Immunoglobulin Heavy-Chain Fluorescence In Situ Hybridization-Chromogenic In Situ Hybridization DNA Probe Split Signal in the Clonality Assessment of Lymphoproliferative Processes on Cytological Samples

Pio Zeppa, MD, PhD[1]; Laura Virginia Sosa Fernandez, BS[2]; Immacolata Cozzolino, MD, PhD[2]; Valentina Ronga, BS[3]; Rita Genesio, BS[3]; Maria Salatiello, BS[2]; Marco Picardi, MD[4]; Umberto Malapelle, BS[2]; Giancarlo Troncone, MD, PhD[2]; and Elena Vigliar, MD[2]

BACKGROUND: The human immunoglobulin heavy-chain (*IGH*) locus at chromosome 14q32 is frequently involved in different translocations of non-Hodgkin lymphoma (NHL), and the detection of any breakage involving the *IGH* locus should identify a B-cell NHL. The split-signal *IGH* fluorescence in situ hybridization-chromogenic in situ hybridization (FISH-CISH) DNA probe is a mixture of 2 fluorochrome-labeled DNAs: a green one that binds the telomeric segment and a red one that binds the centromeric segment, both on the *IGH* breakpoint. In the current study, the authors tested the capability of the *IGH* FISH-CISH DNA probe to detect *IGH* translocations and diagnose B-cell lymphoproliferative processes on cytological samples. **METHODS:** Fifty cytological specimens from cases of lymphoproliferative processes were tested using the split-signal *IGH* FISH-CISH DNA probe and the results were compared with light-chain assessment by flow cytometry (FC), *IGH* status was tested by polymerase chain reaction (PCR), and clinicohistological data. **RESULTS:** The signal score produced comparable results on FISH and CISH analysis and detected 29 positive, 15 negative, and 6 inadequate cases; there were 29 true-positive cases (66%), 9 true-negative cases (20%), 6 false-negative cases (14%), and no false-positive cases (0%). Comparing the sensitivity of the *IGH* FISH-CISH DNA split probe with FC and PCR, the highest sensitivity was obtained by FC, followed by FISH-CISH and PCR. **CONCLUSIONS:** The split-signal *IGH* FISH-CISH DNA probe is effective in detecting any translocation involving the *IGH* locus. This probe can be used on different samples from different B-cell lymphoproliferative processes, although it is not useful for classifying specific entities. ***Cancer (Cancer Cytopathol)* 2012;120:390-400.** © *2012 American Cancer Society*.

KEY WORDS: immunoglobulin heavy-chain (*IGH*), fluorescence in situ hybridization-chromogenic in situ hybridization (FISH-CISH), DNA split probe, flow cytometry, polymerase chain reaction, lymphoproliferative processes, cytology.

INTRODUCTION

Currently, fine-needle cytology (FNC) is used successfully in the diagnosis and classification of non-Hodgkin lymphoma (NHL). Many laboratories have achieved high levels of diagnostic efficacy in the cytological diagnosis and classification of NHL, combining cytological features, phenotype, and molecular data obtained by immunocytochemistry, flow cytometry (FC), polymerase chain reaction (PCR), and fluorescence in situ hybridization (FISH).[1-17]

Corresponding author: Pio Zeppa, MD, PhD, Department of Medicine and Surgery, Azienda Ospedaliera Universitaria, San Giovanni di Dio e Ruggi D'Aragona, Largo Città d'Ippocrate n.1, 84131 Salerno, Italy; Fax: (011) 089 642849; pzeppa@unisa.it

[1]Department of Medicine and Surgery, University of Salerno, Salerno, Italy; [2]Department of Biomorphological and Functional Science, Federico II University, Naples, Italy; [3]Department of Cellular and Molecular Biology and Pathology, Federico II University, Naples, Italy; [4]Department of Biochemistry and Medical Biotechnology, Federico II University, Naples, Italy

Received: December 9, 2011; **Revised:** February 27, 2012; **Accepted:** March 22, 2012

Published online April 19, 2012 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cncy.21203, wileyonlinelibrary.com

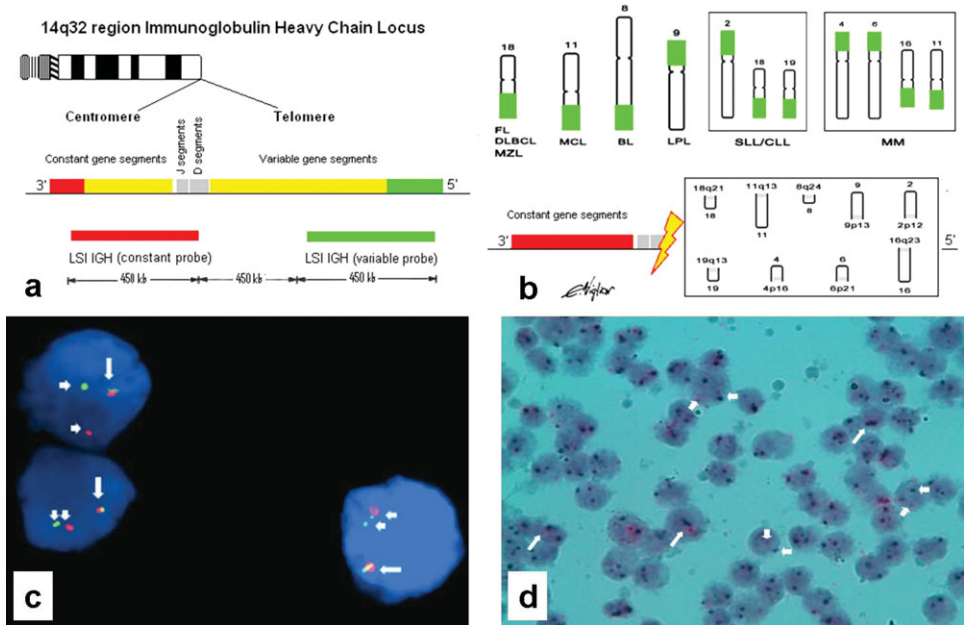

FIGURE 1. (a) A graphic representation of the immunoglobulin heavy-chain (*IGH*) locus hybridized with the split-signal *IGH* fluorescence in situ (FISH) DNA probe is shown. The green-labeled DNA probe (*IGH-Flu*) binds the 612-kilobase (kb) telomeric segment and the red-labeled DNA probe (*IGH-TR*) binds the 460-kb centromeric segment, generating a fusion signal (yellow). LSI indicates locus specific indicator. (b) The *IGH* breakpoint with translocation of 1 segment generating a split signal (red and green) independent of the corresponding chromosomal partners is shown. FL indicates follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; MZL, marginal zone B-cell lymphoma (10%); MCL, mantle cell lymphoma; BL, Burkitt lymphoma; LPL, lymphoplasmacytic lymphoma; SLL/CLL, small lymphocytic lymphoma/chronic lymphocytic leukemia; MM, multiple myeloma. (c) FISH evidence of *IGH* assessment by the split-signal *IGH* FISH DNA probe is shown. The long arrows indicate fusion signals and the short arrows indicate split signals. (d) Chromogenic in situ hybridization (CISH) revelation of the *IGH* FISH DNA probe split signals is shown. Blue signals reveal the green-labeled DNA probe (*IGH-Flu*) and the red signals indicate the red-labeled DNA probe (*IGH-TR*). The long arrows indicate fusion signals and the short arrows indicate split signals.

