Technical Advance

Successful Application of a Direct Detection Slide-Based Sequential Phenotype/Genotype Assay Using Archived Bone Marrow Smears and Paraffin Embedded Tissue Sections

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Identification of genetic abnormalities in pathological samples is critical for accurate diagnosis, risk stratification, detection of minimal residual disease, and assessment of response to therapy. Interphase fluorescence *in situ* hybridization analysis is the standard cytogenetic assay used by many laboratories to detect specific clonal karyotypic aberrations in formalin-fixed, paraffin-embedded tissue. However, direct correlation with immunophenotype or morphology in individual cells is rarely performed because the procedural steps are labor intensive and usually require extensive troubleshooting. In this study, we present a sequential fluorescence *in situ* hybridization-based technique that uses the identical archived bone marrow smears or paraffin-embedded tissue sections previously evaluated by a pathologist for morphological or immunohistochemical characteristics. This approach is relatively straightforward, using uncomplicated pretreatment and hybridization conditions and basic equipment attached to an automated image analyzer with image capture software to record the location of targeted cells for genotypic/phenotype correlation. Furthermore, the method has proved reliable and reproducible on test samples regardless of specimen age, tissue type, or referring institution. (J Mol Diagn 2007, 9:589–597; DOI: 10.2353/jmoldx.2007.070050)

Recent development of targeted molecular therapies has increased the importance of reliable and standardized methods for the identification of genetic abnormalities that are critical for diagnosis, risk stratification, and as-

essment of response to therapy in neoplastic disorders. Conventional cytogenetics remains the “gold standard” assay for detecting acquired karyotypic aberrations in oncology. However, this approach is limited by the availability of fresh tumor material, the growth characteristics of the tumor *in vitro*, and the mitotic index. Fluorescence *in situ* hybridization (FISH) can address these limitations by hybridizing appropriate DNA probes to complementary DNA sequences to detect specific cytogenetic aberrations in interphase nuclei from archived formalin-fixed, paraffin-embedded (FFPE) or frozen tissue samples. Despite these advances, cell morphological and immunological characteristics are lost because the standard conventional cytogenetics and FISH protocols destroy key identifying cell membrane and cytoplasmic features.¹ This challenge limits our ability to consistently detect minimal disease based on recognition of often rare tumor cells by their characteristic phenotype and genotype. Methods for simultaneous or sequential immunohistochemistry (IHC) or morphology/FISH analyses on fresh tissue are well established^{2–8}; however, fresh tumor tissue is not always available for testing, especially in second opinion consultation cases.

Advances in computer and microscope technology have facilitated combinations of molecular cytogenetics and established pathology techniques that use either Romanowsky-stained blood and bone marrows films (ie, Wright-Giemsa and May-Grünwald Giemsa) or various immunocytochemical stains.^{2–10} In addition, automated image capture has lessened tedious, manual cell localization, allowing technical support to focus on assay optimization and tumor cell-specific characterization. In this manuscript, we describe successful methods for optimi-

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zation of sequential IHC or morphology/FISH analyses using archived bone marrow smears or formalin-fixed paraffin-embedded tissue sections obtained from 32 patients.

Materials and Methods

Patient Samples

A feasibility study was conducted to develop a reliable and reproducible method for sequential morphology or IHC and FISH using archived material. This study was approved by the Institutional Review Board of the City of Hope National Medical Center. The samples chosen for this assay were collected from a variety of medical institutions between 1992 through 2006. Examples from seven illustrative patients are presented in detail.

Bone Marrow Smears

Eleven archived Wright-Giemsa bone marrow smears were obtained, including six multiple myeloma smears and one smear of each of the following diseases: acute lymphoblastic lymphoma (ALL), acute myeloid leukemia (AML) with atypical nodular T-cell aggregates, therapy-related AML 3 years after treatment for breast cancer, AML with systemic mastocytosis, and chronic myeloid leukemia.

