Papers

Evaluation of B cell lymphoid infiltrates in bone marrow biopsies by morphology, immunohistochemistry, and molecular analysis

B Maes, R Achten, A Demunter, B Peeters, G Verhoef, C De Wolf-Peeters

Abstract

***Aims*—Morphological criteria to distinguish between reactive and neoplastic B cell lymphocytoid infiltrates in trephines have been defined but are not always reliable. Polymerase chain reaction (PCR) analysis of the CDR3 region of the immunoglobulin heavy chain (IgH) gene which, by demonstrating monoclonality, can provide additional arguments in favour of lymphoid malignancy is now frequently used for the detection and follow up of B cell lymphoma (NHL). The aim of this study was to investigate the usefulness of morphological findings in bone marrow biopsies in comparison with data obtained by PCR analysis.**

***Methods*—Eighty nine bone marrow biopsies displaying lymphoid infiltrates were evaluated by morphology and immunohistochemistry as well as by CDR3-PCR using consensus framework 3 (FRW3) and JH primers.**

***Results*—The presence of a clonal B cell proliferation was demonstrated by PCR analysis in 45 biopsies, including 21 samples considered to be positive, 17 to be suspicious, and seven to be negative by morphology. In the remaining 44 trephines we found no evidence of clonality, although 12 of these trephines were thought to be positive by morphology.**

***Conclusions*—These results, revealing an incomplete correlation between CDR3-PCR data and immunomorphological findings, indicate that molecular analysis may be more sensitive and specific in general. However, false negative PCR results do occur, which emphasises the necessity to combine both diagnostic tools in the evaluation of lymphoid infiltrates.**

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Keywords: bone marrow biopsy; lymphoid infiltrate; polymerase chain reaction

Department of Pathology, University Hospital of Leuven, Minderbroedersstraat 12, 3000 Leuven, Belgium
B Maes
R Achten
A Demunter
C De Wolf-Peeters

Department of Biochemistry, University Hospital of Leuven
B Peeters

Department of Haematology, University Hospital of Leuven
G Verhoef

Correspondence to:
Dr Maes, Laboratory for Histochemistry and Cytochemistry, Minderbroedersstraat 12, 3000 Leuven, Belgium
Brigitte.Maes@uz.kuleuven.ac.be

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The evaluation of bone marrow biopsies has become a standard procedure in the identification, classification, and staging of non-Hodgkin's lymphoma (NHL). In addition to its contribution to diagnosis, the analysis of trephines is also of major importance in monitoring the course of disease and in assessing the response to treatment.[1] The appraisal of bone marrow involvement is traditionally based on morphological examination and has been shown to be prognostically relevant.[2] However, normal bone marrow contains a lymphoid component of 10–16%, which may become hyperplastic in many inflammatory and immune disorders, and may even appear as clear cut aggregates in these conditions. Moreover, bone marrow biopsies of elderly patients are known to display non-malignant aggregates in approximately 50% of cases. On the other hand, immune diseases are associated with an increased incidence of NHL and the frequency of lymphoma in general rises with age.

Because these pitfalls hamper a reliable distinction between reactive and neoplastic conditions, many attempts have been made to define a set of exact morphological guidelines that could unequivocally resolve this issue. Although several criteria, referring to the extent, the size, the location, and the cytology of the infiltrate, have been suggested,[3 4] their application is often problematic, and multiple exceptions to the general rules have been described. In addition, discordance between morphology at the primary lymphoma site and that noted in the bone marrow further complicates a straightforward assessment of bone marrow involvement by NHL. The incidence of such discordance, which seems to bear important prognostic consequences, depends upon the lymphoma subtype and varies between different reports.[5–9]

Immunohistochemical techniques using monoclonal antibodies can provide more information on the extent of bone marrow involvement and on the lineage of the lymphoid cells. The introduction of antibodies specifically immunoreacting with pan-T cell markers such as CD45RO (UCHL1) or CD3 and with the pan-B-cell marker CD20 (L26) has allowed the identification of B cells and T cells in routinely processed, paraffin wax embedded trephines.[10–13] These phenotypic data might help to distinguish between benign and malignant aggregates: homogeneous staining for CD20 favours bone marrow involvement by a

B cell lymphoma, whereas a mixed infiltrate of CD20 and CD45RO/CD3 positive cells suggests a reactive lymphoproliferative process.[14 15] Unfortunately, this criterium is also of limited value because B cell lymphoid nodules of malignant origin are often accompanied by increased numbers of reactive T cells. The use of a more extended panel of antibodies, including anti-Igκ and anti-Igλ, will undoubtedly increase the diagnostic potential, but this is still problematical when using fixed and paraffin wax embedded tissue.

