Interphase Fluorescence In Situ Hybridization Identifies Chromosomal Abnormalities in Plasma Cells From Patients With Monoclonal Gammapathy of Undetermined Significance

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Karyotypic studies in patients with monoclonal gammapathy of undetermined significance (MGUS) have been hampered by a low percentage of bone marrow plasma cells (BMPC), which are predominantly nonproliferating. By combining cytomorphometry and interphase fluorescence in situ hybridization (FISH), we investigated whether or not chromosomal abnormalities occur in BMPC from patients with MGUS. Studying chromosomes 3, 7, 11, and 18, which we found to be frequently aneuploid by FISH in multiple myeloma (MM), we observed three hybridization signals for one of these chromosomes in 19 of 36 patients (52.8%). Gains of chromosome 3 were most common, occurring in 38.9% of patients, followed by gains of chromosomes 11 (25%), 7 (16.7%), and 18 (5.6%). Among BMPC, the frequency of aneuploid cells was 18.9% ± 13.9% (mean ± SD) for chromosome 3, 22.3% ± 9.2% for chromosome 11, 23.2% ± 22.0% for chromosome 7, and 6.1% ± 2.3% for chromosome 18. In five patients, chromosomal abnormalities were shown to be restricted to BMPC expressing cytoplasmic immunoglobulins corresponding to the serum paraprotein. No gain of hybridization signals was observed in normal and reactive plasma cells. In one patient with MGUS, metaphase cytogenetics revealed one abnormal metaphase with 47,XY,-4, and trisomy 4 was also demonstrated in a subpopulation of BMPC by interphase FISH. FISH results from patients with MGUS and newly diagnosed MM at stage IA (n = 14) indicated that aberrations involving ≥2 chromosomes occurred significantly more often in early stage MM (P < .01). With respect to clinical and laboratory features, MGUS patients with and without chromosomal abnormalities were indistinguishable. Our results indicate that MGUS already has the chromosomal characteristics of a plasma cell malignancy.

MATERIALS AND METHODS

Patients. Bone marrow (BM) specimens from 36 patients with MGUS were included in this study. Patients with MGUS fulfilled the diagnostic criteria (serum monoclonal protein less than 3 g/dL; less than 10% plasma cells in the BM; absence of bone lesions on radiographic bone survey; no anemia, renal failure, or hypercalcemia) as previously defined.2,12 Patients with MGUS had a minimum follow-up of 1 year with stable monoclonal protein levels in the serum or urine. In 33 patients with MGUS, the BM was obtained at initial presentation; 3 patients were studied at follow-up (23, 85, and 99 months after diagnosis). Types of the M-component were present in MGUS, we used interphase FISH to investigate copy numbers of chromosomes 3, 7, 11, and 18 in plasma cells from patients with MGUS. We decided to study these chromosomes because results from our previous FISH study9 as well as data from metaphase cytogenetics11 indicated frequent gains of these chromosomes in MM. Based on these observations we hypothesized that aberrations of one or more of these chromosomes should be detectable if chromosomal abnormalities were present in MGUS.

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IgG-kappa in 17 patients, IgG-lambda in 8 patients, IgA-kappa in 3 patients, and IgA-lambda in 5 patients; 1 patient had a biclonal paraprotein (IgA-kappa plus IgG-lambda), and 2 patients had laboratory findings consistent with idiopathic Bence-Jones proteinuria. We also studied 14 patients who had been diagnosed with MM at stage IA according to Durie and Salmon. From these patients, BM samples were obtained at the time of diagnosis.

BM aspirates were performed for diagnostic purposes, and informed consent following institutional guidelines was obtained for the use of an aliquot of the specimens for research purposes. BM aspirates were obtained from the posterior iliac crest of sternum and collected in a heparinized syringe. After dilution with phosphate buffered saline (PBS), mononuclear BM cells were separated by density gradient centrifugation over Ficoll-Hypaque (density = 1.077; Sigma, St Louis, MO). After 2 washes with PBS, cells were treated with Carnoy’s fixative [methanol/glacial acetic acid 3:1 (vol/vol)] and stored at -20°C.

