American Journal of Pathology, Vol. 149, No. 1, July 1996

Incidence of Chromosome Numerical Changes in Multiple Myeloma

Fluorescence in Situ *Hybridization Analysis Using 15 Chromosome-Specific Probes*

Dolores Tabernero,* Jesús F. San Miguel,† Ramón García-Sanz,† Luisa Nájera,‡ María García-Isidoro,* José A. Pérez-Simón,† Marcos González,† Joop Wiegant,§ Anton K. Raap,§ and Alberto Orfão*

From Servicio General de Citometría, and Departamento de Biología Celular,‡ Universidad de Salamanca, and Servicio de Hematología,† Hospital Universitario de Salamanca, Salamanca, Spain, and Sylvius Laboratory,§ University of Leiden, Leiden, The Netherlands*

The presence of complex karyotypes with frequent numerical and structural abnormalities has been reported in 20 to 50% of multiple myeloma (MM) patients. This variability is mainly due to the difficulty of conventional cytogenetics to obtain tumor metaphases representative of all possible neoplastic clones in MM. To gain insight into the real incidence of numerical chromosome changes in MM we have studied by fluorescence* in situ *hybridization technique 15 different human chromosomes, 1, 3, 6, 7, 8, 9, 10, 11, 12, 13, 15, 17, 18, X, and Y, in a series of 52 MM patients. In all cases, the DNA index assessed by a propidium iodide/CD38 double-staining technique with flow cytometry was simultaneously investigated for correlation with fluorescence* in situ *hybridization results. Additional aims of this study were 1) to analyze whether the abnormalities detected were common to all plasma cells or were present in only a subpopulation of tumor cells, 2) to explore changes caused by disease progression, and 3) to establish possible associations among the altered chromosomes. Although the overall incidence of numerical abnormalities was 67%, this frequency increased to 80% in the 41 cases in which 7 or more chromosomes were analyzed. Trisomies were significantly more common than monosomies (84%* versus *16%). Chromosomes 9 and 15 were the most frequently altered (52% and 48% of cases, respectively), with all of their abnormalities corresponding to trisomies. The most frequent losses involved chromosomes 13 (26%) and X in females (32%). Other common numerical changes corresponded to chromosomes 1 (39%), 11 (37%), 6 (32%), 3 (31%), 18 (29%), 7 (28%), and 17 (22%). By contrast, chromosomes 8 (13%), 10 (8%), and 12 (3%) were rarely altered. DNA aneuploidy by flow cytometry was detected in 67% of patients, and a high degree of correlation was observed between the DNA index obtained by flow cytometry and the chromosome index derived from fluorescence* in situ *hybridization studies, calculated according to two mathematical formulas (coefficient of correlation of 0.82 and 0.91 when at least 7 or 12 chromosomes were considered, respectively). The frequency of numeric chromosome aberrations was higher in those patients with progressive disease and, interestingly, trisomy of chromosome 8 was exclusively detected in this latter group of patients. Our study shows that, with the exception of chromosome 8, a possible marker of clonal evolution, the numeric chromosome changes are present in nearly all malignant plasma cells (r > 0.84). Finally, frequent associations between chromosomal aberrations were observed (ie, chromosomes 6, 7, 9,

Partially supported by a grant from the Spanish FIS(95/1475) and DGICYT (PB93–0614). M. García-Isidoro was supported by a grant from the Spanish FIS-SS.

Accepted for publication February 29, 1996.

Address reprint requests to Dr. Alberto Orfão, Servicio de Citometría, Laboratorio de Hematología, Hospital Universitario de Salamanca, Paseo de San Vicente, 58–182, 37007, Salamanca, Spain.

and 17; 7 and 15; and 11 and 17). By excluding them, it was found that two triple combinations of chromosome-specific probes, chromosomes 1 and 9 together with either chromosome 13 or 15, could be a useful marker for detection of residual disease, as it permits the identification of most MM patients displaying numerical changes. (Am J Pathol 1996, 149:153–161)

The availability of cytogenetic information in multiple myeloma patients has long been hampered by the low mitotic activity of the myelomatous plasma cells (PCs) as well as by their poor growth in cell culture, which make it difficult to obtain abnormal metaphase cells for conventional chromosome analysis. Cytogenetic studies have shown a 20 to 50% incidence of abnormal karyotypes in multiple myeloma (MM) patients,[1–4] although no specific chromosome aberration has been established. It should be noted that the majority of these studies have failed to obtain metaphases in many patients[1–4] and therefore could not be analyzed. Moreover, in those cases with normal metaphases it was not clarified whether the metaphases obtained were derived from myelomatous PCs or from normal residual hemopoietic cells that coexist with PCs in the bone marrow of MM patients. Therefore, the reported incidence of MM cases displaying abnormal karyotypes and numerical chromosome changes using conventional cytogenetics is probably underestimated. Procedures that can be applied to both metaphase cells and interphase nuclei, such as the flow cytometry measurement of cell DNA contents and fluorescence *in situ* hybridization (FISH), are very well suited to the analysis of cytogenetic abnormalities in tumor cells. Accordingly, flow cytometric studies have shown that the presence of DNA aneuploidy can be detected in a high proportion of MM patients, its reported incidence being as high as 80%.[5–7] In addition, these studies have shown that hyperdiploidy is much more frequent than hypodiploidy. However, the analysis of cell DNA content by flow cytometry would not allow the detection of individual chromosome changes as the resolution between a diploid and an aneuploid G_0/G_1 peak usually requires a change in cell DNA content of at least 4%[8,9] (the DNA content of the largest human chromosome is approximately that value). On the other hand, interphase ISH with DNA probes specific for the identification of the centromeric regions of human chromosomes has established itself as an accurate technique for the assessment of numerical chromosome abnormalities in both hematological malignancies and solid tumors.[10–12] It overcomes both the disadvantages of conventional cytogenetics as regards the need for abnormal metaphases and those of flow cytometry DNA studies as it allows the analysis of both interphase and metaphase cells with individual chromosome-specific probes. At present, no study has been performed in a large series of MM patients in which the incidence of numerical chromosomal abnormalities is analyzed by FISH for a high number of chromosomes.