From this perspective, ancillary techniques can assess clonality, demonstrating light-chain restriction or specific phenotyping profiles by FC, and detect the immunoglobulin heavy-chain (*IGH*) or T-cell receptor rearrangements by PCR. With regard to FISH, the probes used for the identification of specific translocations are also able to indicate clonality. FISH is generally used, after FNC/FC, in the differential diagnoses of ambiguous cases, such as small lymphocytic lymphoma/chronic lymphocytic leukemia (SLL/CLL) versus mantle cell lymphoma (MCL), which share the coexpression of cluster of differentiation (CD) 5/CD19, or to confirm a specific subtype (eg, Burkitt lymphoma [BL]) by the *IGH/MYC/CEP8* fusion probe to detect the t(8;14) chromosome translocation.[10-17] The human *IGH* locus at chromosome 14q32 is most frequently involved in different NHL translocations (Fig. 1) (Table 1).[18-26] Therefore, the detection of any breakage involving the *IGH* locus at chromosome 14q32 should identify a B-cell NHL independently of the specific subtype. The split-signal IGH FISH-CISH DNA probe is a mixture of 2 fluorochrome-labeled DNAs: a green fluorescein-labeled DNA probe (*IGH-Flu*) that binds to a 612-kilobase (kb) segment telomeric, and a red-labeled DNA probe (*IGH-TR*) that binds to a 460-kb segment centromeric, both to the *IGH* breakpoint. Therefore, the split-signal *IGH* FISH-CISH DNA probe should detect any translocation involving the *IGH* locus at chromosome 14q32, assessing the clonality of the corresponding processes (Figs. 1c and 1d). The objective of the current study was to evaluate the performance characteristics of the *IGH* FISH-CISH DNA probe split signal in assessing B-cell lymphoproliferative processes on cytological samples. To the best of our knowledge, this is the first study that investigates the application of the *IGH* FISH-CISH DNA probe on cytological samples of B-cell lymphoproliferative processes.

Table 1. Most Frequent Translocations Involving the *IGH* Locus

NHL Histotype	Translocation	Percentage	Study
FL	t(14;18)(q32;q21)	70%-95%	Swerdlow 2008[18]
DLBCL	t(14;18)(q32;q21)	30%	Swerdlow 2008[18]
DLBCL	t(14;19)(q32;q12)	8%	Nagel 2009[19]
DLBCL	t(3;14)(p14;q32)	Rare	Fenton 2006[20]
SLL/CLL	t(2;14)(p12;q32), t(14;19)(q32;q13), t(14;18)(q32;q21)	21%	Cavazzini 2008[21] Aoun 2004[22]
MCL	t(11;14)(q13;q32)	75%	Swerdlow 2008[18]
BL	t(8;14)(q24;q32)	75%	Swerdlow 2008[18]
lpl	t(9;14)(p13;q32)	50%	Swerdlow 2008[18]
MZL	t(9;14)(p13;q32)	Rare	Baro 2006[23]
MZL	t(14;18)(q32;q21)	10%	Tsai 2010[24]
MALT	t(3;14)(p14;q32)	10%	Streubel 2005[25]
MM	t(11;14)(q13;q32), t(6;14)(p21;q32), t(4;14)(p16;q32), t(14;16)(q32;q23)	80%-90%	Bergsagel 2001[26]

Abbreviations: BL, Burkitt lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; *IGH*, immunoglobulin heavy-chain; lpl, lymphoplasmacytic lymphoma; MALT, mucosa-associated lymphoid tissue lymphoma; MCL, mantle cell lymphoma; MM, multiple myeloma; MZL, marginal zone B-cell lymphoma; NHL, non-Hodgkin lymphoma; SLL/CLL, small lymphocytic lymphoma/chronic lymphocytic leukemia.

MATERIALS AND METHODS

Fifty consecutive cytological samples of lymphoproliferative processes were collected at the Department of Biomorphological and Functional Science of the Federico II University in Naples, Italy during a 10-month period between September 2010 and June 2011. The series included mainly specimens of lymph nodes and extranodal lymphoproliferative processes involving different sites such as soft tissue or the orbit and obtained by FNC, as well as effusions suspected to be lymphoproliferative processes. The study did not in any way influence patient management because all the necessary tests were performed routinely. In those patients undergoing FNC, the diagnostic procedure and its related risks were discussed with the patients beforehand, including the use of the data for scientific purposes. Informed consent was obtained before patient inclusion in the study and the FNC procedure was then performed as previously described.[5] In all cases, the first pass was used to prepare 2 traditional smears. The first was stained using the Diff-Quik technique and was immediately examined to assess the adequacy of the sample and the second smear was fixed in 96% ethanol and stained using the Papanicolaou method. The remaining material left in the hub of the needle was carefully flushed out with phosphate-buffered saline (PBS) solution and added to a second pass. This material was used both for FC assessment, performed as previously described,[9] and to prepare 1 or 2 air-dried cytospin preparations that were fixed in 96% ethanol for 1 minute and then stored at −20°C for FISH-CISH analysis. Finally in 30 cases in which it was possible, an additional pass was performed and suspended in RNA*later* (Life Technologies Corporation, Carlsbad, Calif) to be used for PCR. This procedure is routinely performed in the study laboratory to confirm FNC/FC diagnoses, mainly when no light-chain restriction is detected or when histology or follow-up are not available. FNC/FC was performed to assess whether the corresponding process was reactive or lymphomatous at light-chain assessment, and for the detection of specific phenotypic profiles, if any. Cases diagnosed as NHL were then subclassified, whenever possible, by evaluating their cytological features and specific phenotypes, according to the most recent World Health Organization classification.[18]

Three cases of Hodgkin lymphoma and 2 cases of peripheral T-cell lymphoma were excluded from the current study. Histology and follow-up were checked at the time of the study by reviewing the former and subsequent histological diagnoses, which were all confirmed by 2 of the authors (P.Z. and I.C.).