In the past decade, molecular techniques have been widely introduced to demonstrate monoclonality in B cell lymphomas and lymphoproliferative disorders. These techniques are often based on the detection of the unique immunoglobulin heavy chain (IgH) VDJ sequence, resulting from the rearrangement of variable (V), diversity (D), and joining (J) region segments, which occurs in every B cell.[16] This rearrangement, carried by its entire neoplastic progeny, is the molecular signature of the B cell lymphoma. Among all molecular techniques investigating this area of the B cell genome, the polymerase chain reaction (PCR) has been particularly useful, especially since the recognition of consensus primers.[17–21] In addition, PCR can be used on DNA extracted from routinely fixed trephine biopsies.[22]

The purpose of our present study was to compare morphological and immunohistochemical findings on trephines containing a lymphoid infiltrate with the results of PCR analysis of the same paraffin wax embedded material, using framework 3 (FRW3) and JH consensus primers, allowing the detection of the CDR3 region.

In addition, we investigated the extent to which these bone marrow biopsy data correlated with the results of bone marrow smear cytology and with the immunophenotypic findings obtained by fluorescence activated cell sorting (FACS) analysis on the corresponding aspirate.

Materials and methods

CASE SELECTION

Eighty nine consecutive bone marrow biopsies were selected from the database of the department of pathology of the University Hospitals of KU Leuven, Belgium. All 89 samples were chosen on the basis of the presence of a lymphoid infiltrate, without knowledge of the clinical presentation or the clinical history of the patient.

After study of the clinical files, 71 cases were found to be from patients with a known B cell lymphoma. These 71 biopsies were taken either at the time of diagnosis or during follow up. An extramedullary biopsy, mostly taken as the diagnostic sample, was available for further investigation in 38 of these 71 cases. Lymphomas were classified according to the REAL classification.[23] In the other cases (n = 33) the diagnosis was based on blood and/or bone marrow smear findings. Eighteen bone marrow biopsies were taken to evaluate various haematological disorders in patients without confirmed NHL.

MORPHOLOGY AND IMMUNOHISTOCHEMISTRY

Bone marrow biopsies were fixed in B5 for three hours and decalcified in formic acid overnight. All 89 paraffin wax embedded trephines were reviewed on haematoxylin and eosin stained sections. Lymphoid infiltrates were analysed for their growth pattern (nodular, interstitial, or diffuse) and cytological characteristics. When nodular aggregates were present, their number, size, and localisation (intertrabecular or paratrabecular) were evaluated as well. In addition, serial sections were stained for the B cell marker CD20 (L26 antibody; Dakopatts, Glostrup, Denmark) and for the T cell marker CD45RO (antibody UCHL1; Dakopatts), using a peroxidase labelled avidin–biotin complex method.

Trephines were thought to be positive for involvement by B cell NHL if more than two aggregates or two large (threshold: six times the size of an adipocyte) aggregates were present, predominantly composed of B cells. Other findings regarded sufficient to support a diagnosis of lymphomatous involvement were infiltration by a diffuse solid B cell proliferation or by infiltrates predominantly composed of large atypical cells.

The presence of two small aggregates or of one large or paratrabecular aggregate, in both cases predominantly composed of B cells, was believed insufficient but still suspicious for a diagnosis of bone marrow involvement by B cell NHL. Similarly, biopsies showing an interstitial increase of B cells or samples with paratrabecular, mixed B and T cell aggregates were also considered suspicious for NHL involvement.

If only one small B cell aggregate or multiple mixed, intertrabecular localised aggregates were noticed, it was agreed that the bone marrow was not involved and was therefore negative.

PCR ANALYSIS

PCR for the amplification of the IgH CDR3 region was performed on all 89 cases to detect clonal rearrangement of the CDR3 region. PCR was also carried out on the extramedullary diagnostic sample in case PCR analysis of the corresponding bone marrow biopsy showed no monoclonality (20 cases).