In the present paper, the incidence of numerical aberrations of 15 different human chromosomes is analyzed by FISH in a series of 52 MM patients. Additional aims of these study were 1) to analyze whether the abnormalities detected were common to all PCs present in the patients' bone marrow (BM) or were present in only a fraction of the myelomatous PCs, 2) to explore whether or not the incidence of chromosomal changes is higher in cases of disease progression, 3) to establish possible associations among the altered chromosomes, and 4) to correlate the FISH results with the DNA index obtained by flow cytometry.

Materials and Methods

Patients

A total of 52 myeloma patients were included in the present study, 44 of whom were untreated patients with symptomatic MM diagnosed according to the criteria of the Chronic Leukemia-Myeloma Task Force[13]; 8 patients had either relapse or progressive disease. The mean age of the series was 66 ± 8 years (range, 55 to 83), with 33 males and 19 females. According to Durie and Salmon's clinical staging system (1975),[14] the patients were distributed as follows: stage I, 5.7%; stage II, 30.8%; stage III, 63.3%. The monoclonal component was IgG in 49% of the cases, IgA in 30%, IgD in 2%, and Bence Jones in the remaining 15% of patients. In one case, no monoclonal serum protein was found. The serum monoclonal light chain was κ in 60% and λ in 40%. In 47% of cases, a urine monoclonal light chain was detected. All patients were treated according to the protocols of the PETHEMA group.[15]

Immunophenotypic Identification of Plasma Cells

The percentage of BM PCs was assessed by two different observers on May-Grünwald-Giemsa-stained smears, the mean value being 45 ± 24%. The immunophenotypical identification of PCs was

based on their strong reactivity for the CD38 (Leu17-phycoerythrin; Becton Dickinson, San José, CA) monoclonal antibody (MAb), the specificity of which has been described elsewhere.[16,17] Analysis of PC reactivity for this surface antigen was performed using direct immunofluorescence. For data acquisition and analysis, the FACScan flow cytometer (Becton Dickinson) equipped with an argon ion laser tuned at 488 nm and 15 mW was used. Results were stored and analyzed for at least 10,000 cells/test, using the LYSYS-II and PAINT-A-GATE-PLUS software programs (Becton Dickinson), respectively.[16,17]

FISH Studies

FISH analysis of numerical chromosome abnormalities was performed on heparin-anticoagulated erythrocyte-lysed whole BM samples according to previously reported methods.[18] Briefly, cells were fixed in Carnoy's medium and dropped onto ethanol/ether (1/1, vol/vol) cleaned slides according to conventional cytogenetics protocols[18] and stored for 24 to 72 hours in the dark (room temperature). The slides were then sequentially incubated with solutions containing 0.1 mg/mL of RNAse A (1 hour at 37°C) and 0.1 mg/ml pepsin (10 minutes at 37°C). They were fixed in 1% acid-free paraformaldehide (10 minutes at room temperature) and dehydrated in ethanol according to previously reported techniques.[19,20] Afterwards, the slides containing both cells, DNA, and 10 ng of each DNA probe were denatured in an 80°C oven for 100 seconds. Biotinylated, fluoresceinated, or digoxigenin-labeled α-satellite DNA probes specific for the centromere of human chromosomes 1 (pUC1.77, Boehringer Mannheim, Mannheim, Germany), 3 (pAE0.68, Boehringer Mannheim), 6 (D6Z1, Oncor, Gaithersburg, MD), 7 (pZ7.6B, Boehringer Mannheim), 8 (pZ8.4, Boehringer Mannheim), 9 (D9Z1, Oncor), 10 (CEP10, Vysis, Framingham, MA), 11 (CEP11, Vysis), 12 (D12Z3, Oncor), 15 (pMC15, Boehringer Mannheim), 17 (pZ17–1.6A, Boehringer Mannheim), 18 (pZXba, Boehringer Mannheim), X (pDMX1, Boehringer Mannheim), and Y (pHY2.1, Boehringer Mannheim) were used in the present study. In addition, a locus-specific DNA probe for chromosome 13 was used (LSI13, Vysis). Upon denaturation, slides were placed at 37°C and hybridized overnight in a humid chamber.

The immunological detection of the biotinylated and digoxigenin-labeled hybridized probes was performed with an immunological blocking incubation with 4 mol/L buffer (30 minutes at 37°C) followed by another incubation (30 minutes at 37°C) with a solution containing avidin-fluorescein isothiocyanate (FITC) (Vector Laboratories, Burlingame, CA) and a mouse anti-digoxigenin MAb conjugated with TRITC (Boehringer Mannheim, Figure 1). Immunological amplification of the signals was performed whenever the fluorescence intensity of the hybridization spots was not strong enough to be clearly identified with the microscope. For that purpose, either a biotinylated anti-avidin goat MAb (Vector) plus avidin-FITC or a tetraethylrhodamine-isothiocyanate-conjugated sheep anti-mouse immunoglobulin antibody[18] were used. Cells were counterstained with DAPI according to previously described methods.[18] The number of hybridization spots was evaluated using a DMRB fluorescence microscope (Leitz, Wezlar, Germany) equipped with a 100× oil objective, which was used for counting hybridization spots/cell in at least 200 cells/sample. In all slides analyzed, the number of unhybridized cells in the areas assessed was lower than 1%, and only those spots with a similar size, intensity, and shape were counted.