**FISH studies.** BM cells were consecutively assessed for cytomorphology and interphase FISH using a modification of the technique published previously. Fixed BM cells were stained with Wright-Giemsa, and cells with the cytomorphologic appearance of plasma cells were documented. After hybridization, the same cells were relocated, and hybridization signals were specifically enumerated in plasma cells. For FISH, alpha-satellite DNA probes specific for chromosomes 3, 4, 7, 11, and 18 were used. The chromosome 3-specific probe (D3Z1, biotin-labeled) was purchased from Oncor (Gaithersburg, MD). Probes for chromosomes 7, 11, and 18 were directly labeled with fluorochromes (either Spectrum-green or Spectrum-orange; Imagingetics, Framingham, MA). In one patient with MGUS and in the normal controls, an alpha-satellite probe specific for chromosome 4 (biotin-labeled; Oncor) was used. Experiments were performed as two-color studies combining two DNA probes labeled with different fluorochromes. For prehybridization, slides were immersed in 0.1 N HCl/0.05% Triton-X-100, washed two times in saline sodium citrate (SSC) and PBS, and treated with formaldehyde (1% in PBS). After washes with PBS and 2× SSC, cells were dehydrated through 70%, 85%, and 100% ethanol. DNA was denatured by incubation with formamide (70% in 2× SSC) at 70°C for 5 minutes. Cells were again dehydrated through ethanol. Hybridization mixture (10 μL) was then applied to each slide, which was coveredslipped and sealed with rubber cement. Hybridization solution contained formamidene (65%; Sigma), 2× SSC, dextran sulfate (10%; Oncor), salmon sperm DNA (100 pg/mL; Sigma), and the specific probe (2 μg/mL). Hybridization was performed overnight at 37°C in a humidified chamber. Posthybridization washes consisted of three rinses in 50% formamide/2× SSC at 45°C and two rinses in 2× SSC at 37°C. To visualize the biotin-labeled probes, avidin-FITC was used. Finally, nuclei were counterstained with DAPI. Cells were analyzed under a fluorescence microscope (Olympus AN-3) equipped with a triple band-pass filter to simultaneously visualize DAPI, FITC/Spectrum-green and Spectrum-orange. Microphotographs were taken with Kodak Ektachrome 1600 color slide films (Eastman Kodak, Rochester, NY).

Our goal was to evaluate as many plasma cells as possible in the BM samples from patients with MGUS. To conclude that no aneuploidy was present, a minimum of 50 plasma cells with counting results below the cutoff level for trisomic cells was required. In many patients, multiple hybridization experiments were performed to obtain results from a sufficient number of plasma cells. In every patient, at least 100 cells with the nuclear morphology of mature myeloid cells and lymphocytes were evaluated as well.

In additional experiments, plasma cells were identified by staining for cytoplasmic immunoglobulins (clg). Staining was performed using goat anti-human antibodies specific for alpha, gamma, kappa, and lambda chains, respectively, of human immunoglobulins (purchased from Southern Biotechnology, Birmingham, AL). Following the manufacturer’s recommendations, 10⁶ fixed cells (Carnoy’s solution) were incubated with TRITC-labeled antibodies (final concentration 5 μg/mL) for 30 minutes on ice. After two washes with PBS, cytoplasmic signals were prepared, and FISH was performed according to the protocol described above. DNA probes were either directly labeled with Spectrum-green or, in case of the biotinylated probe for chromosome 3, detected with FITC-labeled streptavidin. Thus, clg-positive cells (red fluorescence of the cytoplasm) could be selectively analyzed for chromosomal aneuploidy (FISH signals appearing in green fluorescence).

**Statistical analysis.** The χ² test was used where appropriate, and differences between groups of patients were statistically analyzed by using Student’s t test.

**RESULTS**

**Definition of background.** Normal peripheral blood (n = 3) and BM (n = 6) cells were studied to determine the frequency of normal blood cells with three hybridization domains for each DNA probe. Results are summarized in Table 1. Cutoff levels for the diagnosis of trisomy were defined by adding three standard deviations to the mean of the frequency of these cells in normal specimens. Probes for chromosomes 7, 11, and 18 that were directly labeled with fluorochromes gave significantly lower background levels compared with the chromosome 3-specific probe (biotin-labeled, detection with streptavidin-FITC; Table 1). In normal controls, cells with 1 and 0 hybridization signals were below 5% and 1%, respectively, for each chromosome.

**Interphase FISH analysis of plasma cells from patients with MGUS.** Because of the low percentage of plasma cells in the BM from patients with MGUS, we sequentially performed cytomorphologic analysis and FISH to study chromosomal copy numbers in plasma cells. In our previous study, we observed that morphologic features of BM cells were retained after fixation with methanol/acetic acid. Fixed BM samples from patients with MGUS were stained with Wright-Giemsa to document cells with the typical morphology of plasma cells. After hybridization, the same cells were relocated and analyzed for chromosomal abnormalities by interphase FISH (Fig 1).

