Fluorescence *in Situ* Hybridization Analysis of Immunoglobulin Heavy Chain Translocations in Plasma Cell Myeloma Using Intact Paraffin Sections and Simultaneous CD138 Immunofluorescence

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Fluorescence *in situ* hybridization (FISH) studies are much more sensitive than classical cytogenetics for identification of karyotypic abnormalities in plasma cell myeloma. However, FISH analysis of bone marrow samples is often challenging because of a large number of admixed non-neoplastic hematopoietic elements. In this report, we describe a novel method using FISH analysis of intact paraffin sections of formalin-fixed, bone marrow clot preparations with simultaneous CD138 tyramine signal amplification (TSA)-mediated immunofluorescence. We studied 22 cases of plasma cell myeloma for translocations involving the immunoglobulin heavy chain locus that are of known diagnostic and/or prognostic significance. All cases were analyzed using dual color, break-apart immunoglobulin heavy chain probe and dual color, dual fusion probes for t(11;14)(q13;q32) and t(4;14)(p16;q32). TSA-mediated fluorochrome deposition in CD138+ cells was unaltered by protease pretreatment. Translocations were identified in 10 cases, including five with t(11;14)(q13;q32) and three with t(4;14)(p16;q32). When present, abnormalities were identified in a large percentage of CD138+ cells (47 to 93%, median 84%). This technique allows for efficient molecular cytogenetic analysis of plasma cell myeloma using routinely archived paraffin-embedded material. (J Mol Diagn 2006, 8:459–465; DOI: 10.2353/jmoldx.2006.050149)

Conventional cytogenetics and fluorescence *in situ* hybridization (FISH) studies of plasma cell myeloma (PCM; also known as multiple myeloma) have identified a wide variety of recurring numerical and structural karyotypic abnormalities.^{1–4} Approximately 40 to 60% of PCM cases are associated with translocations involving the immunoglobulin heavy chain (*IGH*) locus at chromosome 14q32 and one of several partner genes including *CCND1*

(11q13), *MMSET* (4p16), *CCND3* (6p21), *CMAF* (16q32), and *MAFB* (20q11), among others. Cases of PCM lacking an *IGH* translocation are usually associated with hyperdiploid karyotypes.^{5,6}

In recent years, it has been shown that several of the recurring *IGH* translocations are associated with distinct clinical or pathological features. For example, the t(4;14)(p16;q32), which dysregulates both *FGFR3* and *MMSET* genes, is associated with an adverse prognosis.^{7–9} In contrast, the t(11;14)(q13;q32), involving *IGH* and *CCND1*, is associated with either a neutral or slightly improved prognosis^{7–9} and is frequently associated with lymphoplasmacytic morphology and CD20 expression, leading to a differential diagnosis with non-Hodgkin's lymphomas with plasmacytic differentiation.^{10–12} Identification of these recurring translocations in routine evaluation of PCM therefore provides important information that may assist in the diagnosis and prognostic assessment of such cases. However, techniques for identification of these abnormalities using FISH in formalin-fixed, paraffin-embedded material have not previously been described.

In this report, we describe a novel method of interphase FISH analysis of PCM, using intact paraffin sections of formalin-fixed, bone marrow clot preparations with simultaneous CD138 immunofluorescence. The use of a tyramide signal amplification (TSA) protocol produces a CD138 immunofluorescent label that is highly resistant to proteinase digestion, allowing for optimization of FISH probe hybridization without loss of CD138 signal. This protocol provides several distinct advantages for the molecular cytogenetic evaluation of PCM in routine bone marrow evaluation.

Materials and Methods

Case Selection

Cases of plasma cell myeloma with plasma cells present in a formalin-fixed, paraffin-embedded clot preparation were identified from the case files of the Cleveland Clinic

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Foundation. The aspirate smears and routine hematoxylin and eosin sections of the bone marrow core biopsies and clot sections were reviewed. Differential counts were performed on the aspirate smears in all cases. The diagnosis of plasma cell myeloma was based on World Health Organization criteria.¹³ Twenty of the 22 cases were previously included in a prior study of plasma cell myeloma.¹⁰ This study was approved by the Institutional Review Board of the Cleveland Clinic Foundation.