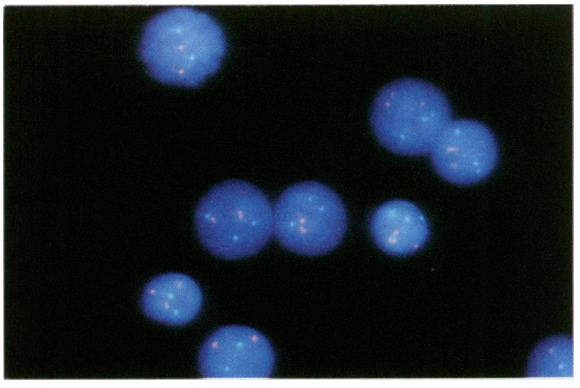

Figure 1. *Double hybridization for chromosomes 7 and 15 in the bone marrow cells of a MM case with trisomy of the two chromosomes. There are cells with two signals of each chromosome (normal cells) and cells with three signals of each chromosome (neoplastic cells).*

Mononuclear cells from 10 age- and sex-matched healthy individuals displaying a normal karyotype were used as controls. The mean percentage of trisomic/monosomic cells in these control samples for the chromosomes assessed was as follows: chromosome 1, 0.1 ± 0.3%/2.1 ± 1.6%; chromosome 3, 0.5 ± 0.7%/3.9 ± 2.7%; chromosome 6, 0.0 ± 0.0%/1.6 ± 1.9%; chromosome 7, 0.1 ± 0.3%/2.1 ± 1.5%; chromosome 8, 0.1 ± 0.3%/1.1 ± 0.9%; chromosome 9, 0.3 ± 0.9%/5.5 ± 2.6%; chromosome 10, 0.1 ± 0.2%/0.9 ± 0.6%; chromosome 11, 0.2 ± 0.2%/0.8 ± 0.6%; chromosome 12, 0.4 ± 0.7%/1.7 ± 2.2%; chromosome 13, 0.5 ± 0.7%/3.0 ± 1.9%; chromosome 15, 0.8 ± 0.7%/1.2 ± 0.8%; chromosome 17, 0.5 ± 0.8%/5.6 ± 2.8%; chromosome 18, 0.8 ± 0.4%/1.3 ± 0.7%; chromosome $X_{females}$, 2.0 ± 1.4%/0.0 ± 0.0%. In addition, the percentage of cells

with more than or less than one spot for chromosomes X and Y in males was 0.1 ± 0.3%/0.7 ± 2.1% and 0.8 ± 1.0%/1.4 ± 1.7%, respectively. A patient was considered to carry a numerical chromosomal abnormality for a chromosome when the percentage of cells displaying a proportion of events with an abnormal number of spots was at percentages higher than the mean value plus two standard deviations of the percentages obtained for that specific chromosome in normal controls. The correlation between the proportion of cells carrying numerical chromosome changes and the percentage of PCs detected by immunological analysis was based on statistical analysis.

The expected amount of total DNA cell content according to the FISH results was assessed by the chromosome index calculated according to two different mathematical formulas:
1) chromosome index = $1 + [(Ch_1 \times 0.043) - (2 \times 0.043)] + [Ch_3 \times 0.035) - (2 \times 0.035)] + [(Ch_6 \times 0.030) - (2 \times 0.030)] + [Ch_7 \times 0.027) - (2 \times 0.027)] + [(Ch_8 \times 0.026)(2 \times 0.026)] + [(Ch_9 \times 0.024) - (2 \times 0.024)] + [(Ch_{10} \times 0.023) - (2 \times 0.023)] + [(Ch_{11} \times 0.024) - (2 \times 0.024)] + [(Ch_{12} \times 0.023) - (2 \times 0.023)] + [Ch_{13} \times 0.019) - (2 \times 0.019)] + [(Ch_{15} \times 0.017) - (2 \times 0.017)] + [(Ch_{17} \times 0.015) - (2 \times 0.015)] + [(Ch_{18} \times 0.014) - (2 \times 0.014)] + [(Ch_x \times 0.027) - (x \times 0.027)] + [(Ch_y \times 0.009) - (y \times 0.009)]$ and 2) corrected chromosome index = $[(Ch_1 \times 0.043) + (Ch_3 \times 0.035) + (Ch_6 \times 0.030) + (Ch_7 \times 0.027) + (Ch_8 \times 0.026) + (Ch_9 \times 0.024) + (Ch_{10} \times 0.023) + (Ch_{11} \times 0.024) + (Ch_{12} \times 0.023) + (Ch_{13} \times 0.019) + (Ch_{15} \times 0.017) + (Ch_{17} \times 0.015) + (Ch_{18} \times 0.014) + (Ch_x \times 0.027) + (Ch_y \times 0.009)] \div [(2 \times 0.043) + (2 \times 0.035) + (2 \times 0.030) + (2 \times 0.027) + (2 \times 0.026) + (2 \times 0.024) + (2 \times 0.023) + (2 \times 0.024) + (2 \times 0.023) + (2 \times 0.019) + (2 \times 0.017) + (2 \times 0.015) + (2 \times 0.014) + (x \times 0.027) + (y \times 0.009)]$, where Ch_n was the number of signals per cell for each probe, x was 2 in women and 1 in men, and y was 0 in women and 1 in men.