The slides were scanned initially on a Bioview Duet image analyzer (Bioview, Rehovot, Israel) using a software algorithm designated IHCx20_TS. This tracking/classifying software automatically photographs the slide and records the coordinates for the user-defined region on the slide. Although the software can be optimized to identify particular cell phenotypes, we chose to focus on manually selected specific cells that best represented the morphology of the diseases under study. Plasma cells, mast cells, T-cell aggregates, and myeloid cells were individually reviewed and classified. After cell localization was complete, the archived smears were placed in xylene to remove the coverslip and mounting media and allowed to air dry. Coverslip removal took 72 to 96 hours, depending on the age of the slide and the original slide processing. Slides were immersed in methanol, incubated in 2× standard saline citrate (SSC), and digested with pepsin/10 mmol/L HCL solution at a concentration of 0.05 mg/ml at 37°C for 10 minutes. Slides were then fixed in 1% formaldehyde, rinsed in 1× PBS, and dehydrated. At this point, the smears were evaluated under a phase contrast microscope to check for adequate removal of the red blood cells. If red blood cells remained, additional pepsin treatments for 5 minutes at a time were repeated until the red blood cells were adequately digested.

FISH Studies

Probes used for FISH analyses included the LSI IGH dual color break apart (*IGH@*), LSI D5S23/D5S721/CEP 9/CEP 15 triple color probe set, the dual color, dual fusion LSI

AML1/ETO (*RUNX1/RUNX1T1*), the CEP X α -satellite/CEP Y α -satellite, the LSI MLL dual color rearrangement probe, the CEP 8/LSI C-MYC, the LSI D7S486 (7q31)/CEP 7, and a custom-made triple color BCR/ABL1/ASS probe set [all obtained from Abbott Molecular (Des Plaines, IL)]. In addition, the LSI D20S108 (20q12) probe (Abbott Molecular) was combined with a noncommercial, "home-brew" probe RP11-11O15 (BACPAC Resources, Oakland, CA) that maps to band 20p12, for a dual color chromosome 20 probe set. All probes were validated, and their sensitivity and specificity were determined per standard procedure.¹¹ Specific cutoff values, confidence intervals, and control cell lines used for each probe are provided in Table 1. The automated platform, detailed biostatistical methods, and sensitivity based on dilution experiments (spiking a known number of cancer cells into a normal cell population) of the T-FISH assay have been described previously.⁷

To detect an *IGH@* translocation or hyperdiploidy in multiple myeloma, 15 μ l of the LSI *IGH* break-apart probe or the LSI D5S23/D5S721/CEP 9/CEP 15 multicolor probe set was applied to each bone marrow smear, coverslipped, and sealed with rubber cement. All slides were co-denatured for 5 minutes on an 80°C hotplate. Probes were prepared and applied according to manufacturer's direction. The slides were placed immediately in a humidified chamber for 24 to 48 hours. Postwashing was slightly modified as follows. The slides were immersed in 2× SSC/0.1% Igepal (Sigma-Aldrich, St. Louis, MO) at 72°C for 2 minutes, transferred to 2× SSC/0.1% Igepal at room temperature for 2 minutes, and allowed to air dry. Ten microliters of 4'-6-diamidino-2-phenylindole 2HCL (Bioview) was applied to the slides and covered with a 22 × 50 coverslip.

Formalin-Fixed, Paraffin-Embedded Sections

FFPE sections (5- μ m thickness) from 21 malignant lymphoma sections were cut and transferred to glass slides. IHC was performed using a CD20 monoclonal antibody (DAKO, Carpinteria, CA) according to standard methods, with EDTA-based high-temperature and pressure antigen retrieval, and detected with 3-amino-9-ethyl carbazole. The slides were coverslipped using aqueous mounting medium (Biomedex Corp., Foster City, CA) before being scanned on the Bioview Duet Image analyzer (Bioview) using the IHCx20_TS- software task. On completion of the scan, slides were immersed in room temperature water for 15 minutes to remove coverslips. If the coverslips failed to come off during the room temperature incubation, the slides were placed in water at 72°C for 15 minutes. All slides were air dried and placed in Carnoy's fixative (3:1 MeOH:acetic acid) for 1 hour, air-dried for 1.5 hours at room temperature, 2× SSC at 37°C for 10 minutes, and immediately transferred to a 1% pepsin solution at 37°C for 10 minutes. Slides were washed in 1× PBS for 5 minutes and dehydrated. Digestion was evaluated with 4'-6-diamidino-2-phenylindole counterstain. None of the lymphoma FISH slides required additional pepsin treatment.