Five tissue sections of 20 μm thickness were dewaxed and digested with proteinase K in a volume of 100 μl of proteinase K buffer (10 mM Tris/HCl, pH 7.6, 1 mM EDTA, and 0.5% Tween 20) at 55°C for 12 hours. A 5 μl aliquot of this lysate was used as a template for the IgH CDR3 PCR analysis. Consensus primers directed at the IgH FRW3 region (5'-CTGTCGACACGGCCGTGTATTACTG-3') and the IgH joining region (5'-AACTGCAGAGGAGACGGTGACC-3') were used. The PCR reaction mixture consisted of: primers (2 mM), dNTPs (200 μM), commercially prepared PCR buffer (Perkin Elmer), $MgCl_2$ (25 mM), and Taq Polymerase (5 U/μl). The total reaction volume was 20 μl. Thermocycler conditions were: denaturation at 94°C, annealing at 59°C, and extension at 72°C, each for 40 seconds, with a total number of 50

cycles. All PCR products were electrophoresed in an 8% polyacrylamide gel and visualised with ethidium bromide. Positive and negative controls were included, consisting of the Namalwa cell line DNA and no DNA, respectively. The sensitivity of this IgH CDR3-PCR method was determined using a dilution series of Namalwa DNA in normal peripheral blood DNA and was found to be 10^{-3}.

BONE MARROW SMEARS AND PHENOTYPING BY FACS ANALYSIS ON BONE MARROW ASPIRATES

Cytology and phenotypic data based on the corresponding bone marrow aspirates were available for 81 and 42 cases, respectively. Cytology was considered positive when the smear (if representative) showed that > 20% of lymphocytes displayed atypical features. Smears were considered suspicious if lymphocytes showed atypia but were < 20%. However, if the patient was known to have NHL, and if the cytological features matched those seen in the original diagnostic smear, the aspirate was diagnosed to be involved by lymphoma. A normal lymphocytosis or a slight (20–30%) increase in lymphocytes lacking atypia was considered negative for NHL involvement.

FACS analysis was performed using a panel of antibodies to B cell and T cell antigens. Immunoglobulin light chain restriction (normal κ to λ ratio, 2 : 1) or coexpression of B cell markers and CD5, were taken as reliable indicators for the presence of neoplastic B cells. The latter criterium was only considered indicative for lymphoma if the CD19/CD5 coexpressing population exceeded 5%, because normal bone marrow can contain up to 1% CD5 expressing B cells, and this can be even higher after bone marrow transplantation.[24 25] A slight deviation from the normal κ to λ ratio (< 3 or > 1) was considered to be suspicious. A normal distribution of lymphocytes between different subsets without light chain restriction and without aberrant coexpression of CD5 was

Table 1 Summary of morphological and molecular data on 89 bone marrow (BM) biopsies and cytological and fluorescence activated cell sorter (FACS) analysis data on the corresponding bone marrow (BM) aspirates