DNA Measurements

DNA measurements were performed in all cases using two different methods. Briefly, between 100 and 200 μl of the BM aspirate sample containing between 0.5×10^6 to 1×10^6 white blood cells were placed in two different tubes, as previously described.[17]

In the first tube, cells were lysed using ammonium chloride, washed in citrate buffer, and resuspended in 200 μl of a sodium citrate buffer. Then, sample preparation was performed following the technique of Vindelov et al,[21] slightly modified.[17] Cells were incubated for 10 minutes at room temperature with 1.8 ml of solution A containing trypsin (30 mg/L). Afterwards, 1.5 ml of solution B containing RNAse (100 mg/L) and a trypsin inhibitor (500 mg/L) were added and the mixture incubated for another 10 minutes at room temperature. Finally, a third incubation for at least 15 minutes at room temperature in the dark was performed after adding 1.5 ml of solution C containing propidium iodide (208 mg/L) to complete a final volume of 5 ml.

In the second tube, BM cells were incubated for 15 minutes with 10 μl of the GR7A4 (CD38) MAb, washed once (5 minutes at 1900 rpm) in phosphate-buffered saline, and incubated for another 15 minutes with MAb anti-mouse immunoglobulin $(F(ab')_2$ fragments; (Dakopatts, Copenhagen, Denmark). Afterwards, 2 ml of ammonium chloride was added and cells were incubated in the dark for 10 minutes. After lysing the erythrocytes, cells were washed once in 1 ml of sodium citrate buffer and resuspended in 200 μl of the same buffer. Then, 1.5 ml of solution B was added and the cells were incubated for 10 minutes. Finally, 1.5 ml of solution C was added and another incubation period of at least 15 minutes was performed in the dark.

In all cases, measurements were performed within 1 hour on a FACScan flow cytometer (Becton Dickinson) using the CellFit software program (Becton Dickinson) for at least 10,000 cells/sample. The electronics of the instrument were adjusted so that the modal channel for the G_0/G_1 diploid nuclei was 200 (fluorescence scaled from channel 0 to 1023) and fluorescence compensation between FITC and propidium iodide established, using a mixture of propidium-iodide-stained chicken erythrocyte nuclei and FITC-labeled beads (CALIBRITE beads, Becton Dickinson). The percentage of CD38 strong positive plasma cells was calculated after gating cell doublets on a FL_2A/FL_2W dot plot using the PAINT-A-GATE-PLUS software.

The criteria of aneuploidy were defined by the presence of two distinct peaks of cells in G_0/G_1 phase in the histogram obtained with the isolated nuclei technique. The diploid cells were the normal residual hemopoietic cells present in the patient BM. All cases had a sufficient number of normal cells for this internal control, and it was not necessary to add other control cells. The CD38/propidium iodide double-staining technique was used in all MM cases to identify which of the G_0/G_1 peaks corresponded to the myelomatous PCs (CD38 strong$^+$) and the normal residual hemopoietic cells (CD38 negative or dim/intermediate positive).

Table 1. *Incidence of Numerical Chromosomal Changes in MM Patients According to the Different Chromosomes Analyzed*

Chromosome abnormalities	Chromosome														
	1	3	6	7	8	9	10	11	12	13	15	17	18	Xm	Xf
Trisomies	14/38	9/29	13/41	13/46	3/39	22/42	3/38	10/30	1/39		10/21	9/41	8/34	1/22	
	36.8%	31%	31.7%	28.3%	7.7%	52.4%	7.9%	33.3%	2.6%		47.6%	22%	23.5%	5.9%	
Monosomies	1/38				2/39			1/30		10/38			2/34		6/19
	2.6%				5.1%			3.3%		26.3%			5.9%		31.5%
Total	15/38	9/29	13/41	13/46	5/39	22/42	3/38	11/30	1/39	10/38	10/21	9/41	10/34	1/22	6/19
	39.4%	31%	31.7%	28.3%	12.8%	52.4%	7.9%	36.6%	2.6%	26.3%	47.6%	22%	29.4%	5.9%	31.5%

Results are expressed both as number of cases from the patients analyzed and as their percentage. Xm, chromosome X in males; Xf, chromosome X in females. No numerical changes were observed for chromosome Y.

Results

The overall incidence of numerical chromosome abnormalities in the MM patients included in the present study was 67%. This incidence significantly increased when 7 or more chromosomes were analyzed (41 patients), reaching 80.5% compared with only 18% in the remaining 11 cases. It should be noted that when more than 7 chromosomes were assessed, the incidence of numerical chromosome abnormalities remained stable. Accordingly, in those cases (n = 30) in which 12 or more chromosomes were analyzed, the incidence was 81.5%. Overall, trisomies were more frequent than monosomies (84% *versus* 16%).

Table 1 shows the distribution of numerical chromosomal abnormalities according to each of the 15 chromosomes analyzed in the present study. Chromosome 9 (52.4%) and chromosome 15 (47.6%) were the most frequently altered, with all of their abnormalities corresponding to trisomies. In contrast, it should be noted that the lowest incidence of numerical chromosomal abnormalities corresponded to chromosomes 12 (2.6%) and 10 (7.9%), with all of the abnormalities of both chromosomes corresponding again to trisomies. Accordingly, from the remaining chromosomes analyzed, most of them displayed trisomies as either the only chromosomal abnormality detected (chromosomes 3, 6, 7, and 17) or the most frequent one (chromosomes 1, 8, 11, and 18). In contrast, monosomies were the only numerical abnormality detected for chromosome 13 (26.3%). As far as chromosome X was concerned, both monosomies and an extra X chromosome were detected. Interestingly, although monosomies were exclusively present in females (31.5%), the only patient displaying an extra X chromosome was a male.

Upon grouping the patients according to the moment at which the FISH study was performed, it was observed that those cases studied at diagnosis (n = 44) showed a lower incidence of trisomies for chromosomes 1, 6, 8, 9, 11, and 18 with respect to those patients in either relapse or progression (n = 8); trisomy of chromosome 8 was exclusively detected in the latter group (Table 2). On the other hand, no significant differences were detected for the incidence of numerical aberrations of the remaining chromosomes analyzed including monosomy 13 (25% *versus* 29%).