Table 1. Sensitivity, Specificity, and Cell Line Controls for FISH Probes Used

Probe	Cutoff for negativity (%)	% Sensitivity (95% CI)	% False negative (95% CI)	% Specificity (95% CI)	% False positive (95% CI)	Control cell line*
LSI IGH dual color break-apart probe	≤2	100 (76.8 to 100)	0 (0 to 23.2)	100 (69.2 to 100)	0 (0 to 30.8)	JVM-2 [†]
LSI D5S23/D5S721, CEP 9, CEP 15	≤2	81.8 (48.2 to 97.7)	18.2 (2.3 to 51.8)	100 (69.2 to 100)	0 (0 to 30.8)	46,XY
LSI MLL dual color rearrangement probe	≤3	100 (80.5 to 100)	0 (0 to 19.1)	100 (71.5 to 100)	0 (0 to 29.5)	Nomo-1 [‡]
BCR/ABL1/ASS (custom preparation)	≤1	97.4 (86.5 to 99.9)	2.6 (0.1 to 13.5)	100 (88.4 to 100)	0 (0 to 11.6)	SD-1 [§]
LSI AML1/ETO dual fusion probe	≤2	100 (79.4 to 100)	0 (0 to 20.6)	100 (79.4 to 100)	0 (0 to 20.6)	Kasumi-1 [¶]
CEP8/LSI CMYC	≤1	100 (69.2 to 100)	0 (0 to 30.8)	100 (73.5 to 100)	0 (0 to 26.5)	46,XY
LSI D7S486(7q31)/CEP 7	≤1	100.0 (69.1 to 100)	0 (0 to 30.9)	100 (71.5 to 100)	0 (0 to 28.5)	46,XY
CEP X α-satellite/CEP Y α-satellite	≤1	100 (83.2 to 100)	0 (0 to 16.8)	100 (83.2 to 100)	0 (0 to 16.8)	46,XY
LSI D20S108 (20q12)	≤2	100 (85.8 to 100)	0 (0 to 14.2)	100 (69.1 to 100)	0 (0 to 30.9)	46,XY

*All cell lines were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, German Collection of Microorganisms and Cell Culture.

[†]JVM-2, human B polymphocytic leukemia cell line with a confirmed *IGH@* rearrangement, t(11;14)(q13;q32).

[‡]Nomo-1, human acute myeloid leukemia with a confirmed *MLL* rearrangement, t(9;11)(p22;q32).

[§]SD-1, human acute lymphoblastic leukemia with a confirmed *BCR/ABL1* fusion, t(9;22)(q34;q11.2).

[¶]Kasumi-1, human acute myeloid leukemia with a confirmed *RUNX1/RUNX1T1* fusion, t(8;21)(q22;q22).

FFPE FISH Studies

Probe was applied (5 to 15 μ l) to each slide, depending on the size of the section. Slides were coverslipped with an 18-mm round coverslip for the 5- μ l probe volume or a 24- × 30-mm coverslip for the 15 μ l probe volume and sealed with rubber cement. Sections were co-denatured for 8 minutes on an 80°C hotplate and placed in a humidified chamber at 37°C overnight. The coverslip was never pulled from the slide to prevent damage to the section. This critical step took between 20 and 60 minutes in 1 × 0.1 mol/L Na₂HPO₄, 0.1 mol/L NaH₂PO₄, and 1% Igepal. Post wash and counterstain were identical to those used for the bone marrow smears.