		BM biopsy		*BM aspirate*				*BM biopsy*		*BM aspirate*	
Case	*NHL subtype*	*M/IHC*	*PCR*	*Smear*	*FACS*	*Case*	*NHL subtype*	*M/IHC*	*PCR*	*Smear*	*FACS*
1	B-CLL	Pos	Pos	Susp	Pos	46	HCL	Pos	Pos	Pos	Pos
2	B-CLL	Pos	Pos	Neg	ND	47	HCL	Susp	Pos	Neg	Susp
3	B-CLL	Pos	Pos	ND	ND						
4	B-CLL	Pos	Neg	Pos	Pos	48	DLBCL	Pos	Pos	Susp	ND
5	B-CLL	Pos	Neg	Pos	Pos	49	DLBCL	Pos	Pos	Neg	ND
6	B-CLL	Pos	Neg	Pos	Pos	50	DLBCL	Pos	Pos	Neg	ND
7	B-CLL	Pos	Neg	Neg	Pos	51	DLBCL	Pos	Pos	Neg	Neg
8	B-CLL	Susp	Pos	Pos	Pos	52	DLBCL	Pos	Neg	Neg	ND
9	B-CLL	Susp	Pos	Pos	Pos	53	DLBCL	Pos	Neg	ND	ND
10	B-CLL	Susp	Pos	Susp	Pos	54	DLBCL	Susp	Pos	Neg	ND
11	B-CLL	Susp	Pos	Susp	Pos	55	DLBCL	Susp	Pos	Neg	ND
12	B-CLL	Susp	Pos	Neg	Pos	56	DLBCL	Susp	Neg	Pos	ND
13	B-CLL	Susp	Pos	Neg	Pos	57	DLBCL	Susp	Neg	Neg	Neg
14	B-CLL	Susp	Pos	Neg	ND	58	DLBCL	Neg	Neg	ND	ND
15	B-CLL	Susp	Pos	Neg	ND						
16	B-CLL	Susp	Neg	Pos	Pos	59	Small cell, nos	Pos	Pos	Pos	Pos
17	B-CLL	Susp	Neg	Pos	Pos	60	Small cell, nos	Pos	Pos	Pos	Pos
18	B-CLL	Susp	Neg	Neg	Neg	61	Small cell, nos	Pos	Pos	Pos	ND
19	B-CLL	Susp	Neg	Neg	Neg	62	Small cell, nos	Pos	Pos	Susp	ND
20	B-CLL	Neg	Pos	Neg	Pos	63	Small cell, nos	Pos	Pos	Neg	Neg
21	B-CLL	Neg	Neg	Pos	ND	64	Small cell, nos	Susp	Pos	Susp	ND
22	B-CLL	Neg	Neg	Neg	Neg	65	Small cell, nos	Susp	Pos	Susp	Pos
						66	Small cell, nos	Susp	Pos	Neg	ND
23	LPL/I	Pos	Pos	Neg	ND	67	Small cell, nos	Susp	Neg	Neg	Pos
24	LPL/I	Neg	Pos	Susp	Pos	68	Small cell, nos	Susp	Neg	Neg	ND
						69	Small cell, nos	Neg	Pos	Neg	ND
25	MCL	Pos	Pos	Susp	Pos	70	Small cell, nos	Neg	Pos	ND	ND
26	MCL	Pos	Pos	Neg	ND	71	Small cell, nos	Neg	Neg	Neg	ND
27	MCL	Pos	Neg	Neg	ND						
28	MCL	Susp	Pos	Neg	Neg	72	No NHL	Pos	Pos	ND	ND
29	MCL	Susp	Neg	Susp	Pos	73	No NHL	Pos	Neg	Susp	ND
30	MCL	Susp	Neg	Neg	ND	74	No NHL	Pos	Neg	Neg	ND
31	MCL	Neg	Pos	Neg	Neg	75	No NHL	Psos	Neg	Neg	ND
						76	No NHL	Susp	Pos	Neg	ND
32	FCL	Pos	Pos	Susp	Susp	77	No NHL	Susp	Pos	ND	ND
33	FCL	Pos	Pos	Neg	ND	78	No NHL	Susp	Neg	Pos	Pos
34	FCL	Pos	Pos	ND	ND	79	No NHL	Susp	Neg	Susp	Neg
35	FCL	Pos	Neg	Pos	ND	80	No NHL	Susp	Neg	Susp	Neg
36	FCL	Pos	Neg	Neg	ND	81	No NHL	Susp	Neg	Neg	Neg
37	FCL	Susp	Neg	Susp	Pos	82	No NHL	Susp	Neg	Neg	Neg
38	FCL	Susp	Neg	Neg	Neg	83	No NHL	Susp	Neg	Neg	ND
39	FCL	Neg	Neg	Neg	Neg	84	No NHL	Susp	Neg	Neg	ND
						85	No NHL	Susp	Neg	ND	ND
40	nodMZCL	Pos	Pos	Pos	Pos	86	No NHL	Neg	Pos	Neg	ND
41	nodMZCL	Susp	Neg	Neg	ND	87	No NHL	Neg	Neg	Neg	ND
42	nodMZCL	Susp	Neg	Neg	ND	88	No NHL	Neg	Neg	Neg	ND
43	nodMZCL	Neg	Pos	Neg	ND	89	No NHL	Neg	Neg	Neg	ND
44	splenMZCL	Neg	Neg	Susp	Neg						
45	splenMZCL	Neg	Neg	Neg	ND						

B-CLL, B cell chronic lymphocytic leukaemia; DLBCL, diffuse large B cell lymphoma; FCL, follicle centre lymphoma; HCL, hairy cell leukaemia; LPL/I, lymphoplasmacytoid lymphoma/immunocytoma; MCL, mantle cell lymphoma; M/IHC, morphology/immunohistochemistry; ND, not done; Neg, negative for NHL involvement; NHL, non-Hodgkin's lymphoma; nodMZCL and splenMZCL, nodal and splenic marginal zone cell lymphoma, respectively; Pos, positive for NHL involvement; Small cell, nos, small cell lymphoma, not otherwise specified; Susp, suspicious for NHL involvement.