The comparison between the percentage of PCs present in the sample as assessed by immunological markers and the proportion of myelomatous PCs displaying numerical chromosome aberrations by FISH showed the existence of a significantly high ($r > 0.84$) degree of correlation for all of the chromosomes analyzed except chromosome 8 (Table 3). Interestingly, an association between the chromosomal abnormalities was observed. Thus, it was observed that trisomy 6 was significantly associated with gains of chromosomes 7 ($P = 0.0002$), 9 ($P = 0.002$), and 17 ($P = 0.003$). In addition, abnormalities of chromosomes 7 and 11 were associated with those of chromosomes 15 ($P = 0.005$) and 17 ($P =$

Table 2. *Incidence of Trisomies in MM Patients at Diagnosis* versus *Relapse/Progression*

	Chromosome													
	1	3	6	7	8	9	10	11	12	13	15	17	18	Xm
Trisomies at diagnosis	10/31	7/23	8/34	10/38	0/33	16/36	3/32	7/24	1/32	0/3	8/15	7/34	5/27	1/18
	32.2%	30.4%	23.5%	26.3%	0%	44.4%	9.3%	29.1%	3.1%	0%	53.3%	20.5%	18.5%	5.5%
Trisomies at relapse/ progression	4/7	2/6	5/7	3/8	3/6	6/6	0/6	3/6	0/7		2/6	2/7	3/7	0/4
	57.1%	33.3%	71.4%	37.5%	50%	100%	0%	50%	0%		33.3%	28.6%	42.9%	0%

Results are expressed as number of cases from the patients analyzed and as number and percentage of cases displaying trisomy. Xm, chromosome X in males.

Table 3. *Correlation between the Percentage of PCs and the Proportion of Cells Displaying Numerical Chromosomal Abnormalities*

Chromosome	Coefficient of correlation	*y* intercept	Slope of the best linear fit
1	0.939	7	0.859
3	0.967	3	0.778
6	0.855	10	0.587
7	0.892	7	0.660
8	−0.548	39	−0.249
9	0.843	10	0.727
10	0.999	0.3	0.854
11	0.966	−4	1.267
13	0.951	−2	1.029
15	0.952	−2	1.032
17	0.885	12	0.642
18	0.882	5	0.968
X	0.945	15	0.565

0.003), respectively. Based on these findings, and after excluding these associations, we investigated which were the simplest combinations of chromosomes that would allow the detection of the highest number of MM patients displaying numerical chromosome changes. In this sense, the combined assessment of chromosomes 1 and 9 together with either chromosome 13 or 15 would allow the identification of all abnormal cases analyzed in this study.

The analysis of PC DNA content was assessed simultaneously by flow cytometry in the 52 patients studied using a propidium iodide/CD38 double-staining technique. DNA aneuploidy was found to be present in 67% of the cases, with 65% corresponding to hyperdiploid MMs and 2% to hypodiploid cases. A significant correlation was observed between the DNA index obtained by flow cytometry and the chromosome index as analyzed by FISH and calculated according to two different mathematical formulas described in Materials and Methods. Accordingly, upon considering the 41 cases in which numerical abnormalities for at least 7 chromosomes were simultaneously explored, the coefficient of correlation was 0.81754 (slope of the best linear fit = 0.99705, *y* intercept = 0.05255, $P < 0.0001$) (Figure 2a). The significance of the correlation increased (coefficient of correlation = 0.91371, slope of the best linear fit = 1.16884, *y* intercept = −0.13199, $P < 0.0001$) when only those cases in which a minimal number of 12 chromosomes was assessed (Figure 2b). Interestingly, similar results were obtained by using each of the two mathematical formulas for the calculation of the chromosome index (Figure 2).

Discussion

In MM patients, complex karyotypes with frequent numerical and structural abnormalities have been reported.[1,2,4,22–25] However, the real incidence of these aberrations remains to be established as quite variable results have been published. This is mainly due to methodological problems inherent in conventional cytogenetic studies that require abnormal

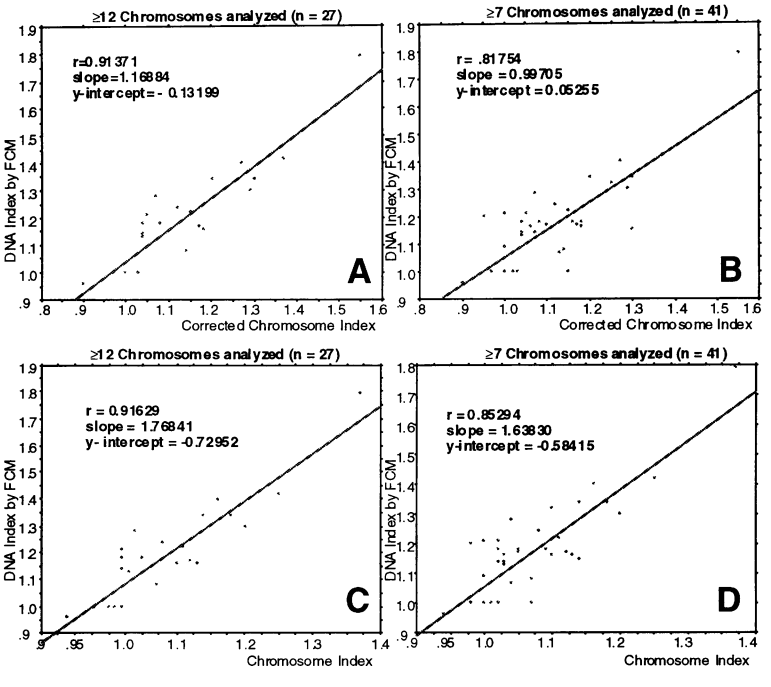

Figure 2. *Correlation between the DNA index obtained by flow cytometry and both the corrected* (A *and* B) *and uncorrected* (C *and* D) *chromosome index obtained by FISH analysis.* A *and* C: *Results obtained for those cases* ($n = 41$) *in which a minimum of 7 chromosomes were explored.* B *and* D: *Correlations found for those cases* ($n = 27$) *in which at least 12 chromosomes were analyzed.*