Re-hybridization

When re-hybridization with a different probe set was indicated, the initial probe was first removed by placing the slides in 2 × SSC for 20 minutes, dehydrated through an ethanol series, and air dried. The second probe was applied and hybridized as described above.

Results

Archived Bone Marrow Smears

Sequential correlation of cytogenetics with phenotype was successfully achieved for all 11 archived Wright-Giemsa-stained bone marrow smears obtained from patients with a variety of hematological disorders (Figure 1, A–F). Morphological (phenotype) evaluation was performed first, followed by FISH (genotypic) analysis using the probes described above. In the six multiple myeloma cases, the slides were scanned initially on the Bioview Duet to map the location of the plasma cells to the slide before destaining and hybridization with the LSI IGH

break-apart probe. Five of six slides from multiple myeloma patients showed an *IGH@* gene rearrangement. The *IGH@*-negative case (patient 1) was then hybridized with a triple combination probe set to exclude hyperdiploidy by determining the copy number of chromosomes 5, 9, and 15 present in the plasma cells. Trisomy 5 was detected in the neoplastic plasma cells (Figure 1A, a–d). The FISH experiments were concordant with the pathology report of persistent multiple myeloma but not with the normal karyotype reported by conventional cytogenetics.

Five additional bone marrow smears from patients with hematological disorders were also investigated. Patient 2 was a 46-year-old female with precursor B acute lymphoblastic leukemia, who was diagnosed and initially treated at another institution. At disease presentation (November 2005), her white blood cell count was 600 K/CMM with 87% blasts, the hemoglobin count was 5.9 G/dl, and the platelet count was 43 K/ μ l. Flow cytometry revealed an immunophenotype positive for CD10, CD19, CD20 (dim), CD34, HLA-DR, TdT, and CD79a with aberrant expression of CD13. Cytogenetic studies performed at the primary center showed a pseudodiploid female karyotype 46,XX,t(7;20)(p13;q11.2),del(20)(q11.2). The patient achieved a complete remission with hyperfractionated cyclophosphamide, vincristine, Adriamycin, and dexamethasone after one cycle of treatment with no evidence of residual disease by pathology and normal conventional cytogenetics. However, the patient remained pancytopenic and required frequent blood transfusions. In June 2006, the cytogenetic aberrations re-appeared with no morphological or flow cytometric evidence of disease relapse. In August 2006, the patient was referred to our institution for advice on the management of her disease. At this time, her bone marrow smear showed variable cellularity with decreased trilineage hematopoiesis and a left shift of both myeloid and erythroid maturation with no morphological evidence of ALL. Bone marrow cyto-

netic studies showed the same abnormal karyotype in 13 of 20 mitotic cells. Because deletion of the long arm of chromosome 20 is commonly observed in both *de novo* and therapy-related myelodysplastic syndromes and rarely observed in ALL, a comparative evaluation of the 2005 ALL presentation smear and the August 2006 bone marrow smear was undertaken to determine the cell lineage (myeloid, lymphoid, or both) of those cells showing the del(20q) clonal abnormality. Figure 1B shows the del(20q) that was present in 94% of the blasts in the ALL presentation study (Figure 1B, a and b) and in the myeloid cells in August 2006 (Figure 1B, c and d) most consistent with an underlying myelodysplasia before her diagnosis of ALL. Taken together, these data helped us to appreciate the pathogenesis of the disease and further supported the recommendation of an allogeneic stem cell transplant to reestablish normal hematopoiesis.

The bone marrow smear of patient 3 was from a male with history of AML who had received a sex-mismatched sibling stem cell transplant 3 months earlier. The bone marrow aspirate smear showed multiple atypical nodular CD3-positive lymphoid aggregates closely associated with eosinophils, raising the question of the origin of the atypical T-cell proliferation after stem cell transplant. The slide was submitted for XY FISH to determine the origin of the lymphoid aggregates. After mapping the location of the atypical nodular aggregates, the suspicious cells were found to be donor (female) derived (Figure 1C), most consistent with benign lymphoid aggregates, possibly related to the patient's graft-versus-host or graft-versus-leukemia response.