Table 2 Correlation of morphological and molecular findings of 89 bone marrow biopsies containing a lymphoid infiltrate

Morphology		IgH CDR3 rearrangement	
		Clonal	Non-clonal
Positive	33	21 (20/1)	12 (9/3)
Suspicious	39	17 (15/2)	22 (14/8)
Negative	17	7 (6/1)	10 (7/3)
Total	89	45 (41/4)	44 (30/14)

Results in parenthesis are those obtained from patients with a known lymphoma/those obtained from patients without a known lymphoma.
IgH, immunoglobulin heavy chain.

considered as indicative of the absence of lymphomatous cells.

Results

The main data on the 89 cases are summarised in table 1.

Seventy one trephines were obtained from patients known to be affected by B cell NHL. These lymphomas were distributed among the different entities recognised in the REAL classification. The series comprised B cell chronic lymphocytic leukaemia (B-CLL; n = 22), lymphoplasmacytoid lymphoma/immunocytoma (n = 2), mantle cell lymphoma (n = 7), follicle centre lymphoma (n = 8), nodal marginal B cell lymphoma (n = 4), splenic marginal zone B cell lymphoma (n = 2), hairy cell leukaemia (n = 2), diffuse large B cell lymphoma (DLBCL; n = 11), and small cell B cell lymphoma not otherwise specified (n = 13).

Based on the morphological findings in the bone marrow biopsy, cases were subdivided into three groups. Table 2 presents the correlation between the morphology and the molecular results.

Group 1 comprises 33 cases diagnosed as positive for NHL involvement by morphology and immunohistochemistry. Twenty nine of these biopsies were taken from patients for whom the diagnosis of NHL was ascertained by other means. In the remaining four patients, a thorough clinical investigation did not reveal any additional evidence in favour of NHL.

PCR detected a clonal lymphoid population in 21 of the 33 trephines (64%), including 20 of 29 cases (69%) with known NHL. One of four trephines obtained from patients lacking extramedullary lymphoma localisations showed a monoclonal pattern by PCR (case 72).

For seven of nine PCR negative trephines, taken from patients known to have lymphoma, an extramedullary diagnostic biopsy was available for further analysis. In contrast with the corresponding trephines, we could show the presence of a clonal PCR pattern in all seven biopsies.

Group 2 is composed of 39 trephines considered suspicious for bone marrow involvement by B cell NHL, by pure morphology and immunohistochemistry. Twenty nine and 10 of these biopsies were taken from patients with and without additional arguments in support of NHL, respectively.

PCR showed monoclonality in 17 of these 39 (44%). If only the 29 cases with a well established lymphoma are considered, this percentage rises to 52% (15 of 29). A monoclonal pattern was obtained by PCR in two of 10 patients who did not show any other sign of lymphoma (cases 76 and 77).

Six of the eight extramedullary diagnostic biopsies available from the 14 PCR negative patients with lymphoma allowed the demonstration of a clonal PCR pattern.

Group 3 is composed of 17 trephines considered negative for bone marrow involvement by B cell NHL. Thirteen of these biopsies were collected from patients with confirmed NHL, whereas the remaining four were taken from patients without known NHL.

PCR analysis produced evidence of monoclonality in seven of these 17 trephines (41%), including six of the 13 (46%) cases with confirmed NHL and one of four (25%) from patients lacking further evidence in favour of NHL (case 86).

For five of the seven PCR negative cases, taken from patients with confirmed NHL, the extramedullary diagnostic tissue was available for further analysis. PCR was performed on this material and found to be positive in four cases.

Eleven of 89 trephines were obtained from patients affected by DLBCL (cases 48 to 58). Only three of these trephines (cases 48, 50, and 51) showed a predominantly large cell infiltrate, whereas five trephines displayed infiltrates mainly composed of small cells (cases 52, 54, and 56 to 58). The lymphoid cells in the remaining three biopsies (cases 49, 53, and 55) were heterogeneous with regard to their size, comprising a mixture of small and large cells.

In the cases in which the lymphoid infiltrate was composed of either predominantly large cells or a mixture of small and large cells (six cases), PCR demonstrated monoclonality except for one case (case 53). Biopsies showing small cell infiltrates (five cases) all showed a polyclonal PCR pattern, except for one case (case 55). The latter biopsy was considered suspicious when morphological criteria were applied.