Fluorescence in Situ Hybridization with Simultaneous CD138 Immunofluorescence

Each case was analyzed using three probes: a dual color, break-apart probe for *IGH* translocations; a dual color, dual-fusion probe for t(11;14)(q13;q32); and a dual color, dual-fusion probe for t(4;14)(p16;q32) (all from Vysis, Downers Grove, IL). Paraffin section interphase FISH studies with simultaneous immunofluorescence was performed using a modification of the immuno-FISH protocol described elsewhere.¹⁴ FISH studies were performed on 5- μ m paraffin sections of formalin-fixed, bone marrow clot preparation with simultaneous CD138-immunofluorescence with tyramide signal amplification (TSA/Alexa 350 kit #7; Molecular Probes, Eugene, OR). Each case was less than 2 years old at the time of analysis. Paraffin slides were baked at 60°C overnight. Slides were deparaffinized by three immersions in xylene for 5 minutes each, followed by two 1-minute immersions in 100% alcohol, two 1-minute immersions in 95% alcohol, and a final 5-minute immersion in molecular grade Milli-Q water (Millipore, Billerica, MA). Slides were heated to 95°C for 40 minutes and allowed to cool for 20 minutes at room temperature. Slides were rinsed three times for 5 minutes each in PBS/0.1% Tween 20. Fifty microliters of 1:20 CD138 antibody (clone BB4; Serotec, Raleigh, NC) was added with incubation for 1 hour in the dark. Slides were washed three times in PBS/0.1% Tween 20. Blocking reagent was added (100 μ l/slide) with incubation in a humidified box for 30 minutes at room temperature. One-hundred microliters of horseradish peroxidase solution was added with a 30-minute incubation at room temperature in a humidified box. Slides were washed three times in PBS/1% Tween 20. One-hundred microliters of tyramide solution was added per slide followed by incubation for 10 minutes at room temperature in the dark. Slides were washed three times. One microliter of proteinase K stock concentrate (Dako, Carpinteria, CA) was added to 5 ml of 50 mmol/L Tris/HCl, pH 7.6, and 150 μ l of the diluted protein K solution was added to the slide. The tyramine amplified signal was resistant to proteinase K digestion, and no loss of CD138 signal intensity was observed with proteinase K digestion times up to 22 minutes. An optimal proteinase K digestion time was therefore established for each specific probe set used to provide maximal probe signal intensity without compromising CD138 staining. Slides were incubated at room temperature for 16 minutes [for t(11;14) and t(4;14) probes] or 19 minutes (*IGH* probe). Slides were washed for 5 minutes in Milli-Q water,

Figure 1. CD138/FISH analysis of *IGH* translocations in CD138+ cell lines. The specificity of the FISH probes in intact paraffin sections was confirmed using the t(11;14)(q13;q32)-positive JVM2 cell line and the t(4;14)(p13;q32)-positive OPM2 cell line. Dashed lines indicate cutoff thresholds for interpretation as a positive result.

dehydrated in alcohol, and allowed to completely air dry. One microliter of probe was added to 2 μ l of water and 7 μ l hybridization buffer (Vysis) in a microcentrifuge tube. Ten microliters of probe solution was added to each slide, and a coverslip was sealed over the slide with rubber cement. The probe and target DNA were allowed to codenature at 73°C for 5 minutes followed by hybridization overnight at 37°C. Next, slides were soaked in 2 $\times$ sodium chloride/sodium citrate (pH 7.0) for 5 minutes at room temperature and coverslips removed. Slides were soaked in 2 $\times$ sodium chloride/sodium citrate/0.1% NP40 for 3 to 5 seconds, rinsed in 2 $\times$ sodium chloride/sodium citrate and then water, and allowed to air dry completely in the dark. Signals were visualized on an Axioskop photomicroscope (Zeiss, Oberkochen, Germany).