FISH Assay

FISH analysis was performed on cytospin preparations prepared from FNC suspension in PBS. The cell suspension was incubated with a lysant buffer for 5 minutes and centrifuged for 8 minutes at 1800 revolutions per minute (rpm). Cytospin preparations were then prepared for each specimen by centrifuging at 500 rpm for 5 minutes and fixing in 96% ethanol, and then stored at −20°C. At the moment of the FISH assay, cytospin preparations were balanced to ambient temperature, observed using a phase-contrast microscope by 2 of the authors (R.G. and V.R.) to confirm the adequacy of the samples, and then incubated with 3.7% formaldehyde for 2 minutes at ambient temperature. The FISH assay was performed using the Cytology FISH Accessory Kit and *IGH* FISH DNA Probe, Split signal (DakoCytomation, Glostrup, Denmark). The *IGH* FISH-CISH DNA probe with split signal is a mixture of 2 fluorochrome-labeled DNAs: a green

fluorescein-labeled DNA probe (*IGH-Flu*) that binds to a 612-kilobase (kb) segment telomeric and a red-labeled DNA probe (*IGH-TR*) that binds to a 460-kb segment centromeric, both to the *IGH* breakpoint. All cytospin preparations were performed according to the manufacturer data sheet. Image acquisition was performed using a fluorescence microscope (Olympus BX61; Olympus, Center Valley, Pa) equipped with DAPI (4′,6-diamidino-2-phenylindole) and Texas Red/fluorescein isothiocyanate (FITC) filters with a Photometrics CCD camera (Photometrics, Tucson, Ariz). Images were viewed more closely using CytoVision software (version 3.93.2; Genus Technologies, Minneapolis, Minn). A total of 200 nuclei from each case were counted. The signal was scored if the appropriate nucleus was intact and not overlapping another one. Two signals separated by a distance less than or equal to twice the diameter of a signal were scored as 1 signal. Cytospin preparations of proven reactive processes and NHLs from former lymph node FNC samples were used as negative and positive controls. With regard to the diagnostic classification, according to the manufacturer's indications, cases in which $>$ 10% of the counted nuclei demonstrated split signals were considered to be positive. The percentage of nuclei with split signals in positive cases $>$ 10% was then calculated and reported.

CISH Assay

The CISH assay was performed using the Dako DuoCISH kit (DakoCytomation) on the cytospin preparations that were already stained to observe, by bright field microscopy, the dual-color chromogenic signals (red and blue signals) achieved with the previous FISH assay. Briefly, cytospin specimens for CISH staining were soaked twice in a fresh Wash Buffer (WB) for 3 minutes, covered with 200 μL of peroxidase block for 5 minutes, and washed twice in WB for 3 minutes. Cytospin preparations were then covered with 200 μL of CISH antibody mix, incubated for 30 minutes in a humid chamber, and washed twice in WB for 3 minutes. In the subsequent steps, cytospin preparations were incubated with 200 μL of red chromogen solution for 10 minutes and then with 200 μL of blue chromogen solution for 10 minutes. Cytospin specimens were then stained with hematoxylin (at a dilution of 1:5) for 5 minutes, washed twice in WB, and air-dried at 37° on a StatSpin ThermoBrite (Iris Sample Processing, Westwood, Mass). A total of 200 nuclei from each case were counted. The signal was scored according to the guidelines provided with the *IGH* FISH-CISH DNA probe.

DNA Extraction and PCR Conditions

DNA was extracted from the cells obtained by FNC, stored in RNA*later* using a commercially available kit (QIAamp DNA Mini Kit; Qiagen Inc, Valencia, Calif) according to the manufacturer's instructions, and resuspended in 40 μL of water (Ambion Nuclease-Free Water-not DEPC-Treated; Life Technologies, Paisley, UK). The quantity of extracted DNA was assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, Del). PCR and analysis of PCR products were performed using a BIOMED-2 protocol.[27,28] We defined a PCR result as positive when 2 of 3 framework-amplified regions demonstrated a monoclonal pattern.[28]

RESULTS

The current series is comprised of 50 cases from 48 patients. For 2 of these cases (cases 8-18 and cases 37-45), there were 2 samples from different anatomical sites that were taken at different times. The 48 patients included 25 men and 23 women, with an age range of 23 to 86 years (median, 60.7 years). The series was comprised of FNC samples obtained from 43 lymph nodes, 2 from soft tissue, 2 from orbital masses, and 3 from effusions (2 pleural and 1 peritoneal) suspected of NHL involvement. The series included patients with a history of NHL (11 patients) and those suspected to have NHL (39 patients). The clinical data are reported in Table 2.

Cytological and FC Results

Cytological features combined with the FC profile produced the following results: 37 positive results, 11 negative results, and 2 inadequate cases (Figs. 2a and 2b and 3a and 3b). Positive cases were then subclassified by combining cytological features with FC data, and included 9 cases of follicular lymphoma (FL), 8 cases of SLL/CLL, 5 cases of diffuse large B-cell lymphoma (DLBCL), 5 cases of MCL, 2 cases of marginal zone lymphoma (MZL), 8 cases of NHL-not otherwise specified (NHL-NOS), and 11 cases of reactive hyperplasia (RH). Comparing the results of the FNC/FC with the final diagnosis obtained by histology and/or follow-up, there were 37 true-positive cases (77%), 9 true-negative cases (19%), 2 false-negative cases (4%), and 0 false-positive cases (0%). The statistical analysis indicated a 95% sensitivity and a 100% specificity.

Table 2. Clinical, Cytological, Flow Cytometry, FISH-CISH, and PCR Results of the Current Study