Cytology and FACS analysis data were available for 81 and 42 cases, respectively, and are also presented in table 1.

Discussion

We studied the correlation between morphological findings and the results of PCR analysis of IgH gene rearrangements in the evaluation of bone marrow biopsies with lymphoid infiltrates.

PCR detected clonal proliferation in 45 of 89 biopsies (50%), clonality being identified in 21 of 33 biopsies considered to be positive for NHL involvement by morphology. The resultant success rate of 63% is in line with data reported on other (extramedullary) paraffin wax embedded, lymphomatous tissues.[17 18]

False negativity has been ascribed to the failure of the consensus primers to bind to their target sequences. We evaluated this hypothesis by applying an identical PCR assay on extramedullary diagnostic tissue available from the same patient. This investigation was

feasible in 20 cases. In 17 of these 20 extramedullary biopsies, monoclonal IgH rearrangements could be demonstrated, which excludes non-binding of the consensus primers as well as interfering chromosomal translocations, deletions, or mutations in the monoclonal IgH gene as satisfactory explanations for PCR negativity. Alternatively, the failure to demonstrate a neoplastic lymphoid population might be caused by degradation of DNA as a result of the B5 fixation and the decalcification procedure. In addition, competitive amplification of background polyclonally rearranged lymphoid DNA may be responsible for the partial success rate.[26] Sampling error could also be responsible for the apparent false negativity of PCR analysis in a certain number of cases.

Several attempts have been made to reduce the number of false negative results by means of a multi-PCR approach. As such, combining PCR techniques demonstrating translocations (t(11;14) and t(14;18)) with PCR analysis of IgH gene rearrangements resulted in a slightly improved clonality detection rate of 66% in mature B cell neoplasms.[27]

Recently, a comparable PCR method detecting monoclonal rearrangements of the immunoglobulin κ light chain (Igκ) gene has been developed by Gong *et al*. It was shown that the additional analysis of Igκ gene rearrangements can improve PCR sensitivity from 66% to 85%.[27] Once the latter combined approach has proved to be routinely applicable to bone marrow biopsies, it will undoubtedly extend the role of PCR analysis in the evaluation of lymphoid infiltrates.

PCR detected a monoclonal lymphoid population in seven of 17 trephines considered to be negative by morphology. In these cases, the infiltrates were usually very small or they consisted of a mixture of B and T cells. Six of these seven samples were obtained from patients who were known to be affected by NHL. These results may reflect the limitations of morphology, despite the use of well defined criteria, and suggest the validity of molecular techniques in the staging and follow up of patients with lymphoma.

On the other hand, the single case (case 86) in which PCR demonstrated monoclonality despite the absence of any other indication favouring lymphoma questions the absolute specificity of PCR analysis, although the true biological importance of this finding is unclear.

Coad *et al* performed a similar study,[28] comparing the morphological characteristics of the bone marrow biopsy with the results of PCR performed on DNA extracted from the concurrently obtained aspirate. To increase the clonality detection rate, they used various primer sets and only included patients with an established diagnosis of NHL. Nevertheless, PCR was positive in only 57% of the morphologically clear cut positive cases. Moreover, 10 of 11 cases with negative PCR results using bone marrow aspirates were positive when trephine samples were used. These results emphasise the superiority of trephine samples over bone marrow aspirates for the implementation of molecular investigations. In fact, bone marrow involvement is often heterogeneous and focal, which may result in the absence of neoplastic lymphocytes in the aspirate, although clearly present in the bone marrow biopsy. Likewise, an increase in reticulin fibres, which is often provoked by lymphomatous proliferations in bone marrow tissue, may result in decreased aspiration of the neoplastic cells. Finally, lymphoma cells may be diluted into normal co-aspirated blood or bone marrow, resulting in an overall lymphoid population below PCR detection limits. From all the above, it is obvious that sample variation between the biopsy and the aspirate is common and most likely to be the main cause of discordant results.[28]

In our study, we confirm these findings by illustrating the higher validity of bone marrow biopsy investigation compared with the cytological evaluation of the aspirate. The latter appeared negative in 14 cases in which the trephine clearly demonstrated involvement by NHL. On the other hand, FACS analysis on the aspirate was able to confirm almost all cytologically positive cases. FACS also revealed lymphomatous involvement in another 13 cases and additionally showed the presence of a monoclonal B cell population in two cases (cases 20 and 24), which were found to be negative by morphology on the trephine. In the latter two cases monoclonality was shown by PCR analysis of the biopsy.