Studying BM samples from 36 consecutive MGUS patients with alpha-satellite probes for chromosomes 3, 7, 11, and 18, we observed a gain of copy numbers for at least one of the chromosomes in plasma cells from 19 patients (52.8%). Results are summarized in Table 2. A gain of chromosomal copy numbers was observed in all patients except one, in whom a gain was observed only on chromosome 18.
We also evaluated BM myeloid cells and lymphocytes from the patients with MGUS. Myeloid cells with three hybridization domains for any chromosome were not observed in any patients. Among lymphocytes, occasional cells with a gain of chromosomes similar to the findings in plasma cells were observed, the frequency of these cells, however, was below the cutoff level for trisomic cells.

**FISH analysis of normal and reactive plasma cells.** Because a subpopulation of plasma cells was aneuploid in MGUS, we evaluated plasma cells from three normal BM specimens and reactive plasma cells (two samples) as additional controls. Reactive plasma cells were obtained from a patient with cirrhosis of the liver (polyclonal hypergamaglobulinemia and plasmacytosis) and from a pleural effusion from a patient with pulmonary embolism. Using cytomorphology and FISH, 50 plasma cells from normal BM specimens and at least 100 reactive plasma cells were studied with each chromosome-specific DNA probe. In normal BM, we did not observe a single plasma cell with three hybridization signals in any experiment. In one experiment with reactive plasma cells, 1 of 137 cells (0.7%) had three signals for chromosome 3. Otherwise, no reactive plasma cells with gain of hybridization signals were found. We thus conclude

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**Fig 1.** Cytomorphology and interphase FISH of a bone marrow plasma cell from a patient with MGUS. The plasma cell was documented and relocated after hybridization with alpha-satellite DNA probes for chromosomes 11 (labeled with Spectrum-orange; red signals) and 7 (labeled with Spectrum-green; green signals). Three hybridization signals are present for chromosome 11. Nuclear DNA was counterstained with DAPI.

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**Fig 3.** Metaphase cytogenetics and interphase FISH analysis of bone marrow cells from a patient with MGUS. (A) G-banding revealed one abnormal metaphase with 47,XY,+4. (B) Interphase FISH with a chromosome 4-specific probe (biotin-labeled, visualized with avidin-FITC) shows three hybridization signals indicating trisomy 4 in a plasma cell. Two signals were detected with the chromosome 11-specific probe (labeled with Spectrum-orange).
We wanted to determine whether or not aneuploidy occurred and MI, only in plasma cells expressing cIg corresponding to the tion was addressed by the simultaneous analysis for cIg and serum paraprotein (cIg-restricted plasma cells). This ques-
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that the cutoff levels as defined with normal peripheral blood and BM cells (Table 1) are also appropriate for plasma cells.

FISH analysis of Ig-restricted plasma cells in MGUS.

We wanted to determine whether or not aneuploidy occurred only in plasma cells expressing cIg corresponding to the serum paraprotein (cIg-restricted plasma cells). This question was addressed by simultaneous analysis for cIg and interface FISH. Five patients were studied (patients HF, MI, KJ, SR, and RA from Table 2), and as shown in Fig 2, chromosomal aneuploidy was exclusively observed in the cIg-restricted plasma cell population. This technique was also applied to two patients with MGUS, in whom no aneu-

ploidy was detected by cytomorphology and FISH. The analysis of cIg-restricted plasma cells (IgG-lambda and IgA-

lambda, respectively) again provided no evidence for aneu-

ploidy.

By staining for kappa and lambda, predominance of light chain-restricted plasma cells was demonstrated in all patients with MGUS, regardless of whether aneuploidy was detected by FISH. We can thus rule out the possibility that failure to detect aneuploidy was caused by an unexpectedly low frequency of transformed plasma cells.

Metaphase cytogenetics and Interface FISH. In one patient with MGUS and disomic results for chromosomes 3, 7, 11, and 18 were obtained by interface FISH analysis of plasma cells, conventional cytogenetics (G-banding analysis) revealed one abnormal out of 20 analyzed metaphases showing 47,XY,+4. We reanalyzed this patient’s plasma cells by interface FISH using an alpha-satellite probe for chromosome 4 (Fig 3): By evaluating 143 plasma cells, a gain of

hybridization domains indicating trisomy 4 was observed in 6.9% (cutoff level for trisomic cells 1.0%).

Interphase FISH results in MM at stage IA. To investigate whether differences of chromosomal abnormalities exist between MGUS and early stage MM, we studied 14 patients with MM at stage IA for abnormalities of chromosomes 3, 7, 11, and 18. Eleven of 14 patients (78.6%) were found to have aneuploid plasma cells, which is more common than the aneuploidy rate observed in MGUS (52.8%), although the difference does not reach statistical significance. However, there were 9 of 14 early stage MM patients with aberrations of ≥2 chromosomes as opposed to only 8 of 36 patients with MGUS (P < .01). Figure 4 shows a comparison between early stage MM and MGUS with respect to the incidence of individual chromosomal abnormalities as well as the percentage of aneuploid plasma cells in cases with chromosomal abnormalities. In general, gains of the chromosomes under investigation were more common in early stage MM than in MGUS (Fig 4A), and for chromosome 3, the difference was statistically significant. However, the proportion of plasma cells with gain of hybridization signals for chromosomes 3, 7, and 11 was very similar between MGUS and early stage MM (Fig 4B). In MM, the percentage of plasma cells with gain of chromosome 18 was higher than in MGUS, but aneuploidy of chromosome 18 in MGUS was too infrequent to perform a meaningful statistical analysis.