Case	Age, Years/Sex	Sample/Site	FNC/FC	FISH-CISH (*IGH*-DNA Probe)	PCR	Histology or Follow-Up
1	68/Man	Axillary LN	FL (p)	Positive (35%)	+	FL (H)
2	61/Man	Supraclavicular LN	RH	Negative	-	DLBCL (F)
3	56/Man	Inguinal LN	FL (p)	Positive (30%)	+	FL (H)
4	63/Woman	Orbit	SLL/CLL (p)	Inadequate	NP	SLL/CLL (F)
5	79/Man	Pleural effusion	RH	Negative	-	RH (F)
6	52/Woman	Inguinal LN	RH	Negative (<10%)	NP	FL (F)
7	75/Man	Submandibular LN	DLBCL (p)	Positive (90%)	+	DLBCL (H)
8	75/Man	Laterocervical LN	SLL/CLL (p)	Positive (15%)	+	SLL/CLL (F)
9	25/Woman	Laterocervical LN	DLBCL (p)	Inadequate	+	DLBCL (H)
10	82/Woman	Submandibular LN	NHL-NOS (p)	Positive (80%)	+	DLBCL (H)
11	86/Woman	Lomboaortic LN	MZL (p)	Positive (20%)	NP	MZL (H)
12	44/Man	Pleural effusion	RH	Negative (<10%)	NP	RH (F)
13	74/Woman	Soft tissue of arm	NHL-NOS (r)	Inadequate	-	FL (F)
14	45/Woman	Inguinal LN	FL	Positive (40%)	+	FL (F)
15	48/Woman	Iliac LN	Inadequate (p)[a]	Inadequate	NP	DLBCL (F)
16	86/Woman	Lomboaortic LN	MZL (p)	Positive (20%)	+	MZL (H)
17	72/Woman	Laterocervical LN	MCL (p)	Positive (40%)	NP	MCL (H)
18	75/Man	Inguinal LN	SLL/CLL (p)	Positive (20%)	-	SLL/CLL (H)
19	42/Man	Soft tissue of arm	MCL (r)	Positive (30%)	NP	MCL (H)
20	68/Woman	Laterocervical LN	SLL/CLL (p)	Positive (40%)	+	SLL/CLL (H)
21	46/Man	Axillary LN	NHL-NOS (r)	Positive (98%)	+	FL (F)
22	60/Woman	Axillary LN	NHL-NOS (r)	Inadequate	NP	DLBCL (F)
23	69/Man	Submandibular LN	RH	Negative (<10%)	-	RH (F)
24	79/Woman	Iliac LN	DLBCL (r)	Positive (60%)	NP	DLBCL (F)
25	54/Man	Inguinal LN	FL (r)	Positive (15%)	-	FL (F)
26	65/Man	Inguinal LN	DLBCL (r)	Positive (30%)	NP	DLBCL (F)
27	81/Woman	Axillary LN	FL (p)	Positive (20%)	+	FL (F)
28	52/Man	Inguinal LN	RH	Negative (<10%)	-	RH (F)
29	57/Man	Supraclavicular LN	FL (p)	Positive (40%)	+	FL (F)
30	59/Woman	Inguinal LN	DLBCL (r)	Negative (<10%)	+	DLBCL (F)
31	33/Man	Inguinal LN	RH	Negative (<10%)	-	RH (F)
32	52/Man	Laterocervical LN	RH	Negative (<10%)	NP	RH (F)
33	57/Man	Supraclavicular LN	FL (p)	Positive (15%)	NP	FL (H)
34	56/Woman	Peritoneal effusion	RH	Negative (<10%)	-	RH (F)
35	23/Woman	Laterocervical LN	RH	Negative (<10%)	NP	RH (F)
36	82/Man	Inguinal LN	SLL/CLL (r)	Positive (80%)	NP	SLL/CLL (F)
37	41/Woman	Lomboaortic LN	NHL-NOS (p)	Positive (20%)	+	FL (F)
38	53/Man	Inguinal LN	Inadequate[a]	Positive (20%)	NP	FL (H)
39	56/Woman	Supraclavicular LN	MCL (p)	Positive (95%)	+	MCL (H)
40	64/Woman	Submandibular LN	SLL/CLL (p)	Negative (<10%)	NP	SLL/CLL (F)
41	76/Man	Axillary LN	NHL-NOS (p)	Inadequate	+	NHL-NOS (H)
42	68/Woman	Submandibular LN	FL (p)	Positive (15%)	NP	FL (H)
43	67/Man	Orbit	NHL-NOS (p)	Positive (30%)	NP	NHL-NOS (F)
44	57/Man	Laterocervical LN	FL (r)	Positive (50%)	-	FL (F)
45	41/Woman	Iliac LN	SLL/CLL (p)	Positive (20%)	-	SLL/CLL (F)
46	50/Man	Laterocervical LN	RH	Negative (<10%)	-	RH (F)
47	47/Woman	Axillary LN	MCL (p)	Negative (<10%)	+	MCL (H)
48	81/Woman	Inguinal LN	SLL/CLL (p)	Negative (<10%)	NP	SLL/CLL (F)
49	59/Man	Laterocervical LN	MCL (r)	Positive (30%)	+	MCL (F)
50	75/Man	Axillary LN	NHL-NOS (p)	Positive (40%)	NP	DLBCL (F)

Abbreviations: +, positive; -, negative; DLBCL, diffuse large B-cell lymphoma; F, follow-up or histology performed elsewhere; FC, flow cytometry; FISH-CISH, fluorescence in situ hybridization-chromogenic in situ hybridization; FL, follicular lymphoma; FNC, fine-needle cytology; H, histology; *IGH*, immunoglobulin heavy-chain; LN, lymph node; MCL, mantle cell lymphoma; MZL, marginal zone B-cell lymphoma; NHL-NOS, non-Hodgkin lymphoma-not otherwise specified; NP, not performed; p, primary; PCR, polymerase chain reaction; r, recurrence; RH, reactive hyperplasia; SLL/CLL, small lymphocytic lymphoma/chronic lymphocytic leukemia.

[a] FNC was suggestive for NHL, and FC was inadequate.

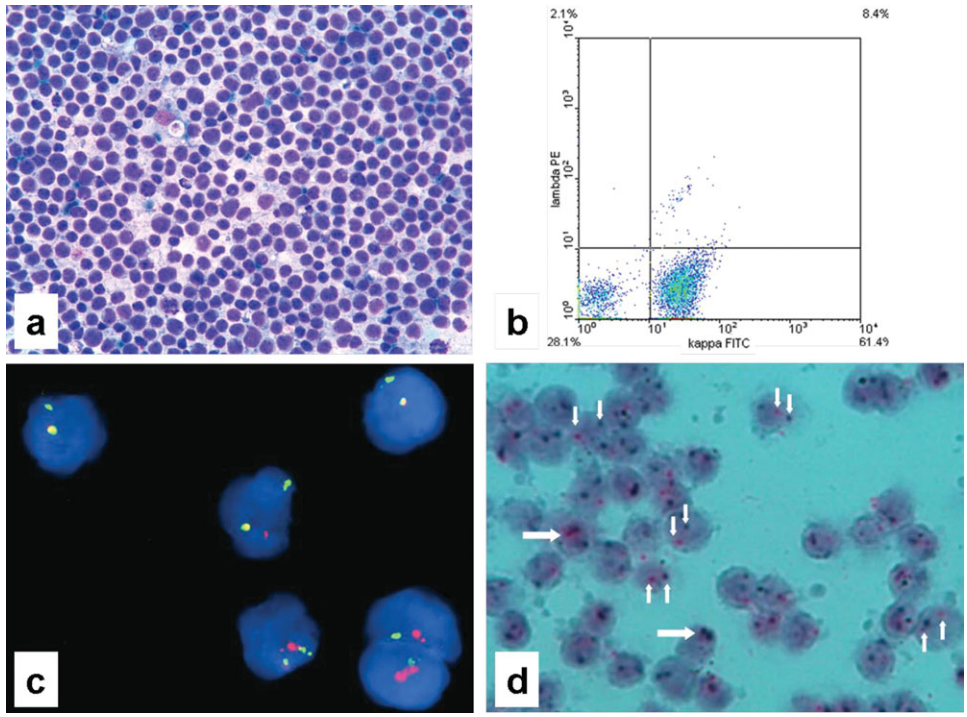

FIGURE 2. Results of cytology, flow cytometry (FC), fluorescence in situ hybridization (FISH), chromogenic in situ hybridization (CISH) in case 1 are shown. (a) A smear demonstrating an atypical monomorphous population of medium-sized cells with irregularly shaped and cleaved nuclei, granular chromatin, and inconspicuous nucleoli is shown (Diff-Quik stain, × 430). (b) FC light-chain restriction in the lower right quadrant is shown. PE indicates Phycoerythrin; FITC, fluorescein isothiocyanate. (c) FISH evidence of immunoglobulin heavy-chain (*IGH*) rearrangement by the split signals of the *IGH* FISH DNA probe is shown. (d) CISH revelation of the *IGH* FISH DNA probe split signals is shown. Note the split blue and red signals in the nuclei.