Plasma cells were specifically identified by cytoplasmic CD138 staining under 4'-6-diamidino-2-phenylindole filter. One-hundred to 200 plasma cells were counted. For dual fusion probes, nuclei were scored as positive (at least one fusion signal present) or negative (all other patterns). For the *IGH* break-apart probe, nuclei were scored as positive (separate red and green signals at least three signal widths apart) or negative. As negative controls, intact paraffin sections of five cases of reactive lymphoid hyperplasia were analyzed. Cutoff thresholds for interpretation as a positive result were established at four standard deviations above the mean of the negative controls, yielding cutoffs of >5% for the *IGH* and t(11;14)(q13;q32) probes and >10% for the t(4;14)(q13;q32) probe.

Conventional Cytogenetics

Classical cytogenetic studies of the bone marrow were performed at the Cleveland Clinic Foundation ($n = 7$) or at an outside reference laboratory (Dynagene, Houston, TX) ($n = 15$). For cases performed at the Cleveland Clinic Foundation, samples were cultured for 24 hours without mitogens. Parallel cultures stimulated with granulocyte-macrophage colony stimulating factor, tetradecanoyl phorbol 12-myristate 13-acetate, or conditioned medium III (derived from human bladder carcinoma cell line 5637) were also used. In cases analyzed at the reference laboratory, 48-hour unstimulated or 72-hour cultures stimu-

Figure 2. Paraffin section FISH analysis with CD138 immunofluorescence. Under a 4'-6-Diamidino-2-phenylindole filter, CD138+ plasma cells are identified by their intense blue cytoplasmic staining. Other bone marrow elements remain unlabeled.

lated with lipopolysaccharide were used. Chromosomes were identified by trypsin-Giemsa banding and classified according to the International System for Cytogenetic Nomenclature.¹⁵

Results

FISH Analysis of IGH Translocations in CD138+ Cell Lines

We first confirmed the utility of the *IGH*, t(11;14)(q13;q32), and t(4;14)(p16;q32) probes in intact paraffin sections by analyzing formalin-fixed, paraffin-embedded cell block preparations of two well-characterized CD138+ cell lines. The JVM2 cell line is positive for the t(11;14)(q13;q32)¹⁶ whereas the OPM2 cell line is known to contain the t(4;14)(p16;q32).^{17,18} As expected, the JVM2 cell line was positive using the *IGH* break-apart

probe and the t(11;14)(q13;q32) probe but negative for t(4;14)(p16;q32), and the OPM2 cell line was positive using the *IGH* break-apart and t(4;14)(p16;q32) probes but negative for t(11;14)(q13;q32) (Figure 1).

FISH Analysis of IGH Translocations in Plasma Cell Myeloma

FISH studies were performed next on 22 cases of plasma cell myeloma (Figures 2 and 3). The percentage of plasma cells ranged from 6 to 88% (median 51%). The number of CD138+ cells observed by immunofluorescence was consistent with the plasma cell percentage identified in aspirate smears. Ten cases (45%) were positive for *IGH* translocations, as detected by the *IGH* break-apart probe. Analysis using the translocation-specific probes identified five of these cases as t(11;14)(q13;q32) and three cases as t(4;14)(p16;q32) (Table 1). In the remaining two *IGH* translocation-positive cases, the fusion partner genes could not be identified. Each of the cases containing t(11;14)(q13;q32) or t(4;14)(p16;q32) were also positive using the *IGH* break-apart probe. When present, translocations were noted in a large percentage of the CD138+ plasma cells counted (47 to 93%, median 84%), regardless of the overall percentage of plasma cells in the bone marrow (Figure 4).

Comparison with Classical Cytogenetic Analysis

Following classical cytogenetic studies, 10 of the cases displayed abnormal karyotypes, seven of which were hyperdiploid. None of the 10 cases positive for *IGH* rearrangements by FISH showed evidence of 14q32 abnormalities by classical cytogenetics (Table 1).

