ORIGINAL ARTICLE

Role of polymerase chain reaction and immunocytochemistry in the cytological assessment of lymphoid proliferations

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J Clin Pathol 2006;59:1160–1165. doi: 10.1136/jcp.2005.032987

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Accepted for publication
11 October 2005
Published Online First
13 March 2006

Background: Fine-needle aspiration cytology (FNAC) is used as a screening test to evaluate lymphadenopathy. The combined use of genetic analysis and flow cytometry for immunophenotyping has increased the accuracy of diagnosis and correct categorisation of lymphomas on cytological preparations.

Aim: To show the utility of immunocytochemistry and polymerase chain reaction (PCR) in the evaluation of cytological preparations of lymph nodes.

Methods: Fine needle aspirates were obtained from 33 patients (initial presentation, n = 27; recurrence, n = 6). Routine examination was undertaken using immunocytochemistry and DNA PCR to detect clonality and specific translocations. The cytodiagnosis and subclassification of lymphoma was correlated with histological diagnosis in the available follow-up biopsies.

Results: 14 patients had a cytological diagnosis of non-Hodgkin's lymphoma (NHL), 4 had suspected NHL, 2 had atypical lymphoid proliferation and 13 had reactive hyperplasia. A World Health Organization (WHO) subtype was suggested in 8 patients. Incorporating the results of immunoglobulin heavy chain (IgH) and T-cell receptor (TCR) gene rearrangements enabled diagnosis of lymphoma in 17 patients, including 5 of the 6 patients suspected to have NHL or an atypical lymphoid proliferation. Identification of the translocations t(14;18) and t(2;5) helped WHO categorisation in 3 of the patients. The cytological findings were confirmed in 12 out of the 13 patients for whom histological follow-up was available. Seven of the 18 lymphoma patients were managed without a subsequent biopsy. We made one false-positive diagnosis of B-cell NHL on cytology.

Conclusion: The use of immunocytochemistry and PCR is valuable in the definitive diagnosis and subtyping of malignant lymphomas on cytological preparations. The use of these techniques may avoid lymph node biopsies in some cases and allow definitive treatment based on aspirate findings alone.

Fine-needle aspiration cytology (FNAC) is widely used in the initial evaluation of lymphadenopathy. It is most useful in the identification of metastatic carcinoma, but there is continued scepticism about its role in the management of lymphomas, with most centres in the UK not using FNAC for diagnosis and typing of lymphomas. Rather, FNAC is usually used as a screening tool to identify those lymph nodes that should be excised for definitive diagnosis and typing of lymphomas. The European Society of Medical Oncology guidelines recommend open biopsy rather than FNAC for diagnosis and treatment of Hodgkin's and non-Hodgkin's lymphomas (NHLs).^{1,2} The factors that support the continued use of histology for primary diagnosis of lymphomas are inability to provide an accurate Revised European–American classification of Lymphoid neoplasms (REAL) or World Health Organization (WHO) subtype, low diagnostic sensitivity of FNAC for certain types of lymphomas, limited haematological experience of cytopathologists, poor cellularity of specimens, sampling errors and limited availability of archival FNAC material.^{3,4} The adoption of the WHO classification of lymphomas, with its emphasis on cytomorphology rather than architectural pattern, with the use of fluorescence in situ hybridisation (FISH), polymerase chain reaction (PCR) and flow cytometry for immunophenotypical and genetic analysis, has enhanced the role of FNAC in the diagnosis of lymphomas.^{5–10}

We evaluate new lymphadenopathy by FNAC, performing subsequent excision biopsy if there is a cytological diagnosis

of malignant lymphoma, suspected lymphoma or an atypical lymphoid proliferation on cytological preparations. A precise subclassification is not required.

The aim of this study is to show the clinical utility of immunocytochemistry and PCR as an adjunct to morphology in the evaluation of lymphoid proliferations in a setting where FNAC is used as a screening test.