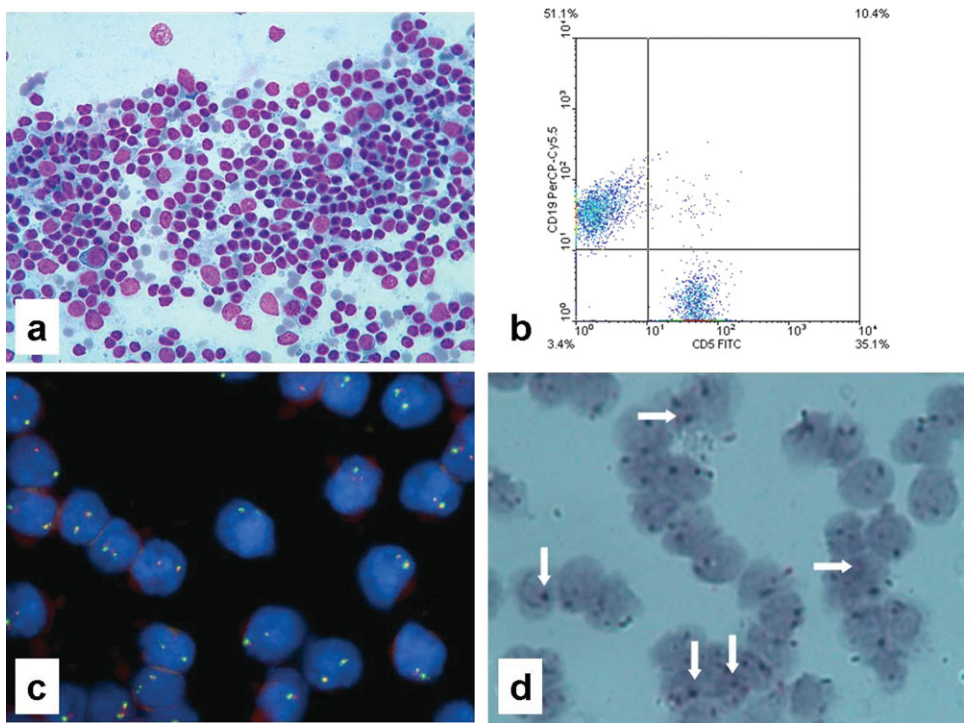

FIGURE 3. Results of cytology, flow cytometry (FC), fluorescence in situ hybridization (FISH), chromogenic in situ hybridization (CISH) in case 5 (a reactive pleural effusion) are shown. (a) Cytological features demonstrate a relatively polymorphous cell population of small, medium, and large lymphoid cells (Diff-Quik stain, × 430). (b) FC demonstrates 2 positive cell populations: cluster of differentiation (CD) 5 and CD19. PerCP indicates Peridinin-chlorophyll-protein complex; FITC, fluorescein isothiocyanate. (c) FISH evidence of *IGH* integrity by the split-signal *IGH* FISH DNA probe is shown. Note the fusion signals in the nuclei. (d) CISH revelation of the *IGH* FISH DNA probe split signals is shown. Note the joint or overlapping blue and red signals in the nuclei.

Table 3. FISH-CISH False-Negative Results and Possible Explanations Compared With FNC/FC and Histology/Clinical Follow-Up

No. of FISH-CISH False-Negative Cases	FNC/FC	Histology or Follow-Up	Possible Explanations
2	RH	DLBCL	Lack of t(14;18)
6	FL	FL	Insufficient number of split signals detected (<10%)
30	DLBCL	DLBCL	Lack of t(14;18)
40	SLL/CLL	SLL/CLL	Lack of any translocation involving the *IGH* locus
47	MCL	MCL	Insufficient no. of split signals (<10%) detected
48	SLL/CLL	SLL/CLL	Lack of any translocation involving the *IGH* locus

Abbreviations: DLBCL, diffuse large B-cell lymphoma; FC, flow cytometry; FISH-CISH, fluorescence in situ hybridization-chromogenic in situ hybridization; FL, follicular lymphoma; FNC, fine-needle cytology; *IGH*, immunoglobulin heavy-chain; MCL, mantle cell lymphoma; RH, reactive hyperplasia; SLL/CLL, small lymphocytic lymphoma/chronic lymphocytic leukemia.

The positive predictive value was 100% and the negative predictive value was 82%.

FISH-CISH Results

The signal score, performed according to the guidelines provided, produced comparable results at the time of FISH and CISH analysis. There was disagreement in only 1 case (case 8) in which the FISH score of split signals was < 10% but was found to be 15% at CISH. Comparing inadequate cases obtained by FISH-CISH and FC, 1 case was found to have an inadequate result with both techniques (case 15); the remaining 5 cases, which were determined to be inadequate at the time of FISH-CISH analysis, were diagnosed by FC. FISH-CISH analysis produced the following results: 29 positive, 15 negative, and 6 inadequate cases (Figs. 2c and 2d and 3c and 3d). Comparing FISH-CISH results with the final diagnoses of all the cases obtained by histology and/or follow-up, there were 29 true-positive cases (66%), 9 true-negative cases (20%), 6 false-negative cases (14%), and 0 false-positive cases (0%). On statistical analysis, sensitivity was found to be 83%, specificity was 100%, the positive predictive value was 100%, and the negative predictive value was 60%. Focusing on the 6 false-negative cases (Table 3), the corresponding NHL were DLBCL (2 cases), SLL/CLL (2 cases), FL (1 case), and MCL (1 case); 2 of these cases also were found to be false-negative on FC (1 DLBCL and 1 FL). The FISH-CISH results are summarized in Table 2.

PCR Results

A sizeable amount of DNA (range, 8 ng/μL-40 ng/μL) was obtained from 30 of the 50 cases; 18 of these were found to be monoclonal and 12 were found to be polyclonal on PCR analysis. For the remaining 20 cases, PCR could not be performed. When comparing PCR data with the final diagnosis made on the basis of histology and/or follow-up, there were 18 true-positive cases (60%), 6 true-negative cases (20%), 6 false-negative cases (20%), and 0 false-positive cases (0%). The statistical analysis demonstrated a sensitivity of 75% and a 100% specificity. The positive predictive value was 100% and the negative predictive value was 50%.