Discussion

For many years, classical cytogenetic studies have played an important role in the diagnosis, classification,

Figure 3. FISH analysis of *IGH* translocations in plasma cell myeloma. **A:** Analysis with a break-apart probe spanning the *IGH* locus identifies one pair of juxtposed red (centromeric) and green (telomeric) signals, and one pair of split red and green signals consistent with an *IGH* translocation. **B:** Further analysis with a dual fusion probe for t(11;14)(q13;q32) identifies two separate red (*CCND1*) and green (*IGH*) signals, indicating absence of the *IGH/CCND1* translocation. **C:** A dual fusion probe for t(4;14)(p13;q32) shows separate red (*MMSET*) and green (*IGH*) signals, and two juxtposed (fusion) signals, identifying this *IGH* translocation as t(4;14)(p13;q32).

Table 1. FISH and Classical Cytogenetic Studies in Plasma Cell Myeloma

% Bone marrow plasma cells	IGH FISH (% nuclei)	t(11;14) FISH (% nuclei)	t(4;14) FISH (% nuclei)	Cytogenetics
10	Pos (83%)	Pos (87%)	Neg (2%)	46,XY[20]
14	Pos (68%)	Pos (62%)	Neg (0%)	46,XY[20]
60	Pos (78%)	Pos (86%)	Neg (0%)	46,XX[19]
44	Pos (88%)	Pos (89%)	Neg (0%)	46,XY[20]
80	Pos (90%)	Pos (93%)	Neg (0%)	46,XX[20]
20	Pos (73%)	Neg (0%)	Pos (76%)	46,XX[20]
40	Pos (85%)	Neg (0%)	Pos (83%)	46,XX[20]
80	Pos (89%)	Neg (0%)	Pos (62%)	46,XY[20]
30	Pos (87%)	Neg (3%)	Neg (9%)	45,XY,add(1)(p22), -20[2]/46,XY[18]
60	Pos (47%)	Neg (1%)	Neg (3%)	50-54,XY,+3,add(4)(p1?2),+5,+add(7)(q3),add(8)(p23),+11,del(12)(q12q21),-13,-13,+15,+15,del(17)(p11.2),+add(19)(q12),+22,+22,+2-3mar[cp4]/46,XY[16]
49	Neg (0%)	Neg (0%)	Neg (0%)	55,X,-X,del(1)(p22p33),+3,+5,+7,+9,+9,+11,+15,+15,+21,+22[5]/46,XX[15]
80	Neg (0%)	Neg (0%)	Neg (0%)	46,XY[20]
33	Neg (3%)	Neg (1%)	Neg (3%)	46,XY[20]
53	Neg (0%)	Neg (0%)	Neg (0%)	56-57,XX,add(6)(q15),+add(6),+7,+9,add(10)(q26),+11,+14,+15,+15,+19,+21,+22,+mar[cp8]/46,XX[12]
88	Neg (0%)	Neg (4%)	Neg (0%)	44-46,X,-X,del(1)(p13p22),-2,+add(3)(p12),der(4)t(2;4)(q14.1;q35),del(5)(q31q33),add(6)(p23),-8,+9,del(11)(q14),+add(11)(q23),+14,add(14)(p11.2),der(14)t(X;14)(q13;p13),add(17)(q21),+18,i(18)(q10),-20,-21,add(22)(q11.2),+1-2mar[cp16]/46,XX[1]
70	Neg (2%)	Neg (0%)	Neg (0%)	50-52,X,-Y[6],+3[6],add(4)(p16)[6],+7[6],add(8)(p23)[4],+9,+11[5],+15[5],-16[6],+19[6],+19[6],-21[6],+2mar[6]/cp6/46,XY[14]
74	Neg (4%)	Neg (0%)	Neg (0%)	52,X,-X,add(2)(q37),+3,+5,t(8;9)(p21;q13),+9,+11,+15,+19,+21[2]/51-53,idem,-19[2],-22[1],+1-3mar[3]/cp3/46,XX[15]
70	Neg (0%)	Neg (0%)	Neg (0%)	49-50,XY,add(1)(p22),+3,der(5)t(5;22)(q3?3;q11.2),+7,dic(7;16)(q36;p13.3),del(8)(p21),+9,+11,+der(11)del(11)(q13q14),del(11)(q24),der(12;22)(q10;q10),+15,+15,-22[cp5]/46,XY[15]
53	Neg (0%)	Neg (0%)	Neg (0%)	53-54,X,+X,-Y,dic(1;?)(p12;?),+3,+7,del(8)(p11.2p21),+9,-10,+11,+15,+add(16)(q22),add(18)(q23),+add(19)(q13.1),+21,-22,+1-2mar[cp3]/46,XY[17]
47	Neg (1%)	Neg (0%)	Neg (0%)	43-45,XY,-Y[5],+add(1)(p12)[7],del(3)(p13p21)[7],-4[7],add(7)(q22)[5],-8[5],-10[7],-14[6],add(15)(p11.2)[7],add(16)(q12.1)[6],add(22)(q11.2)[7],+mar[7]/cp7/46,XY[13]
20	Neg (0%)	Neg (0%)	Neg (0%)	46,XX[20]
6	Neg (0%)	Neg (0%)	Neg (0%)	46,XY[20]