Clinical and laboratory features of patients with MGUS. We compared MGUS patients with and without chromosomal abnormalities by interface FISH with respect to clinical and laboratory features. As shown in Table 3, no statisti-
Fig. 2. FISH analysis of plasma cells stained for cytoplasmic immunoglobulin (clg) expression (kappa and lambda light chains, gamma and alpha heavy chains). At least 100 clg-restricted plasma cells were analyzed, and 60 cells expressing clg not representing the serum paraprotein were evaluated. Patient initials correspond to patients in Table 2. Bars indicate the percentage of aneuploid cells by FISH (gain of hybridization signals for a particular chromosome as indicated by +3, +7, and +11, respectively) among clg-expressing plasma cells. It is evident that aneuploidy was detected only among clg-restricted plasma cells.

DISCUSSION

Metaphase cytogenetic studies in monoclonal gammopathies have been hampered by the low proliferative activity of the involved B-cell clone. Interphase FISH, which allows the assessment of chromosomal abnormalities in cells independent of their proliferative capacity, has greatly improved the detection of chromosomal abnormalities in MM. In this study, we investigated whether chromosomal abnormalities are also a characteristic of plasma cells in MGUS. The presence of aneuploid cells in MGUS has been previously suggested when cells with an abnormal DNA content were detected by flow cytometry in four of nine patients with MGUS. By using interphase FISH for chromosomes 3, 7, 11, and 18, we provide evidence that chromosomal aneuploidy is present in more than half of the patients with MGUS (52.8%). It is tempting to speculate that the proportion of MGUS patients with chromosomally abnormal plasma cells might be even higher if more FISH probes were used. Support for this hypothesis is provided by results from the patient in whom both metaphase and interphase cytogenetics demonstrated trisomy 4, but absence of abnormalities of chromosomes 3, 7, 11, and 18. It needs to be emphasized that the approach of using alpha-satellite DNA probes primarily allows the detection of numeric chromosomal aberrations, but it is not suited for the assessment of distinct structural aberrations involving these chromosomes. Specific translocations may be of greater biologic significance than numeric aberrations, analogous to the observations in other hematologic malignancies. Nevertheless, further studies of chromosomes 3, 7, and 11 as well as genes located on these chromosomes are warranted to determine the significance of the described chromosomal abnormalities in MGUS and MM.

It was a consistent finding in patients with MGUS that chromosomal abnormalities were confined to a subpopulation of BM plasma cells. This may, at least in part, be explained by the presence of both normal and transformed plasma cells in the bone marrow of patients with MGUS. Simultaneous analysis of clg and FISH demonstrated that chromosomal abnormalities were present only in the clg-restricted plasma cells. It is still worth noting that the clg-restricted plasma cell population consisted of both disomic and aneuploid cells. Chromosomal abnormalities in a distinct
population of plasma cells in MGUS could also indicate that the described aberrations are secondary changes occurring as a consequence of a yet unidentified primary transforming event. The observation of aneuploidy in subpopulations of plasma cells also in early stage MM is in agreement with this interpretation. Plasma cells from patients with MM at stage I, however, tend to have abnormalities of two or more chromosomes, a finding that is rather infrequent in MGUS. Although we did not have consecutive samples from patients that progressed from MGUS to MM, this result is consistent with the view that accumulation of chromosomal abnormalities parallels disease progression and/or transformation.

For a long time, MGUS was considered as a "benign" condition. After an appropriately long follow-up period, however, it has become clear that MGUS may progress to MM or other lymphoproliferative disorders in a significant proportion of patients. Based on the results presented in this report we conclude that MGUS has the chromosomal characteristics of a plasma cell malignancy, which manifests itself as a symptomatic disease only in some patients. It is unclear which factors contribute to the long clinical stability observed in patients with MGUS. At present, it also remains unclear whether the detection of chromosomal abnormalities will help identify MGUS patients with increased risk of progression to MM. At the time of this analysis, MGUS patients with and without chromosomal abnormalities by FISH were indistinguishable with respect to clinical and laboratory features. Prospective follow-up is needed to determine the clinical value of cytogenetic abnormalities in monoclonal gammopathies.

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