metaphase cells, which in turn are difficult to obtain in MM patients due to the low proliferative rate of myelomatous PCs.[26] Moreover, as residual normal BM cells display a higher rate of cell turnover than malignant PCs,[26] metaphases derived from normal cells may lead to an overestimation of normal karyotypes in MM patients. Accordingly, interphase cytogenetics using FISH represents a major advantage in these cases, as it allows the determination of numerical chromosome aberrations in nondividing or interphase cells as well as in metaphases.[27–32] Under the best cytokine stimulation conditions (ie, granulocyte/macrophage colony-stimulating factor plus interleukin-6),[3] the reported frequencies of numerical changes assessed by conventional cytogenetic analysis range from 20 to 47%.[1–4] By contrast, flow cytometry studies of cell DNA contents clearly show that between 50 and 80% of MM patients display DNA aneuploidy.[5–7]

To gain insight into the real incidence of numerical chromosome changes as well as into the cause of the discrepancies observed between conventional cytogenetics and flow cytometry studies in MM, we have analyzed by FISH the incidence of numerical chromosome abnormalities in a group of 52 MM patients. Although the overall frequency was 67%, it should be noted that when 7 or more chromosomes were analyzed it increased to 80%. Therefore, according to these results, most patients with MM have numerical changes. In the present FISH study it was observed that trisomies were significantly more common than monosomies, which is consistent with the cell DNA content studies by flow cytometry showing a similar predominance of hyperdiploidy over hypodiploidy.[5–7] Flow cytometry has now been used for more than 20 years for the detection of abnormal cell DNA contents in many types of tumors including MM patients. Although several attempts have been made to correlate the flow cytometry DNA aneuploidy with the cytogenetic results, this type of study has long been hampered by the aforementioned problems inherent in cytogenetic studies. To study whether or not the amount of abnormal total cell DNA contents obtained by flow cytometry really reflects numerical chromosome changes, the FISH results were correlated with the DNA index obtained by flow cytometry for the same patients. For that purpose, two different mathematical formulas were used in which the relative DNA content weight of each numerical chromosome abnormality detected was taken into account. Interestingly, a high degree of correlation was obtained between both types of information, clearly indicating that the flow cytometric DNA index obtained with the techniques used largely reflects the numerical chromosome abnormalities present in MM patients. However, it should be noted that in approximately one-half of MM patients displaying a diploid flow cytometry DNA histogram, numerical chromosome changes exist, but either the gains and losses of chromosomes were balanced or, more frequently, they were single aberrations that in both cases remain undetectable by conventional flow cytometry.

Apparently MM patients do not display specific chromosomal abnormalities. However, in the present study it is shown that there is a preferential involvement of some chromosomes, such as gains of chromosomes 9 and 15 detected in one-half of the patients and losses of chromosome 13 observed in one-fourth of the patients. Interestingly, these chromosomes are known to carry genes that may play an important role in the pathogenesis of the disease such as p16 in chromosome 9[33] and Rb in chromosome 13.[34] By contrast, chromosomes 12, 10, and 8 are rarely involved in MM. These findings, although at a higher incidence, confirm previous reports based on cytogenetic studies.[1–4] The most striking result concerning individual chromosomes in our FISH study is the high frequency at which three spots for the chromosome 1 probe were detected (37%), which contrasts with the lower incidence reported by Facon (2%)[23] and Sawyer (1%)[4] using conventional cytogenetics. However, structural aberrations of chromosome 1 have been reported in nearly one-half of MM patients with abnormal karyotypes.[35] In addition, the existence of three copies of the long arm of chromosome 1, either reflecting direct 1q duplications, isochromosomes 1q, or extra copies translocated to different chromosomes, is a common feature in many types of cancer.[36,37] Moreover, Philip et al[38] have reported the existence of three copies for 1q21-q3 in 31% of his myeloma patients. The α-centromeric probe used in the present study for the identification of chromosome 1 was initially isolated from human satellite III DNA after an *Eco*RI digestion.[10] Satellite III DNA consists of relatively short oligonucleotide tandem repeats that are arranged to chromosome-specific repeats of a higher order leading to unique sequences of different lengths in the heterochromatic peri(centric) region of the human chromosomes 1, 9, 16, and Y.[11,12] The hybridization signal is specific for chromosome 1 when appropriate stringent conditions are used. Therefore, the results obtained in the present paper for chromosome 1 would reflect the detection of extra copies of 1q whenever the heterochromatic pericentric region is also involved. An alternative explanation for this discrepancy between FISH and conventional cytogenetics, regarding trisomy 1, would be that those

cases displaying such aberration would have an especially low PC proliferative index during cell culture, which would make cytogenetic analysis difficult. One additional observation was the higher frequency of numeric chromosome aberrations in those patients studied at relapse or displaying a progressive disease state, which is in accordance with previous cytogenetic observations.[1,2,4] Nevertheless, it should be noted that sequential samples from individual patients were not analyzed in the present study. Interestingly, none of the cases analyzed at diagnosis displayed trisomy 8, whereas one-half of those in relapse/progression showed this aberration. This observation would support the notion that trisomy 8 in MM patients would be a secondary event and a marker of clonal evolution as it has been suggested for other hematological malignancies such as the acute leukemias following a myelodysplastic or a myeloproliferative syndrome.[30,39,40] Additional studies in which sequential samples are examined are needed to elucidate this question.