MATERIALS AND METHODS

Fine-needle aspirate samples

The study comprised a retrospective analysis of 33 lymph node fine-needle aspirate (FNA) samples between January 2003 and May 2005. The cases included reactive lymphoid hyperplasia and NHL. Informed consent was obtained from patients before aspiration.

Free-hand FNA was carried out on peripheral lymph nodes in 29 patients. Two to three passes were made using a 23-G needle, a 10-ml syringe and a Cameco syringe holder. A computed tomography-guided FNA of abdominal or mediastinal lymph nodes was obtained from the other four patients. A cytotechnologist assessed specimen adequacy

Abbreviations: DLBCL, diffuse large B cell lymphoma; FISH, fluorescence in situ hybridisation; FNA, fine-needle aspiration; FNAC, fine-needle aspiration cytology; IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain; NHL, non-Hodgkin's lymphoma; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TCR, T cell receptor; WHO, World Health Organization

Figure 1 (A–C) Patient 6. (A) Diff Quick-stained smear from low-grade follicular lymphoma shows medium-sized and small lymphoid cells with relatively few large lymphoid cells. (B) κ immunostaining on the surface or in the cytoplasm of tumour cells. (C) λ immunostaining is positive on the surface or in the cytoplasm of few cells only. (D–F) Patient 14. (D) Diff Quick-stained smear from atypical marginal-zone hyperplasia reported as malignant lymphoma on cytological examination shows preponderance of large centroblast-type cells. (E) κ immunostaining is positive in few cells. (F) λ immunostain positivity in large cells suggests λ light chain restriction. (G–I) Patient 3. (G) Diff Quick-stained smear from large B cell lymphoma arising in post-transplant lymphoproliferative disorder shows large cells with prominent apoptosis. (H) κ and (I) λ immunostains show nuclear staining and hence are not interpretable.

immediately in these cases. In 27 patients FNA was carried out for initial evaluation of lymphadenopathy and six patients had a known history of lymphoma.

Morphological assessment

The lymph node aspirates were reported as benign, atypical, suspicious or diagnostic of malignant lymphoma.

Immunocytochemistry

To prepare material for immunocytochemistry, we washed out the syringe with phosphate-buffered saline (PBS, pH 7.0; Sigma, Steinheim, Germany) or Roswell Park Memorial Institute (RPMI)-1640 (Sigma) medium. Cells were recovered by centrifugation and resuspended in ammonium chloride (BDH, Poole, UK) to lyse red blood cells. After further centrifugation, the cells were suspended in 3% polyethylene glycol (Merck, Hoddlesdon, UK) in 50% alcohol. Immunocytochemistry was carried out on cytopsins prepared from this suspension using the streptavidin avidin–biotin complex or horseradish peroxidase (Dako, DakoCytomation, Glostrup, Denmark) method. The antibody panel included CD20 (Dako; 1:20), CD3 (Dako; 1:50), CD5 (Novocastra, Gateshead, UK; 1:5), CD30 (Dako; 1:10), CD15 (Dako; 1:10), bcl-2 (Dako; 1:100), cyclin D1 (Labvision, Fremont,

California, USA; 1:50), CD10 (Novocastra; 1:5), MIB-1 (Dako; 1:50) and κ and λ light chains (Dako; 1:5000). The antibodies used for diagnosis of lymphoma and subclassification in individual patients depended on the morphological results.

κ and λ light chain staining was carried out on all samples to assess the clonality.

DNA preparation, PCR and sequencing

After immunocytochemistry, the residual fixed cells were used for PCR. B Cell or T cell receptor (TCR) gene rearrangements were requested in all cases. Specific translocations were looked for in patients with suggestive clinical history and morphological or immunocytochemical findings.

High-molecular-weight genomic DNA was isolated from the FNA using the Puregene DNA purification kit (Gentra Systems, Minneapolis, Minnesota, USA). Immunoglobulin heavy chain (IgH) and T Cell receptor (TCR) γ gene rearrangements were carried out as previously described.¹¹ Briefly, DNA was amplified for complete VDJH (Variable, V_H , diversity, DH Joining, J_H) rearrangements with three different sets of family-specific primers covering the three framework regions to detect B cell clones. TCR γ was used to amplify the DNA and assess the presence of a clonal T cell population.