False-Negative Results and Histotypes

The evaluation of the *IGH* FISH-CISH DNA probe as well as the FC and PCR data compared with the final histological diagnoses did not demonstrate any false-positive cases. There were 6 false-negative cases using FISH-CISH (Table 3), 2 using FC, and 6 using PCR. The specific subtypes of NHL resulting in false-negative findings with the different procedures were distributed as follows: 2 DLBCL cases, 1 FL case, 2 SLL/CLL cases, and 1 MCL case on FISH-CISH; 1 case of DLBCL and 1 case of FL on FC; and 3 cases of FL, 2 SLL/CLL cases, and 1 case of DLBCL on PCR.

DISCUSSION

The use of FNC has become widespread in the diagnosis of lymphoma because the combined application of cytological features, FC, PCR, and FISH allows for a correct diagnosis and classification in a high percentage of cases.[1-17,29-40] However, in all these studies, there were still several inconclusive and false-negative results.[6-9,29-36] Apart from high-grade lymphomas with evident

cytological atypia in low-grade NHL, this goal may be achieved by the demonstration of light-chain restriction and/or specific pathological phenotypes on FC, or by heavy-chain or light-chain rearrangement on PCR. FISH has been demonstrated to be a powerful tool in the identification of specific and/or additional aberrant translocations.[10-13,37-39] FISH analysis on interphase nuclei is generally performed using 2 different types of probes: the fusion-signal FISH probe and the split-signal FISH probe. The fusion-signal FISH approach uses 2 fluoresceinated probes that hybridize 2 regions proximal to the breakpoint of 2 chromosomes involved in a supposedly reciprocal translocation. In the absence of any chromosome aberration, 2 pairs of distinct 2-color signals are generally detected. Conversely, in the case of a specific translocation involving the labeled loci, the 2 differently labeled probes will be juxtaposed giving fusion signals. Split-signal FISH probes hybridize 2 regions of the same chromosome proximal to a supposed breakpoint. In normal cells, the 2 hybridized regions are proximal to each other and 2 fusion signals are generated. In the case of a translocation involving the *IGH* locus, whatever the fate of the sequence detached, 2 distinct split signals are observed. The split-signal FISH has some advantages over the fusion-signal FISH; in fact, the detection of a translocation is independent of the partner genes involved and is useful for detecting translocations involving multiple partner genes, as is the case of the *IGH* locus. Another generally considered advantage of split-signal FISH is the absence of the false-positive cases that have been reported using the fusion-signal FISH probes.[41,42]

Because the majority of low-grade NHL cases are characterized by specific translocations, the FISH technique is highly effective in the classification of specific NHL subtypes whether applied on histological or cytological samples.[10-17] Moreover, the identification of multiple reciprocal translocations such as 14 to18 plus 8 to 14 has added prognostic and predictive value to the method.[40] FISH probes with fusion signals are generally used on the basis of microscopic and phenotypic features; in fact, in a flow chart by Zhang et al regarding FNA of lymph nodes,[11] FISH is the last step of a diagnostic algorithm generally used in ambiguous situations (ie, SLL/CLL vs MCL, etc). As reported earlier, the split-signal IGH FISH-CISH DNA probe should detect any translocation involving the *IGH* locus at chromosome 14q32, despite any other chromosome being involved, as shown in Figure 1b. Therefore, this probe promises to be highly sensitive but not specific for any of the corresponding entities. In fact, on histological samples, this *IGH* split probe, other than in FL and MCL, resulted in positive findings in 14% of MM cases, 13% of CLL cases, and 50% of DLBCL cases.[43,44] In the current study, the *IGH* FISH DNA probe identified 29 NHL cases (78% of all NHL cases), namely 11 FL, 5 SLL/CLL, 4 MCL, 4 DLBCL, 2 MZL, and 3 NHL-NOS cases; therefore, we demonstrated that the probe can identify most instances of B-cell NHL, independent of the specific subtype. Because *IGH* translocations are generally detectable in only 50% to 60% of all B-cell lymphomas,[18-26] the rate of positivity in the current study (78%) is unusually high. However, when examining the corresponding subtypes (Table 1), much of the latter is characterized by *IGH* translocations; therefore, the corresponding high positivity rate should not be surprising. With regard to false-negative findings, these were obtained in different subtypes (Table 3). In 2 SLL/CLL cases (cases 40 and 48) and 2 DLBCL cases (cases 2 and 30), false-negative results were most likely determined by the absence of any translocation involving the *IGH* locus and in 1 FL case (case 6) and 1 MCL case (case 47) by the low rate of split signals detected ($<$ 10%) (Table 3). Finally, the possible relation between false-negative findings and aberrant breakpoints was also considered.[19] However the *IGH* split-signal DNA probe spans much of the constant and variable regions of the *IGH*; therefore, we believe that split signals should be generated even in the case of aberrant breakpoints. Comparing the sensitivity of FISH-CISH with the other methods used in clonality assessment, we obtained the highest sensitivity with FC followed by FISH. With regard to PCR values, the low sensitivity may depend on the small number of cases (30 cases); the different passes used to obtain additional material; and the high degree of specificity that it is required for PCR assessment in our laboratory, as reported above. With regard to the *IGH* FISH DNA probe, the current study is to our knowledge the first in which the probe has been used and it is possible that, with increasing experience, sensitivity could improve as well. In some studies,[10-12] PCR and FISH have produced complementary results that are sometimes mutually exclusive, thereby contributing synergistically to the diagnosis. In the current study, 3 of the 6 cases found to be inadequate on FISH analysis were diagnosed by PCR. These data

appear to support the complementary role of the 2 methods, whereas in the remaining 3 cases that were found to be inadequate on FISH, PCR was not performed for the reason discussed above. The size and the quality of the samples represent other aspects of these techniques that may influence their sensitivity and efficacy. In fact, FC needs vital suspended cells; moreover, to obtain a gate of at least 5000 vital cells (the standard size of the samples for a reliable FC analysis), > 200,000 suspended cells are generally required.[45,46] PCR can detect clonality using very small amounts of DNA; in fact, it has been calculated that just a few cells might be sufficient to detect IGH rearrangement.[47] Concerning the sensitivity of PCR, in the current study, we defined as positive any result in which 2 of 3 framework-amplified regions demonstrated a monoclonal pattern. Therefore, we did not obtain false-positive results, but this cutoff was in part responsible for the 4 false-negative findings. Finally, for the FISH analysis, only 200 well-preserved nuclei are required and this characteristic confers additional feasibility to the method. Despite the cytospin evaluation reported for this study, in 2 of 6 cases determined to be inadequate on FISH, cellularity was not high in the corresponding cytospin preparations and, together with the remaining 4 inadequate cases in which cellularity was sufficient, it was inadequate at the time of FISH-CISH evaluation. A possible explanation might be the length of time the lymphoid cells had remained in PBS before cytospin preparation. A prolonged PBS permanence might have affected the hybridization efficiency by hampering individual cell evaluation, clear signal detection, and straightforward cell scoring, as suggested by da Cunha Santos et al.[10]