Laï et al[3] have shown that in the majority of MM patients with an abnormal karyotype, there is a mixture of normal and abnormal metaphases and only 3 out of 129 patients had exclusively abnormal cells. However, whether the chromosome abnormalities detected are common to all PCs present in each patient's BM or they are present in only a subpopulation of myelomatous PCs remains to be elucidated. Our data show that, with the exception of chromosome 8, the numeric chromosome changes are present in nearly all malignant PCs. As mentioned before, trisomy 8 was exclusively detected in patients studied either at relapse or progression; thus, it is logical that this abnormality should be present in only a subpopulation of PCs, which would account for the low correlation observed between total number of PCs and PCs with trisomy 8.

Interestingly, the present study shows that there were some associations between the detected chromosomal abnormalities. By excluding these associations we were able to define two triple combinations of chromosome-specific probes (chromosomes 1 and 9 together with either chromosome 13 or 15) that would allow the detection of most myeloma patients displaying numerical changes. Accordingly, the use of these triple combinations by FISH could become a useful marker tool for both the analysis of clonal involvement of B cells and B cell precursors as well as for the detection of residual disease in myeloma patients. Its sensitivity limit ranges from 10^{-2} to 10^{-5} depending on which chromosomes display numerical changes.

References

1. Dewald GW, Kyle RA, Hicks GA, Greipp PRP: The clinical significance of cytogenetic studies in 100 patients with multiple myeloma, plasma cell leukemia, or amyloidosis. Blood 1985, 66:380–387
2. Durie BGM, Vela EE, Baum V: Cytogenetics abnormalities in multiple myeloma. Epidemiology and Biology in Multiple Myeloma. New York, Springer-Verlag, 1991, pp 137–141
3. Laï JL, Zandecki M, Mary JY, Bernardi F, Izydorczyk V, Flactif M, Morel P, Jouet JP, Bauters F, Facon T: Improved cytogenetics in multiple myeloma: a study of 151 patients including 117 patients at diagnosis. Blood 1995, 85:2490–2497
4. Sawyer JR, Waldron JA, Jagannath S, Barlogie B: Cytogenetic findings in 200 patients with multiple myeloma. Cancer Genet Cytogenet (in press)
5. Latreille J, Barlogie B, Johnston DA, Drewinko B, Alexanian R: Ploidy and proliferative characteristics in monoclonal gammopathies. Blood 1982, 59:43–51
6. Shimazaki C, Gotoh H, Ashihara E, Oku N, Inaba T, Murakami S, Itoh K, Ura Y, Nakagawa M, Fujita N: Immunophenotype and DNA contents of myeloma cells in primary plasma cell leukemia. Am J Hematol 1992, 39:159–162
7. García-Sanz R, Orfão A, González M, Moro MJ, Hernández JM, Ortega F, Borrego D, Carnero M, Casanova F, Jiménez R, Portero JA, San Miguel JF: Prognosic implications of DNA aneuploidy in a series of 156 untreated MM patients. Br J Haematol 1995, 90:106–112
8. Shankey TV, Ravinovitch PS, Bagwell B, Bauer KD, Duque RE, Hedley DW, Mayall BH, Wheeless LL: Guidelines for implementation of clinical DNA cytometry. Cytometry 1993, 14:472–477
9. Orfão A, González M, Ciudad J, López-Berges MC, López A, Vidriales B, Macedo A, San Miguel JF: Cell cycle and DNA aneuploidy: biological bases and terminology. DNA Cytometric Analysis. Edited by A. Sampedro, A Orfão. Oviedo, Spain, Ediciones de la Universidad de Oviedo, 1993, p 13–24
10. Cooke HJ, Hindley M: Cloning of human satellite III DNA: different components are on different chromosomes. Nucleic Acids Res 1979, 6:3177–3197
11. Prosser J, Frommer M, Paul C, Vincent PC: Sequence relationship of three human satellite DNAs. J Mol Biol 1986, 187:145–155
12. Grady D, Ratliff RL, Robinson DL, McCanlies EC, Meyne J, Moyzis RK: Highly conserved repetitive DNA sequences are present at human centromeres. Proc Natl Acad Sci USA 1992, 89:1695–1699
13. Chronic Leukemia-Myeloma Task Force, National Cancer Institute: Proposed guidelines for protocol studies. II. Plasma cell myeloma. Cancer Chemother Rep 1973, 4:145–158
14. Durie BGM, Salmon SE: A clinical staging system for multiple myeloma: correlation of measured myeloma cell mass with presenting clinical features, response to treatment and survival. Cancer 1975, 36:842–854
15. Bladé J, San Miguel JF, Alcalá A, Maldonado J, Sanz

MA, García-Conde J, Moro MJ, Alonso C, Besalduch J, Zubizarreta A, Besses C, González Brito G, Hernández-Martín J, Fernández-Calvo J, Rubio D, Ortega F, Jiménez R, Colominas P, Faura MV, Font L, Tortosa J, Domingo A, Fontanillas M, Rozman C, Estapé C: Alternating combination VCMP/VBAP chemotherapy *versus* melphalan/prednisone in the treatment of multiple myeloma: a randomized multicentric study of 487 patients. J Clin Oncol 1993, 11:1165–1171