PCR products were analysed on an ABI 310 gene analyser (Applied Biosystems, Warrington, Cheshire, UK), with clonal populations defined as one or two peaks.

PCR assays for the detection of *bcl-2*¹¹ and anaplastic lymphoma kinase-nucleophosmin (*ALK-NPM*)¹² are as described previously.

Amplification of incomplete DJH rearrangements was carried out as described previously.¹¹ Amplifiable DNA was determined using primers to the human β globin gene.

Products were purified using the QIAquick PCR purification kit (Qiagen, Crawley, West Sussex, UK) and sequenced directly using 3' J_H consensus primer in an automated ABI 377 DNA Sequencer, using Big-Dye terminators (Applied Biosystems).

RESULTS

Thirteen patients were reported as having benign or reactive lymphoid hyperplasia (table 1). All were polyclonal on immunocytochemistry and 11 patients showed no immunoglobulin or TCR gene rearrangements. Amplifiable DNA was not found in the other two patients. Follow-up excision biopsies conducted in two patients showed reactive hyperplasia, thus confirming the cytological diagnosis. Lymph node biopsy was not performed in the remaining 11 patients in the 6 to 18-month follow-up period.

In all, 14 patients were reported as having malignant lymphoma, 4 patients had suspected NHL and 2 had atypical lymphoid proliferation. WHO subtype was suggested in 8 patients. κ and λ light chain staining showed a polyclonal B cell population in 21 patients, of whom 7 were diagnosed with B cell lymphoma and 8 with a monoclonal B cell population, including 1 false-positive case of lymphoma (fig 1).

IgH gene rearrangements were detected in nine patients, immunoglobulin light chain (IgL) rearrangement in one patient and TCR gene rearrangements in two patients (fig 2). IgH and IgL rearrangements were detected in five patients with suspicious or malignant cytomorphology, but no IgL restriction was seen on immunocytochemistry. The *bcl-2/t(14;18)* translocation was detected in four patients and *Alk/t(2;5)* in one patient. The PCR findings enabled a specific diagnosis and WHO subtyping of peripheral T cell lymphoma: not otherwise specified (*n* = 2), anaplastic large-cell lymphoma (*n* = 1), diffuse large B cell lymphoma (DLBCL, *n* = 5) and follicular lymphoma (*n* = 4) in cases where immunocytochemical data were incomplete or did not allow a definitive diagnosis. Amplifiable DNA was not obtained in three patients with lymphomas.

The cytological diagnosis of recurrent lymphoma was confirmed by histological examination in only two of five patients. Subsequent histological results were available in 9 other patients diagnosed with malignant lymphoma, or atypical or suspected of lymphoma. One HIV-positive patient was diagnosed with atypical marginal zone hyperplasia. This patient had a false-positive cytological diagnosis of malignant lymphoma, with a clonal B cell population being detected on immunocytochemistry and PCR (fig 1). However, PCR carried out on the lymph node biopsy specimen and bone marrow samples failed to show a monoclonal population. At 12 months, follow-up of this patient showed a benign clinical course. In four patients, histology corroborated the cytological diagnosis (incorporating PCR results) and subtype.

In all, 4 (28.4%) of the 13 newly diagnosed lymphomas and 3 of the 5 (60%) recurrent lymphomas were treated without histological verification of the FNA diagnosis.

Overall, in eight patients the results of PCR were essential for cytological diagnosis and WHO categorisation of lymphoma (table 2). Assessment of clonality in reactive lymphoid hyperplasia was most useful when considering a T cell lymphoma in the differential diagnosis (*n* = 2). In

malignant aspirates with necrotic debris, PCR was necessary to identify monoclonal B cells. Two DLBCL aspirates were negative for immunoglobulin gene rearrangement (false negative) but showed *t(14;18)* translocations.