and prognostic assessment of acute leukemias and non-Hodgkin's lymphomas.¹⁹⁻²¹ In contrast, the evaluation of plasma cell myeloma by conventional cytogenetics has been problematic. Because of poor *in vitro* proliferation of

malignant plasma cells, conventional cytogenetic studies demonstrate an abnormal karyotype in only approximately one-third of cases of plasma cell myeloma, despite flow cytometric and FISH evidence that cytogenetic abnormalities are nearly universal in PCM.²⁻⁴ Moreover, even when abnormal karyotypes are obtained, conventional cytogenetics may not be sufficient to detect clinically significant abnormalities. For example, the t(4;14)(p16;q32), which is associated with a poor prognosis, is cryptic by classical cytogenetics and requires molecular techniques for identification.^{17,22,23} The t(14;16)(q32;q23), which also appears to be associated with an adverse prognosis, may also be difficult to detect by classical cytogenetics.^{9,24} For these reasons, FISH analysis has emerged as the technique of choice for evaluation of cytogenetic studies in PCM.

FISH studies performed on bone marrow aspirates involving plasma cell myeloma are technically challenging, however, because the neoplastic plasma cells may represent only a small proportion of the total nuclei

Figure 4. Percentage of abnormal CD138+ nuclei in translocation-positive myeloma. When abnormalities were present, they were seen in a large percentage of CD138+ nuclei scored (median = 84%). The percentage of abnormal CD138+ nuclei seen was similar across the range of bone marrow plasma cell counts tested.

present. To overcome this difficulty, several techniques have been advocated. Some studies have performed FISH on cytologic preparations of bone marrow aspirate with simultaneous immunofluorescence for cytoplasmic kappa or lambda immunoglobulin light chains to allow for selective analysis of monotypic plasma cells ("clg-FISH").^{6,9,25} Others have used antibody-based sorting techniques to purify plasma cells from the bone marrow aspirate before FISH analysis.^{7,26,27} Recently, another technique was proposed using an initial May-Grunwald Giemsa stain with mapping of plasma cells by image analysis software, followed for subsequent FISH analysis of the previously mapped plasma cells.²⁸ However, cell sorting and image analysis techniques both involve cumbersome protocols not routinely performed in most pathology laboratories. Importantly, none of the previously published techniques have been applicable to routinely archived, formalin-fixed, paraffin-embedded material.

In this study, we have described a novel technique for FISH analysis of plasma cell myeloma using intact paraffin sections of formalin-fixed, bone marrow clot preparations and simultaneous CD138 immunofluorescence. The use of combined immunofluorescence and FISH was reported by Weber-Matthiesen et al in 1992 as a procedure termed FICTION (fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms).²⁹ This procedure has been applied to a wide variety of materials including cytologic preparations, peripheral blood, and both frozen and paraffin sections.³⁰⁻³³ Traditional FICTION techniques have been somewhat difficult to employ in formalin-fixed, paraffin-embedded tissue sections because efficient probe hybridization to target DNA requires removal of interfering fixed proteins and reagent proteins deposited during an immunofluorescence step if used in the procedure before *in situ* hybridization. Proteinase treatment to optimize FISH probe hybridization, however, also leads to removal of the protein antigen being stained by immunofluorescence or the antigen/antibody complex. Several protocols have been described in the literature in an attempt to "balance" the need for efficient FISH probe hybridization while retaining immunofluorescent signal.^{34,35} In the current protocol, the immunofluorescent staining is produced using a tyramide-signal amplification protocol that results in a proteinase-resistant signal. Proteinase treatment can therefore be altered as needed to produce bright FISH probe signals without diminution of CD138 signal.