Finally, we would like to stress our findings in 11 cases in which a diagnosis of DLBCL was established on a diagnostic extramedullary sample. Three cases showed concordant morphology in the trephine compared with the diagnostic tissue (predominantly large cells), whereas in five bone marrow biopsies infiltrates predominantly consisted of small cells and three comprised a mixture of small and large cells. PCR analysis of these samples demonstrated monoclonality in all trephines, which showed the presence of large atypical cells (three concordant cases and three mixed large and small cell cases), except for one case. In contrast, in four of five cases characterised by small cell infiltrates, PCR showed a polyclonal pattern. Based on these data, one may question the importance of small cell infiltrates in the bone marrow biopsy of patients with DLBCL, because they might not represent genuine lymphomatous involvement. Hence, the histological criteria for distinguishing benign from neoplastic infiltrates may lack actual relevance in these cases.

The assessment of the clinical outcome of patients with discordant lymphoma, as well as the determination of the prognosis in these cases, was beyond the scope of our study. Nevertheless, other authors have noted that the outcome in patients with DLBCL with a predominantly small cell bone marrow infiltrate does not differ from that seen in those patients without bone marrow involvement. On the other hand, patients with DLBCL and a trephine displaying a large cell component, either homogeneous or admixed with small lymphoid cells, tended to perform significantly worse.[5 8] The clinical implications of discord-

ant disease, as well as the biological mechanism underlying this phenomenon, require elucidation in larger studies. Our results indicate that the latter should comprise molecular techniques to distinguish between reactive and malignant small cell infiltrates to avoid overestimation of the true meaning of discordant infiltrates in consecutive bone marrow biopsies during follow up.

In conclusion, our study demonstrates the validity of PCR in distinguishing benign from malignant lymphoid infiltrates in bone marrow biopsies. However, because false negative PCR results do occur, morphology is still the gold standard in evaluating bone marrow involvement by NHL. For this reason PCR can only be considered as a useful complementary investigation.