DISCUSSION

We evaluated 33 lymph node FNA samples using morphological examination with immunocytochemistry and PCR. We assessed the contribution of these modalities in the diagnosis and subclassification of NHLs. The cytological diagnoses incorporating the results of immunocytochemistry and PCR included anaplastic large-cell lymphoma (*n* = 1), peripheral T cell lymphoma, not otherwise specified (*n* = 2), and probable Burkitt's lymphoma (*n* = 1), but most samples had DLBCL (*n* = 6) and follicular lymphoma (*n* = 4). There was one false-positive cytodiagnosis of malignant lymphoma and no false-negative diagnoses, although lymph node biopsy was not carried out in most of the cases in which the aspirate was reported as benign.

The adoption of the WHO classification and the use of immunophenotyping or molecular techniques have changed the way FNA of lymph nodes are reported. Diagnostic algorithms for lymphoma suggested by some cytopathologists recommend the use of FNA for primary diagnosis and classification, performing biopsy only where necessary, but this has not gained acceptance outside large centres.^{13–15}

Several studies in the past few years have documented the high sensitivity and specificity of cytology for diagnosis and subtyping of lymphomas when ancillary techniques are used.^{3–10 16 17} In the context of screening patients with lymphadenopathy, it is acceptable to report the FNA as malignant lymphoma, suspected of lymphoma, atypical lymphoid proliferation or benign. A subsequent biopsy for precise subtyping is mandatory for the first two reporting categories and would depend on the clinical circumstances for the atypical or benign cytological diagnoses. When FNA is used in lieu of biopsy, it is essential to provide an accurate diagnosis according to the WHO criteria, incorporating immunophenotypical and genotypical information for treatment and prognosis. Excision biopsy provides ample material for ancillary investigations and is the gold standard for lymphomas, but may not be feasible in all patients, particularly the few who require emergency treatment. Despite haematologists and oncologists (at the Royal Victoria Hospital and the Belfast City Hospital, Belfast, UK) relying on histological diagnosis of lymphomas, more than a third of the patients with lymphoma in this study were treated on the basis of cytological diagnosis alone. With the limited amount of material available from aspirates, careful consideration of clinical parameters and morphology is mandatory before ancillary investigations. Specimen handling and preservation in these situations assumes great importance.

Immunocytochemistry on cytospin smears has the advantage of direct visualisation of the antigen on the tumour cells and has been shown to be comparable to flow cytometry in which such direct visualisation is lost.^{18–20} Flow cytometry has the advantages of multiparametric analysis, automation and objective interpretation. Clonality and characteristic translocations in lymphomas have been detected in FNA specimens using Southern blot analysis, FISH and PCR successfully.^{21–28}

We found immunocytochemistry to be generally reliable but difficult to interpret in cases with necrotic debris. The immunocytochemical panel was limited by sample cellularity and guided by the clinical situation and the morphology. Larger panels were used when material was obtained from deep-seated lesions, where obtaining tissue sample for histology was difficult or samples were not available. Previous studies have used immunocytochemistry for