Some studies have successfully used the FISH method in destained slides as well[10,15,37]; we did not, but it is conceivable that the *IGH* FISH-CISH DNA probe should also be suitable on small samples and archived material. As reported earlier, the *IGH* FISH DNA probe may be assessed by CISH further because the 2 fluorochrome-labeled DNA fragments may be detected using corresponding monoclonal antibodies. In our experience, CISH evaluation after FISH offered additional advantages: first, it allowed a double check of the former FISH data; in addition, the persistence of a signal beyond the decay of the fluorescence allowed a protracted reaction. In fact, in a case that was negative on the FISH assay (case 8), the CISH analysis demonstrated detectable split signals in 15% of the nuclei and allowed for the reclassification of the case as positive, thereby improving the sensitivity of the method. In conclusion, the split-signal *IGH* FISH-CISH DNA probe is an effective tool for the detection of any translocation involving the *IGH* locus and hence the clonality on B-cell lymphoproliferative processes. It can also be used on different samples from different B-cell lymphoproliferative processes, although it is not useful for subclassifying NHL. The split-signal *IGH* FISH-CISH DNA probe might be considered in the early steps of the cytological diagnosis of NHL, mainly when other procedures are ineffective or unavailable.

FUNDING SUPPORT

No specific funding was disclosed.

CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

REFERENCES

1. Dey P. Role of ancillary techniques in diagnosing and subclassifying non-Hodgkin's lymphomas on fine needle aspiration cytology. *Cytopathology*. 2006;17:275-287.
2. Robins DB, Katz RL, Swan F Jr, Atkinson EN, Ordonez NG, Huh YO. Immunotyping of lymphoma by fine-needle aspiration. A comparative study of cytospin preparations and flow cytometry. *Am J Clin Pathol*. 1994;101:569-576.
3. Zardawi IM, Jain S, Bennet G. Flow-cytometric algorithm on fine-needle aspirates for the clinical workup of patients with lymphoadenopathy. *Diagn Cytopathol*. 1998;19:274-278.
4. Landgren O, Porwit MacDonald A, Tani E. A prospective comparison of fine-needle aspiration cytology and histopathology in the diagnosis and classification of lymphomas. *Hematol J*. 2004;5:69-76.
5. Zeppa P, Marino G, Troncone G, et al. Fine-needle cytology and flow cytometry immunophenotyping and subclassification of non-Hodgkin lymphoma. A critical review of 307 cases with technical suggestions. *Cancer (Cancer Cytopathol)*. 2004;102:55-65.
6. Bangerter M, Brudler O, Heinrich B, Griesshamnuer M. Fine needle aspiration cytology and flow cytometry in the diagnosis and subclassification of non-Hodgkin's lymphoma based on the World Health Organization classification. *Acta Cytol*. 2007;51:390-398.
7. Wakely PE Jr. Fine-needle aspiration cytopathology in diagnosis and classification of malignant lymphoma: accurate and reliable? *Diagn Cytopathol*. 2000;22:120-125.
8. Cartagena N Jr, Katz RL, Hirsch-Ginsberg C, Childs CC, Ordonez NG, Cabanillas F. Accuracy of diagnosis of malignant lymphoma by combining fine-needle aspiration cytomorphology with immunocytochemistry and in selected cases, Southern blotting of aspirated cells: a tissue-controlled study of 86 patients. *Diagn Cytopathol*. 1992;8:456-464.
9. Zeppa P, Vigliar E, Cozzolino I, et al. Fine needle aspiration cytology and flow cytometry immunophenotyping of non-Hodgkin lymphoma: can we do better? *Cytopathology*. 2010;21:300-310.
10. da Cunha Santos G, Ko HM, Geddie WR, et al. Targeted use of fluorescence in situ hybridization (FISH) in cytospin