Using this approach, analysis of 22 cases of PCM identified *IGH* translocations in ten cases (45%), including five cases with t(11;14)(q13;q32) (23%), three with t(4;14)(p16;q32) (14%), and two with as yet unidentified translocation partner genes. The incidence of each of these abnormalities is consistent with that reported by others using clg-FISH or purified plasma cell preparations.^{6,7,9,27,36} While ten of the cases showed abnormal karyotypes by classical cytogenetic studies, none of the *IGH* translocations were identified by this technique, consistent with the known superiority of FISH for detection of these abnormalities. Scoring of only CD138-positive plasma cells allows for efficient detection of myeloma-

associated abnormalities, which may be demonstrated in a very high proportion of plasma cells counted (median 84%), even when the overall percentage of plasma cells in the marrow is low. This technique offers several advantages over other proposed protocols. Unlike techniques using cytoplasmic immunoglobulin light chain staining, prior knowledge of the restricted light chain involved is not required. Specialized procedures such as cell sorting or computerized image analysis are unnecessary. Most importantly, this technique is readily used to routinely archived, formalin-fixed, paraffin-embedded bone marrow clot preparations. This method therefore allows for not only prospective analysis but also retrospective studies of previously archived paraffin-embedded material.

It should be noted that this technique has been developed for the molecular cytogenetic assessment of specimens that are clearly diagnostic of plasma cell myeloma (either newly diagnosed or relapsed/refractory) using standard morphological and immunophenotypic criteria. Because each of the myeloma-associated *IGH* translocations are also found in monoclonal gammopathy of undetermined significance,^{2,37} the identification of a small percentage of cells with these abnormalities in specimens that are not clearly diagnostic of myeloma by standard criteria would be of little diagnostic utility. In addition, this protocol was not intended for analysis of minimal residual disease, and we have not evaluated the potential of this technique in that setting. Because FISH analysis in intact paraffin sections typically requires higher cutoff thresholds than those procedures using whole cell suspensions or isolated intact nuclei, it is likely that paraffin section material would not be optimal for minimal residual disease analysis.

These results demonstrate the practical utility of an initial screening approach with a break-apart probe to detect *IGH* translocations in routine practice. Overall, approximately 40 to 60% of cases will be expected to contain an *IGH* translocation.¹⁻⁴ Cases lacking an *IGH* translocation have been shown to be associated with hyperdiploid karyotypes, and some studies have reported that *IGH* translocation-negative cases are associated with a superior prognosis.^{5,6,36} The demonstration of an absence of *IGH* translocations therefore provides potential prognostic information. Cases found to harbor an *IGH* translocation could be reflexed to further analysis using dual fusion probes specific to t(11;14)(q13;q32) and t(4;14)(p16;q32), the two most common recurring *IGH* translocations. If this analysis indicated the *IGH* translocation did not represent either of these abnormalities, additional studies for less common abnormalities could then be performed, such as t(14;16)(q32;q32) or others. This approach allows for efficient identification of these abnormalities while keeping the number of FISH studies performed minimized whenever possible.

In conclusion, FISH with simultaneous TSA-mediated immunofluorescence is a valuable technique for analysis of *IGH* translocations in PCM using routinely archived paraffin-embedded material. This technique may be readily adapted to include detection of other clinically significant abnormalities in plasma cell myeloma (such as other *IGH* translocations, chromosome 13q abnormali-

ties, or 17p abnormalities). Similarly, FISH with simultaneous TSA-mediated immunofluorescence could also be applied to essentially any type of paraffin-embedded tissues where immunophenotype-genotype correlation is desired.

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