Patient 4 was diagnosed with chronic myeloid leukemia in February 2003 and immediately placed on 400 mg of imatinib mesylate/day. His dose was escalated to 600 mg daily 6 months later when the t(9;22)-positive cells did not clear from his peripheral blood. However, within 3 weeks the patient became severely neutropenic, requiring hospitalization. After a slow recovery, the patient was restarted on 400 mg of imatinib, but his counts quickly dropped again, and imatinib was halted. Cytogenetically, the bone marrow revealed three apparently "nonrelated" karyotypically aberrant clones: a t(9;22)-positive clone (sole aberration); a Philadelphia chromosome-negative, monosomy 7-positive clone; and a Philadelphia chromosome-negative, trisomy 8-positive clone. After performing a morphological area scan to map the location of the white blood cells to the smear, the slides were destined

for multiple sequential FISH analyses (Figure 1D). First, the slide was hybridized with a triple color BCR/ABL1/ASS probe set (BCR labeled with spectrum green, ABL1 labeled with spectrum red, and ASS1 labeled with spectrum aqua) (Figure 1D, a). After the BCR/ABL1/ASS1 analysis was complete, the slide was re-analyzed with dual color + 8/CMYC probe (Figure 1D, b). Re-hybridization was necessary because the triple color BCR/ABL1/ASS1 probe set already had the 9q34/ASS1 probe labeled with spectrum aqua, precluding simultaneous BCR/ABL1/ASS1 with + 8. A final analysis with the LSI D7S486 (7q31)/CEP 7 was run to assess monosomy 7 (Figure 1D, c). As seen in Figure 1D, individual BCR/ABL1-positive, monosomy 7-positive, and trisomy 8-positive cells were detected in independent myeloid cell populations.

In a case of t(8;21) (RUNX1/RUNX1T1)-positive AML with systemic mastocytosis, mast cells coexisted with the blasts in the bone marrow. The relationship between the mast cells and the leukemic clone was unclear, in particular whether the mast cells were a part of the neoplastic clone. Using the single remaining archived bone marrow smear for FISH analysis, the slide was scanned to localize the position of mast cells on the slide and destined and hybridized with the LSI AML1/ETO probe set to detect the t(8;21) in the interphase mast cells. Figure 1E shows the presence of the RUNX1/RUNX1T1 fusion gene in the bone marrow mast cells, indicating their derivation from the leukemic clone. The t(8;21) could still be detected in the bone marrow mast cells even when the patient was in a durable remission after allogeneic stem cell transplant and conventional cytogenetic studies were normal.⁵

Patient 6 was a 69-year-old female diagnosed with breast cancer in 2002 and treated subsequently with epirubicin, cytoxan, and taxotere. In 2005, she was referred to our institution with a diagnosis of therapy-related myelodysplasia. A cytogenetic report from an outside laboratory reported an inv(11)(p15q23) clonal aberration, but MLL FISH studies were not performed. To confirm the presence of an MLL translocation and its baseline FISH signal pattern, sequential morphology/FISH was performed on the diagnostic bone marrow slide. The patient was negative for MLL translocation, but the trisomic signal pattern indicated either a more complex chromosome 11 rearrangement or a tandem duplication of the MLL gene (data not shown). Thus, these data provided the