preparations: results of 298 fine needle aspirates of B-cell non-Hodgkin lymphoma. *Cancer (Cancer Cytopathol)*. 2010;118:250-258.
11. Zhang S, Abreo F, Lowery-Nordberg M, Veillon DM, Cotelingam JD. The role of fluorescence in situ hybridization and polymerase chain reaction in the diagnosis and classification of lymphoproliferative disorders on fine-needle aspiration. *Cancer (Cancer Cytopathol)*. 2010;118:105-112.
12. Monaco SE, Teot LA, Felgar RE, Surti U, Cai G. Fluorescence in situ hybridization studies on direct smears: an approach to enhance the fine-needle aspiration biopsy diagnosis of B-cell non-Hodgkin lymphomas. *Cancer (Cancer Cytopathol)*. 2009;117:338-348.
13. Caraway NP, Thomas E, Khanna A, et al. Chromosomal abnormalities detected by multicolor fluorescence in situ hybridization in fine-needle aspirates from patients with small lymphocytic lymphoma are useful for predicting survival. *Cancer (Cancer Cytopathol)*. 2008;114:315-322.
14. Richmond J, Bryant R, Trotman W, Beatty B, Lunde J. FISH detection of t(14;18) in follicular lymphoma on Papanicolaou-stained archival cytology slides. *Cancer (Cancer Cytopathol)*. 2006;108:198-204.
15. Kishimoto K, Kitamura T, Fujita K, Tate G, Mitsuya T. Cytologic differential diagnosis of follicular lymphoma grades 1 and 2 from reactive follicular hyperplasia: cytologic features of fine-needle aspiration smears with Pap stain and fluorescence in situ hybridization analysis to detect t(14;18)(q32;q21) chromosomal translocation. *Diagn Cytopathol*. 2006;34:11-17.
16. Caraway NP, Gu J, Lin P, Romaguera JE, Glassman A, Katz R. The utility of interphase fluorescence in situ hybridization for the detection of the translocation t(11;14)(q13;q32) in the diagnosis of mantle cell lymphoma on fine-needle aspiration specimens. *Cancer (Cancer Cytopathol)*. 2005;105:110-118.
17. Safley AM, Buckley PJ, Creager AJ, et al. The value of fluorescence in situ hybridization and polymerase chain reaction in the diagnosis of B-cell non-Hodgkin lymphoma by fine-needle aspiration. *Arch Pathol Lab Med*. 2004;128:1395-1403.
18. Swerdlow SH, Campo E, Harris NL, et al, eds. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Vol 2. Lyon, France: IARC; 2008.
19. Nagel I, Akasaka T, Klapper W, et al. Identification of the gene encoding cyclin E1 (CCNE1) as a novel IGH translocation partner in t(14;19)(q32;q12) in diffuse large B-cell lymphoma. *Haematologica*. 2009;94:1020-1023.
20. Fenton JA, Schuuring E, Barrans SL, et al. t(3;14)(p14;q32) results in aberrant expression of FOXP1 in a case of diffuse large B-cell lymphoma. *Genes Chromosomes Cancer*. 2006;45:164-168.
21. Cavazzini F, Hernandez JA, Gozzetti A, et al. Chromosome 14q32 translocations involving the immunoglobulin heavy chain locus in chronic lymphocytic leukaemia identify a disease subset with poor prognosis. *Br J Haematol*. 2008;142:529-537.
22. Aoun P, Blair HE, Smith LM, et al. Fluorescence in situ hybridization detection of cytogenetic abnormalities in B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma. *Leuk Lymphoma*. 2004;45:1595-1603.
23. Baro C, Salido M, Domingo A, et al. Translocation t(9;14)(p13;q32) in cases of splenic marginal zone lymphoma. *Haematologica*. 2006;91:1289-1291.
24. Tsai AG, Lu Z, Lieber MR. The t(14;18)(q32;q21)/IGH-MALT1 translocation in MALT lymphomas is a CpG-type translocation, but the t(11;18)(q21;q21)/API2-MALT1 translocation in MALT lymphomas is not. *Blood*. 2010;115:3640-3641.
25. Streubel B, Vinatzer U, Lamprecht A, Raderer M, Chott A. T(3;14)(p14.1;q32) involving IGH and FOXP1 is a novel recurrent chromosomal aberration in MALT lymphoma. *Leukemia*. 2005;19:652-658.
26. Bergsagel PL, Kuehl WM. Chromosome translocations in multiple myeloma. *Oncogene*. 2001;20:5611-5622.
27. Berget E, Helgeland L, Molven A, Vintermyr OK. Detection of clonality in follicular lymphoma using formalin-fixed, paraffin-embedded tissue samples and BIOMED-2 immunoglobulin primers. *J Clin Pathol*. 2011;64:37-41.
28. van Dongen JJ, Langerak AW, Bruggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia*. 2003;17: 2257-2317.
29. Young NA, Al-Saleem T. Diagnosis of lymphoma by fine-needle aspiration cytology using the revised European-American classification of lymphoid neoplasms. *Cancer (Cancer Cytopathol)*. 1999;87:325-345.
30. Nicol TL, Silberman M, Rosenthal DL, Borowitz MJ. The accuracy of combined cytopathologic and flow cytometric analysis of fine-needle aspirates of lymph nodes. *Am J Clin Pathol*. 2000;114:18-28.
31. Siebert JD, Weeks MLT, List LW, et al. Utility of flow cytometry immunophenotyping for the diagnosis and classification of lymphoma in community hospital clinical needle aspiration/biopsies. *Arch Pathol Lab Med*. 2000;124:1792-1799.
32. Dong HY, Harris NL, Preffer FI, Pitman MB. Fine-needle aspiration biopsy in the diagnosis and classification of primary and recurrent lymphoma: a retrospective analysis of the utility of cytomorphology and flow cytometry. *Mod Pathol*. 2001;14:472-481.
33. Kaleem Z, White G, Vollmer RT. Critical analysis and diagnostic usefulness of limited immunophenotyping of B-cell non-Hodgkin lymphomas by flow cytometry. *Am J Clin Pathol*. 2001;115:136-142.
34. Young NA, Al-Saleem TI, Ehya H, Smith MR. Utilization of fine-needle aspiration cytology and flow cytometry in the diagnosis and subclassification of primary and recurrent lymphoma. *Cancer (Cancer Cytopathol)*. 1998;84:252-261.
35. Jeffers MD, Milton J, Herriot R, McKean M. Fine needle aspiration cytology in the investigation of non-Hodgkin's lymphoma. *J Clin Pathol*. 1998;51:189-196.
36. Meda BA, Buss DH, Woodruff RD, et al. Diagnosis and subclassification of primary and recurrent lymphoma. The usefulness and limitations of combined fine-needle aspiration cytomorphology and flow cytometry. *Am J Clin Pathol*. 2000;113:688-699.
37. Bentz JS, Rowe LR, Anderson SR, Gupta PK, McGrath CM. Rapid detection of the t(11;14) translocation in mantle cell lymphoma by interphase fluorescence in situ hybridization on archival cytopathologic material. *Cancer (Cancer Cytopathol)*. 2004;102: 124-131.
38. Gong Y, Caraway N, Gu J, et al. Evaluation of interphase fluorescence in situ hybridization for the t(14;18)(q32;q21) translocation in the diagnosis of follicular lymphoma on fine-needle aspirates: a comparison with flow cytometry immunophenotyping. *Cancer (Cancer Cytopathol)*. 2003;99:385-393.
39. Caraway NP, Du Y, Zhang HZ, Hayes K, Glassman AB, Katz RL. Numeric chromosomal abnormalities in small lymphocytic and transformed large cell lymphomas detected by fluorescence in situ hybridization of fine-needle aspiration biopsies. *Cancer (Cancer Cytopathol)*. 2000;90:126-132.
40. Elkins CT, Wakely PE Jr. Cytopathology of "double-hit" non-Hodgkin lymphoma. *Cancer (Cancer Cytopathol)*. 2011;119:263-271.

41. van Rijk A, Mason D, Jones M, et al. Translocation detection in lymphoma diagnosis by split-signal FISH: a standardised approach. *J Hematop*. 2008;1:119-126.
42. van der Burg M, Poulsen TS, Hunger SP, et al. Split-signal FISH for detection of chromosome aberrations in acute lymphoblastic leukemia. *Leukemia*. 2004;18:895-908.
43. Hwang Y, Lee JY, Mun YC, Seong CM, Chung WS, Huh J. Various patterns of IgH deletion identified by FISH using combined IgH and IgH/CCND1 probes in multiple myeloma and chronic lymphocytic leukemia. *Int J Lab Hematol*. 2011;33:299-304.
44. Bernicot I, Douet-Guilbert N, Le Bris MJ, Herry A, Morel F, De Braekeleer M. Molecular cytogenetics of IGH rearrangements in non-Hodgkin B-cell lymphoma. *Cytogenet Genome Res*. 2007;118:345-352.
45. Jorgensen JL. State of the Art Symposium: flow cytometry in the diagnosis of lymphoproliferative disorders by fine-needle aspiration. *Cancer (Cancer Cytopathol)*. 2005;105:443-451.
46. Katz RL. Modern approach to lymphoma diagnosis by fine-needle aspiration: restoring respect to a valuable procedure. *Cancer (Cancer Cytopathol)*. 2005;105:429-431.
47. Roth J, Daus H, Trumper L, Gause A, Salamon-Looijen M, Pfreundschuh M. Detection of immunoglobulin heavy-chain gene rearrangement at the single-cell level in malignant lymphomas: no rearrangement is found in Hodgkin and Reed-Sternberg cells. *Int J Cancer*. 1994;57:799-804.