Table 1 Results of diagnosis of lymphoid proliferations

Patient no	Morphology	Immunocytochemistry	PCR	Cytological diagnosis	Histological diagnosis	I/R	Clinical data
1	Recurrent ALCL	ND	t(2;5)	Recurrent ALCL	Recurrent ALCL	R	
2	PTCL, NOS	ND	TCR+	Recurrent PTCL	Recurrent PTCL	R	
3	PTLD, atypical lymphoid proliferation	P, CD20+, CD10-, MIB-1 ↑	IgH+	Large B cell lymphoma	ND	I	3-year post cardiac transplant
4	Suspected of FL	M, CD20+, CD3-,	IgH+	FL	Grade II FL	I	
5	Malignant lymphoma, NHL	P	IgH+	Large B cell lymphoma	ND	I	Unsuitable for biopsy
6	LG B-NHL	M, CD20+, bcl-2+, CD10-, bcl6-, cyclin D1-	IgH+, t(14;18)	B-NHL, ?FL	Grade II FL	I	
7	Atypical lymphoproliferative disorder	Polyclonal B cells	TCR+	PTCL	PTCL	I	
8	Recurrent NHL	ND	IgH+	Large B cell NHL	ND	R	Previous DLBCL and Kaposi's sarcoma
9	LG B-NHL? FL	P	No DNA	LG B-NHL	ND	R	Previous FL
10	SLL/CLL	M	No DNA	LG B-NHL, ?CLL/SLL	ND	R	Previous CLL/SLL
11	LG B-NHL? FL	M, CD20+, bcl-2+, CD10-	IgH+, t(14;18)	LG B-NHL, FL	ND	I	Abdominal mass. Died, no treatment
12	Benign	P	IgH/TCR-	Benign	Reactive hyperplasia	I	? adult Still's disease
13	Benign	P	IgH/TCR-	Benign	ND	I	Oriental
14	Malignant lymphoma	M CD20+	IgH+	Malignant lymphoma	Atypical marginal zone hyperplasia	I	HIV+, no lymphoma 12 months later
15	Benign	P	IgH-	Benign	ND	R	Previous gastric MALT
16	Benign	P, CD20+, CD3+	IgH/TCR-	Benign	Dermatopathic lymphadenitis	I	
17	Benign	P	IgH-	Benign	ND	I	
18	Benign	P	No DNA	Benign	ND	I	
19	Benign	P	No DNA	Benign	ND	I	
20	Benign	P	IgH/TCR-	Benign	ND	I	
21	Benign	P	IgH/TCR-	Benign	ND	I	
22	Suspected of NHL	P CD 20+ large cells	No DNA	Suspicious of large B cell NHL	DLBCL	I	
23	Benign	P	IgH-	Benign	ND	I	
24	Suppurative lymphadenitis	P, AFB-	IgH-	Benign	ND	I	
25	Benign	P	IgH-	Benign	ND	I	
26	Benign	P	IgH-	Benign	ND	I	
27	HG B-NHL	M CD20+, CD30+, MIB-1 ↑, cyclin D1 -, CD10 -	No DNA	Large B cell lymphoma	ND	I	Mediastinal mass Stridor
28	Suspected of LG B-NHL	P, CD20+	IgL+	LG B-NHL	Grade I FL	I	
29	Suspected of LG B-NHL	ND	IgH+	LG B-NHL	ND	I	
30	Large B-NHL	P, CD20+, CD3-, CD10 -	No DNA	HG B-NHL	BL	I	
	? DLBCL						
	?BL						
31	LG B-NHL	M, CD20+, CD10+, bcl-2+, CD5-, MIB1 ↓	IgH+	LG B-NHL	FL	I	Small core needle bx abdomen
32	HG B-NHL	M	IgH-, t(14;18)+	Large B cell NHL	ND	I	
33	HG B-NHL	P, CD20+, CD30+, CD3 -	IgH- t(14;18)+	HG B-NHL	DLBCL	I	Mediastinal core bx

↑, high; ↓, low; ?, probable; bx, biopsy.

AFB, acid fast bacilli; ALCL, anaplastic large-cell lymphoma; BL, Burkitt's lymphoma; CLL, chronic lymphocytic leukaemia; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; HG, high grade; I, initial presentation; Ig, immunoglobulin; IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain; LG, low grade; M, monoclonal; MALT, mucosa associated lymphoid tissue; MZL, marginal zone lymphoma; ND, not done; NHL, non-Hodgkin's lymphoma; NOS, not otherwise specified; P, polyclonal; PTCL, peripheral T cell lymphoma; PTLD, post-transplant lymphoproliferative disorder; R, recurrence; SLL, small lymphocytic leukaemia; TCR, T cell antigen receptor.

diagnosis,²⁶ but not WHO subclassification of NHL. Our study shows that PCR may refine the cytological diagnosis of malignant lymphoma, particularly when immunophenotyping data are limited. Assessment of clonality by PCR was

Table 2 Contribution of polymerase chain reaction to diagnosis

Clonality for lymphoma diagnosis	5
Specific translocations	5
Supporting ICC assessment of clonality	15
No contribution	7
False negative*	2
False positive	1