Figure 1. Representative examples of sequential morphology and IHC/FISH. All brightfield images were captured with a 20× objective. All fluorescent images were captured with a 63× dry objective and a triple band pass filter or aqua filter. **A:** Example of IGH-negative plasma cell using the dual fusion IGH break-apart probe (Abbott Molecular) with corresponding morphological images. The presence of two red/green fusion signals is the expected normal pattern, indicating no evidence of an IGH@ gene rearrangement (**a** and **b**). Hybridization with the hyperdiploid probe set showed a clonal population of cells with a 3G/2B/2R pattern, indicating trisomy 5 (three green signals, two blue signals for disomy 9 and two red signals for disomy 15) (**c** and **d**). **B:** Representative FISH and morphological images showing a deleted 20q12 population with one red (20q12) and two green (20p12) signals in the majority of leukemic blasts at presentation (**a** and **b**) and in a myeloid cell at follow-up while in clinical remission (**c** and **d**). **C:** FISH and morphological pictures of an atypical lymphoid aggregate from patient 3. The presence of two green signals (XX) is consistent with female (donor) sex chromosomes. There was no evidence of male (XY) cells among the suspicious lymphoid aggregates. **D:** Sequential images from patient 4 representing the BCR/ABL1-positive (one red/blue, one green, one red/green, one blue pattern) (**a**), trisomy 8-negative (two red/two green pattern) (**b**), monosomy 7-negative (two red/two green pattern) clone (**c**), and corresponding morphological image (**d**). Sequential images representing BCR/ABL1-negative (two red-blue/two green pattern) (**e**), trisomy 8-positive (three red/three green pattern) (**f**), monosomy 7-negative (two red/two green pattern) clone (**g**), and corresponding morphological image (**h**). Sequential images representing BCR/ABL1-negative (two red-blue/two green pattern) (**i**), trisomy 8-negative (two red/two green pattern) (**j**), monosomy 7-positive (one red/one green pattern) clone (**k**), and corresponding morphological image (**l**). **E:** Mast cell with positive pattern for t(8;21) (patient 5), one red, one green, and two fusion signals (**a**), with morphological image (**b**). **F:** Representative images of IGH@ FISH on a CD20-positive lymphocyte (patient 7). The one fusion, one red, and one green pattern indicates the presence of an IGH@ rearrangement (**a**). **b:** The immunohistochemical stain used to select this cell.

appropriate FISH signal pattern to monitor the patient's clinical course in interphase cells without the need for conventional (metaphase) cytogenetics.

Paraffin-Embedded Sections: IHC/FISH Studies

Twenty-one samples (5 known controls and 16 T-cell-rich B-cell lymphoma specimens) were submitted for sequential IHC/FISH analysis to limit the FISH study to CD20-positive cells. CD20 staining was detected with 3-amino-9 ethyl-carbozole. Diaminobenzadine was not a suitable detection reagent because its autofluorescent characteristics increased background and obscured the true FISH signal patterns. According to the referring institution, these samples were all negative for *BCL2/IGH@* PCR.

Successful hybridization was achieved with 19 of 21 samples (Figure 1F); two remaining samples were inadequate for evaluation. Three of 16 samples were positive for *IGH@* rearrangement in the targeted cell population as was the positive control. The four negative controls were *IGH@* negative. Two samples with adequate FISH signals outside the limits of the imaging system fluorescent objective of the Duet showed a negative *IGH@* translocation signal pattern by the standard manual scoring method; however, because CD20 IHC correlation was imperfect with the manual scoring method and the malignant cell population made up a small percentage of the tumor, an *IGH@* translocation could not be completely excluded.

Discussion

Current clinical management decisions depend on accurate assessment of the morphological, immunological, and genetic characteristics of a neoplastic disorder. The need to identify and correlate these individual tumor features becomes a challenge when fresh tumor material is not available or is severely limited. This report describes a reliable, reproducible, and quantitative FISH method to correlate phenotype (morphology or immunophenotype) with genotype (cytogenetic aberrations) using archived bone marrow smears and core biopsies, bone marrow clot sections, and paraffin-embedded tissues that were collected, fixed, and stored at multiple medical centers for periods from 1 month to more than 14 years. This approach is especially useful where sampling variability is a concern or focal disease is suspected. By analyzing the exact cells deemed atypical by the pathologist, one can determine the extent of disease involvement. Moreover, this sequential method has value that can apply to individual patient care as well as retrospective and prospective multi-institutional studies. The automated image tracking/classifying software arranges the morphology or IHC images from the entire slide before and after FISH analysis for quick data retrieval, preservation, and documentation.