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1 Bartl R, Frisch B, Burkhardt R, *et al*. Lymphoproliferations in the bone marrow: identification and evolution, classification and staging. *J Clin Pathol* 1984;**37**:233–54.
2 Bartl R, Frisch B, Burkhardt R, *et al*. Assessment of bone marrow histology in the malignant lymphomas (non-Hodgkin's): correlation with clinical factors for diagnosis, prognosis, classification and staging. *Br J Haematol* 1982;**51**:511–30
3 Bain BJ, Clark DM, Lampert IA. *Bone marrow pathology*, 2nd ed. London: Blackwell Scientific Publications, 1996.
4 Foucar K. Bone marrow pathology. In: *Integrative hematopathology*, 1st ed. Chicago: ASCP Press, 1995:403–5.
5 Conlan MG, Bast M, Armitage JO, *et al*. Bone marrow involvement by non-Hodgkin's lymphoma: the clinical significance of morphologic discordance between the lymph node and bone marrow. *J Clin Oncol* 1990;**8**:1163–72.
6 Crisan D, Mattson JC. Discordant morphologic features in bone marrow involvement by malignant lymphomas: use of gene rearrangement patterns for diagnosis. *Am J Hematol* 1995;**49**:299–309.
7 Fisher M, Jacobson JO, Ault KA, *et al*. Diffuse large cell lymphoma with discordant bone marrow histology. Clinical features and biologic implications. *Cancer* 1989;**64**:1879–87.
8 Hodges GF, Lenhardt TM, Cotelingam JD. Bone marrow involvement in large-cell lymphoma. *Am J Clin Pathol* 1994;**101**:305–11.
9 Kluin PM, van Krieken JH, Kleiverda K, *et al*. Discordant morphologic characteristics of B-cell lymphomas in bone marrow and lymph node biopsies. *Am J Clin Pathol* 1990;**94**:59–66.
10 Kubic VL, Brunning RD. Immunohistochemical evaluation of neoplasms in bone marrow biopsies using monoclonal antibodies reactive in paraffin-embedded tissue. *Mod Pathol* 1989;**2**:618–29.
11 Linder J, Ye YL, Harrington DS, *et al*. Monoclonal antibodies marking T lymphocytes in paraffin-embedded tissue. *Am J Pathol* 1987;**127**:1–8.
12 Norton AJ, Isaacson PG. Monoclonal antibody L26: an antibody that is reactive with normal and neoplastic B lymphocytes in routinely fixed and paraffin wax embedded tissues. *J Clin Pathol* 1987;**40**:1405–12.
13 Pileri S, Falini B, Sabattini E, *et al*. Immunohistochemistry of malignant lymphomas. Advantages and limitations of the new monoclonal antibodies working in paraffin sections. *Haematologica* 1991;**76**:226–34.
14 Bluth RF, Casey TT, McCurley TL. Differentiation of reactive from neoplastic small-cell lymphoid aggregates in paraffin-embedded marrow particle preparations using L-26 (CD20) and UCHL-1 (CD45RO) monoclonal antibodies. *Am J Clin Pathol* 1993;**l99**:150–6.
15 Horny HP, Engst U, Walz RS, *et al.* In situ immunophenotyping of lymphocytes in human bone marrow: an immunohistochemical study. *Br J Haematol* 1989;**71**:313–21.
16 Waldmann TA. The arrangement of Ig and T-cell receptor genes in human proliferative disorders. *Adv Immunol* 1987;**40**:247–321.
17 Achille A, Scarpa A, Montresor M, *et al*. Routine application of polymerase chain reaction in the diagnosis of monoclonality of B-cell lymphoid proliferations. *Diagn Mol Pathol* 1995;**4**:14–24.
18 Inghirami G, Szabolcs MJ, Yee HT, *et al*. Detection of immunoglobin gene rearrangement of B-cell non-Hodgkin's lymphomas and leukemias in fresh, unfixed and formalin-fixed, paraffin-embedded tissue by polymerase chain reaction. *Lab Invest* 1993;**68**:746–57.
19 Kevin J, Trainor KJ, Brisco MJ, *et al*. Monoclonality in B-lymphoproliferative disorders detected at the DNA level. *Blood* 1990;**75**:2220–2.
20 Wan JH, Trainor KJ, Brisco MJ, *et al*. Monoclonality in B cell lymphoma detected in paraffin wax embedded sections using the polymerase chain reaction. *J Clin Pathol* 1990,**43**:888–90.
21 Yamada M, Hudson S, Tournay O, *et al*. Detection of minimal disease in hematopoietic malignancies of the B-cell lineage by using third-complementarity-determining region (CDR-III)-specific probes. *Proc Natl Acad Sci U S A* 1989,**86**:5123–7.
22 Pittaluga S, Tierens A, Dodoo YL, *et al*. How reliable is histologic examination of bone marrow trephine biopsy specimens for the staging of non-Hodgkin lymphoma? A study of hairy cell leukemia and mantle cell lymphoma involvement of the bone marrow trephine specimen by histologic, immunohistochemical, and polymerase chain reaction techniques. *Am J Clin Pathol* 1999,**111**:179–84.
23 Harris NL, Jaffe ES, Stein H, *et al.* A revised European-American classification of lymphoid neoplasms: a proposal from the international lymphoma study group. *Blood* 1994,**84**:1361–92.
24 Freedman A, Boyd A, Bieber F, *et al*. Normal cellular counterparts of B cell chronic lymphocytic leukemia. *Blood* 1987;**70**:418–27.
25 Bomberger C, Singh-Jairam M, Rodey G, *et al*. Lymphoid reconstitution after autologous PBSC transplantation with FACS-sorted CD34+ hematopoietic progenitors. *Blood* 1998;**91**:2588–600.
26 Coad JE, Olson DJ, Lander TA, *et al*. The molecular assessment of clonality in lymphoproliferative disorders. I. Immunoglobulin gene rearrangements. *Mol Diagn* 1996,**1**:335–55.
27 Gong JZ, Zheng S, Chiarle R, *et al.* Detection of immunoglobulin kappa light chain rearrangements by polymerase chain reaction: an improved method for detecting clonal B-cell lymphoproliferative disorders. *Am J Pathol* 1999,**155**:355–63.
28 Coad JE, Olson DJ, Christensen DR, *et al.* Correlation of PCR-detected clonal gene rearrangements with bone marrow morphology in patients with B-lineage lymphomas. *Am J Surg Pathol* 1997,**12**:1047–56.