ICC, immunocompetent cells

*These two cases were t(14;18) positive.

most helpful for the T cell lymphomas and in the B cell NHLs that were morphologically suspicious but lacked IgL restriction. This is in agreement with the findings of Katz *et al*,²⁹ who investigated the role of genotyping in 63 patients with lymphoproliferative disorder, but contrasts with the study by Stewart *et al*,²⁶ who found PCR to be unhelpful or misleading in a large number of lymph node aspirates. In our study, there was concordance in assessment of clonality between PCR and immunocytochemistry in 18 of 23 (78.2%) samples, and we believe that PCR is a valuable adjunct to immunophenotyping in cases in which a monoclonal population is not identified despite clinical or cytological suspicion of a lymphoma. Clonality was not detected by PCR in two cases of DLBCL. Possible explanations for this include a background of abundant polyclonal cells masking the monoclonal population and hypermutation in the rearranged V_H,

Figure 2 Polymerase chain reaction analysis of immunoglobulin heavy chain (VH-JH) rearrangements from patient 1. Gene scanning of polyclonal and monoclonal cell populations, showing the typical polyclonal gaussian curves and monoclonal peaks. The approximate distribution of the polyclonal gaussian curves is indicated in base pairs. (A) VH-Fr1 products (310–360 bp); (B) VH-Fr2 products (250–295 bp); and (C) VH-Fr3 products (100–170 bp) for sample 1, showing a polyclonal population.

hindering efficient binding of the primers.²⁷ Furthermore, the t(14;18) chromosomal translocation, including the IgH locus, can render one allele unsuitable for VDJ amplification, causing false-negative results.²⁸ Interestingly, clonality in these two cases of DLBCL was supported by demonstration of t(14;18).

A single false-positive diagnosis of malignant lymphoma was made in an HIV-positive patient. This was diagnosed as an atypical marginal-zone hyperplasia on histological examination, with no evidence of monoclonality in the excised lymph node or subsequent bone marrow sample. Maroto *et al*³⁰ reported monoclonal IgH bands in 6% of aspirates from reactive lymphoid processes.

We also found PCR to be particularly helpful in the WHO categorisation of lymphoma in five aspirates in which t(2;5) and t(14;18) translocations and TCR gene rearrangement were identified in 1, 2 and 2 cases, respectively. Morphology and the limited immunophenotypic analysis of cytospin smears helped us to subclassify eight other cases as large B cell lymphoma (n = 5), small lymphocytic lymphoma or chronic lymphocytic leukaemia (n = 1) and low-grade follicular lymphoma (n = 2). Dong *et al*³ reported 77% success in WHO subtyping of lymphoma on aspirates, with best results in cases of small lymphocytic lymphoma, lymphoplasmacytic lymphoma, Burkitt's lymphoma and mantle-cell lymphoma. In their study, subclassification was less than optimal initially, with DLBCL and follicular lymphoma, but improved greatly after cell size, proliferation fraction and CD10 staining were taken into account. As in the study of Nicol *et al*,⁹ many of our patients were reported to have low-grade or high-grade NHL, depending on the preponderance of small or large cells in the aspirates. Although the terms "high grade" and "low grade" are not included in the WHO classification, we believe that this distinction is clinically useful. Although it is possible to comment on the cell size and presence or absence of follicular centre cell phenotype, it is difficult for cytopathologists to label cases as DLBCL or follicular lymphoma without evaluating the architecture. In this respect, an understanding that the WHO classification emphasises the correlation of cytomorphology, immunophenotyping and genotyping as opposed to the architectural

features is critical for cytopathologists to change terminology in the reports.

In conclusion, our study shows that morphology, in conjunction with immunocytochemistry and PCR on material from FNA, has the potential to provide a clinically relevant diagnosis and subtype of NHL in many cases. This may avoid biopsy in deep-seated lesions in which obtaining a tissue sample for histological diagnosis is problematic.

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Competing interests: None declared.

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