Initially, our laboratory sought to develop a plasma cell-specific FISH assay that was capable of detecting prognostic cytogenetic aberrations in multiple myeloma and related plasma cell disorders.⁴ Genetic character-

ization of tumor cells, however, has broad applicability for both clinical and basic research questions. Patient 2 (Figure 1B) illustrates the importance of correlating morphology and cytogenetic markers in archived disease presentation samples in comparison with current pathology material in a patient seeking a second opinion. Therapy-related myelodysplasia was excluded, and definition of an underlying myelodysplasia before her acute lymphoblastic leukemia provided a clearer picture of the evolution of the patient's hematological malignancy; it also provided additional data supporting the recommendation of an allogeneic stem cell transplant to reestablish normal hematopoiesis. Similar studies could provide valuable clinical data at selected protocol time points to monitor treatment. When cytogenetic studies are not performed at disease presentation yet are available at relapse of disease, this assay with the appropriate DNA FISH probes could detect or confirm the same aberration in the original diagnostic slide. Because three to four hybridizations are possible before nonspecific background fluorescence becomes a problem, this "retrospective" approach may also be used to discriminate between therapy-related versus relapse of *de novo* disease or perhaps clonal evolution by re-hybridization of same slide with multiple FISH probes. Alternatively, if the original leukemia cytogenetic studies were noninformative or not available, sequential morphology/FISH testing could be used to detect "good versus high genetic risk" cytogenetic markers that stratify patients to standard, high-dose, or stem cell transplantation therapeutic options.¹²⁻¹⁶ Likewise, in multiple myeloma, the presence of 1p/1q chromosome imbalances, hypo- or hyperdiploidy, -13/del(13q), del(17p)/*TP53* deletions, and/or *IGH@* gene rearrangements have been useful in assessing genetic risk with outcome and in stratifying patients to specific therapeutic regimens.¹⁷⁻²³

Several recent investigations have reported the emergence of Philadelphia chromosome-negative clones in patients receiving tyrosine kinase inhibitors (TKIs).²⁴⁻²⁷ The observed "chromosome instability" may be associated with the disease itself, that is, the result of *BCR-ABL1* expression leading to additional karyotypic alterations and general genomic instability,²⁸ or TKIs may permit expression of other less proliferative clones that arose from prior stem cell damage,²⁹ or TKIs may have a deleterious effect on nonmalignant (*BCR/ABL1*-negative) cells.³⁰ In patient 4, the sequential morphology/FISH assay confirmed the conventional cytogenetic findings of three distinct clones, assigned cell lineage to each clone (all myeloid), and quantified the clonal populations. These clonal findings are disturbing; however, the clinical significance is unknown. Monosomy 7 and/or trisomy 8 are both common secondary aberrations in Ph+ chronic myeloid leukemia, indicating clonal evolution of disease, but these aberrations alone are also nonrandom primary karyotypic aberrations in acute leukemia. Because long-term follow-up studies for patients receiving TKIs are not yet available and a small percentage of chronic myeloid leukemia patients on TKIs will develop Philadelphia chromosome-negative acute myeloid leukemia,²⁷ there is a

need to monitor patients receiving TKIs closely to define those patients who might be at eventual risk.

An alternative to sequential morphology/FISH is the related sequential IHC/FISH method. Sequential IHC/FISH has the advantage of targeting a broader range of cells at the single-cell level through the use of antibodies. As shown in the CD20-positive cell in patient 7, this method may also be used to determine the frequency of a rare or specific cytogenetic aberration in a selected tumor type. Alternatively, CD34-positive cells could be targeted for specific cytogenetic aberrations in hematopoietic stem cells collected for autologous stem cell transplantation, or IHC/FISH using CD19 and/or CD10 might be used to distinguish residual disease in Philadelphia chromosome-positive ALL from the presence of "hematogones" in regenerating bone marrow after chemotherapy. The limitations of IHC/FISH apply equally to both simultaneous and sequential methods. Antibodies have variable sensitivity and signal strength, requiring optimization of each antibody for fixation, dilution, and diluents, which is time consuming and costly. Weakly stained cells may be missed or misclassified using the IHC tracking/classifying software.

