ On the other hand, the prognostic significance of the individual chromosomal abnormalities that were identified by our FISH panel has been established.^{7,12} The fact that, in general, most MM can be accurately diagnosed based on cell morphology and flow cytometric immunophenotyping analyses underscores that prognostic stratification is more important than genetic diagnosis in managing patients with MM. Therefore, our study further supports the appropriateness of selecting these prognostically significant probes in the MM FISH panel.

In conclusion, our investigation reveals that the present cell-targeting approach (ie, cell sorting by CD138 microbeads) in conjunction with the MM FISH panel is able to generate a higher detection rate compared with FISH performed on untargeted cells in identifying prognostically significant genomic aberrations in MM. The frequencies of genomic abnormalities observed are similar to previously reported data obtained with the standard cell-targeting method. Our cell-targeting approach is also expected to detect minimal residual disease at levels above the cutoff values of the probes used because only enriched CD138⁺ cell populations will be analyzed. Further studies are warranted to address whether this FISH panel is efficient in detecting genomic aberrations in monoclonal gammopathy of undetermined significance. We believe that this study has demonstrated the efficiency of our cell-targeting FISH panel approach and argues for its potential widespread use as an alternative method for the design of risk-adapted treatment strategies.

Acknowledgments

We thank Ms. Kathy Moran for her help with the statistical analysis.

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