16. San Miguel JF, González M, Gascón A, Moro MJ, Hernández JM, Ortega F, Jiménez R, Guerras L, Romero M, Casanova F, Sanz MA, Sánchez J, Portero JA, Orfão A: Immunophenotype heterogeneity of multiple myeloma: influence on the biology and clinical course of the disease. Br J Haematol 1991, 77:185–190
17. Orfão A, García-Sanz R, López-Berges MC, Vidriales MB, González M, Caballero MD, San Miguel JF: A new method for the analysis of plasma cell DNA content in multiple myeloma samples using a CD38/propidium iodide double staining technique. Cytometry 1994, 17: 332–339
18. Tabernero MD, San Miguel JF, García VL, García-Isidoro M, Wiegnant J, Ciudad J, González M, Ríos A, Raap A, Orfão A: Clinical, biological and immunophenotypical characteristics of B-cell chronic lymphocytic leukemia with trisomy 12 by fluorescence *in situ* hybridization. Cytometry 1995, 22:217–222
19. Wiegant J, Ried T, Nederlof PM, Van der Ploeg M, Tanke HJ, Raap AK: *In situ* hybridization with fluoresceinated DNA. Nucleic Acids Res 1991, 19: 3237–3241
20. Arnoldus EPJ, Noordemeer IA, Peters ACB, Boormolen JHC, Bots TAM, Raap AK, Van der Ploeg M: Interphase cytogenetics of brain tumors. Genes Chromosomes & Cancer 1991, 3:101–107
21. Vindelov LL, Christehnsen IBJ, Jensen G, Nissen NI: Limits of detection of nuclear DNA abnormalities by flow cytometric DNA analysis: results obtained by a set of methods for sample storage, staining and internal standardization. Cytometry 1983, 3:332–338
22. Ferti A, Panani A, Arakapis G, Raptis S: Cytogenetic studies in multiple myeloma. Cancer Genet Cytogenet 1994, 12:247–253
23. Facon T, Laï JL, Nataf E, Preudhomme C, Zandecki M, Hammad M, Wattel E, Jouet JP, Bauters F: Improved cytogenetic analysis of bone marrow plasma cells after cytokine stimulation in multiple myeloma: a report of 46 patients. Br J Haematol 1993, 84:743–745
24. Weh HJ, Gutensohn K, Selbach J, Kruse R, Waker-Backhaus G, Seeger D, Fiedler W, Fett W, Hossfeld DK: Karyotype in multiple myeloma and plasma cell leukaemia. Eur J Cancer 1993, 29A:1269–1273
25. Gould J, Alexanian R, Goodrace A, Pathak S, Hecht B, Barlogie B: Plasma cell karyotype in multiple myeloma. Blood 1988, 71:453–456
26. San Miguel JF, García-Sanz R, González M, Moro MJ, Hernández JM, Ortega F, Borrego D, Carnero M, Casanova F, Jiménez R, Portero JA, Orfão A: A new staging system for multiple myeloma based on the number of S-phase plasma cells. Blood 1995, 85:448–455
27. Anastasi J, LeBeau MM, Vardiman JW, Fernald AA, Larson RA, Rowley JD: Detection of trisomy 12 in chronic lymphocytic leukemia by fluorescence *in situ* hybridization to interphase cells: a simple and sensitive method. Blood 1992, 79:1796–1801
28. Dohner H, Polh S, Bulgay-Mörschel M, Stilgenbauer S, Bentz M, Lichter P: Trisomy 12 in chronic lymphocytic leukemia: a metaphase and interphase cytogenetic analysis. Leukemia 1993, 7:516–520
29. Poddinghe PJ, Moesker O, Smeets D, Awwad BH, Ramaekers FC, Hopman AH: Interphase cytogenetics of hematological cancer: comparison of classical karyotyping and *in situ* hybridization using a panel of eleven chromosome-specific DNA probes. Cancer Res 1991, 51:1959–1967
30. Kibbelaar RE, van Kamp H, Dreef EJ, Wessels JW, Beverstock GC, Raap AK, Fibbe WE, den Ottolander GJ, Kluin PM: Detection of trisomy 8 in hematological disorders by *in situ* hybridization. Cytogenet Cell Genet 1991, 56:132–136
31. Drach J, Angerler J, Schuster J, Nowotny H, Scherrer R, Jäger U, Heizn R, Geissler K, Lechner K, Ludwig H, Huber H: Interphase cytogenetic studies in multiple myeloma and monoclonal gammopathy of undetermined significance. Blood 1994, 84:677
32. Facon T, Flactif M, Zandecki M, Bernardi F, Bauters F, Laï JL: Fluorescence *in situ* hybridization improves the detection of cytogenetic abnormalities in multiple myeloma. Blood 1994, 84:2076
33. Hirama T, Koeffler HP: Role of the cyclin-dependent kinase inhibitors in the development of cancer. Blood 1995, 86:841–854
34. Lee WH, Bookstein RE, Lee EYHP: Molecular biology of the human retinoblastoma gene. Tumour Supressor Genes. Edited by G. Klein. New York, Marcel Dekker, 1990, pp 169–199
35. Heim S, Mitelman F: Chronic lymphoproliferative disorders. Cancer Cytogenetics. Edited by R. Alan. New York, Liss, 1987, pp 175–180
36. Atkin NB: Chromosome 1 aberrations in cancer. Cancer Genet Cytogenet 1986, 21:279–285
37. Olah E, Balogh E, Kovaks I, Kiss A: Abnormalities of chromosome 1 in relation to human malignant disease. Cancer Genet Cytogenet 1989, 43:179–194
38. Façon T, Lai JL, Nataf E, Preudhomme C, Zandecki M, Hammad M, Wattel E, Jonet JP, Bauters F: Improved cytogenetic analysis of bone marrow plasma cells after cytokine stimulation in multiple myeloma. Br J Haematol 1993, 84:743–745
39. Hagemeijer A: Chromosome abnormalities in CML. Bailliere's Clinical Hematology: Chronic Myeloid Leukemia. Edited by JM Goldman. London, Bailliere's Tindall, 1987, pp 963–981
40. Nowell PC, Besa EC: Prognostic significance of single chromosome abnormalities in preleukemic states. Cancer Genet Cytogenet 1989, 42:1–7