Sequential IHC/FISH has a number of advantages over simultaneous IHC/FISH studies, a technique also known as FICTION³⁻⁵ or simultaneous fluorescence immunophenotyping and interphase cytogenetics as a tool for oncology. An alternative approach has recently been described using simultaneous CD138 immunofluorescence.³¹ First, if multiple genetic loci are to be investigated, simultaneous studies require a cumbersome and complicated labeling system.⁵ Because the probes must be directly labeled, probe selection is somewhat limited to those >100 kb for consistent results. Home-brew probes, developed from bacterial artificial chromosome clones and plasmid artificial clones, are often smaller (usually <100 kb) and typically require indirect labeling and detection to increase signal strength. For probes <2 kb or for hybridization in paraffin-embedded tissue, tyramide signal amplification is necessary.³² Alternatively, in sequential IHC/FISH, IHC images are independent of the FISH images, so the slide can be re-hybridized with either directly or indirectly labeled probes without danger of immunofluorescent fading or degradation. Second, simultaneous analysis also requires fairly robust antibody staining. Weak antibodies may give false-negative or inconclusive results, whereas chromogenic detection is more stable and will allow identification of weaker antibodies through horseradish peroxidase complex. Because most pathology laboratories use chromogenic substrates, additional work is not necessary because the previously processed slide can be hybridized. Third, a potential research advantage of the sequential assay over the simultaneous method is its ability to correlate gene expression in a cell with a specific genetic abnormality.³³ In a prior study, an *FGFR3* antibody was used to show the level of *FGFR3* expression in t(4;14)(p16;q32)-positive plasma cells before and after transfection with short hairpin RNAs marked with green fluorescent protein. The fact that green fluorescent

protein-marked transfected cells show green fluorescence over the entire cell precluded the use of the simultaneous IHC/FISH assay. Fourth, the simultaneous labeling method has not been evaluated for the detection of minimal residual disease.

The BIOMED-2 concerted action has significantly improved PCR testing for clonality or residual disease testing in B-cell malignancies; however, when using FFPE samples, variations in formalin fixation and paraffin embedding affect the PCR results.³⁴⁻³⁶ Using fresh/frozen tissue, the reported frequency of IGH rearrangements detected by PCR in B-cell malignancies is 72 to 90%.³⁴ However, the reported range for IGH rearrangements using FFPE tissue is lower because the fixation and embedding procedure frequently fragments the DNA precluding high quality DNA for amplification (~300 to 600 bp).³⁶ Our goal was to correlate a specific immunophenotype with IGH status at the single-cell level, a correlation that cannot be achieved by PCR. Furthermore, for the T-cell rich B-cell lymphoma samples described in this study, PCR may not be the optimal clonality test to use because of the limited number of B cells present. Finally, an added advantage to the assay described herein, is the ability to re-hybridize the identical cell of interest to determine the partner gene involved in the IGH gene rearrangement.

The use of archived material presents technically challenging issues for pathology applications. For FISH-based assays, proper aging and pepsin pretreatment are key steps. We found that antigen retrieval with EDTA buffer under high temperature and pressure allowed us to standardize the pepsin incubation for FFPE tissue up to 14 years old from a variety of institutions. Bone marrow smears proved more challenging because the pepsin treatment varied depending on age of specimen and red blood cell density. Although we used an automated platform, this protocol could be applied with any imaging system that has brightfield/fluorescent capabilities and the technical time to invest in locating and recording coordinates of cells of interest. In some cases, this might even be preferable because a higher magnification and oil objective could be used for both brightfield and fluorescence analysis, resulting in better quality images. Nevertheless, the power of the sequential technique described in this study is its ability to correlate suspicious or unusual pathology findings in a wide range of archival material regardless of age with a definite cytogenetic abnormality. This result far outweighs any of the technical concerns encountered. Furthermore, this integrated pathological/genetic approach allows one to focus on designing targeted or individualized therapeutic strategies according to the specific features of the tumor cells.

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