SHORT REPORT

Splitting bone marrow trephines into frozen and fixed fragments allows parallel histological and molecular detection of B cell malignant infiltrates

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Clonality analysis of the immunoglobulin heavy chain (*IgH*) gene is helpful in identifying malignant B cell infiltrates in the bone marrow and is usually carried out on separate aspirates or on the same formalin-fixed decalcified bone marrow specimen. To determine whether the removal of the decalcification step would improve the molecular analysis, we first studied 12 bone marrow specimens with lymphoma infiltration split into a fixed and a small frozen fragment. Both the detection rate of *IgH* gene monoclonality and DNA quality were found to be superior in the frozen part than in the fixed part. Conversely, to evaluate whether the split would compromise histological analysis, we selected a series of 134 bone marrow specimens obtained from patients with small B cell lymphoma and showing *IgH* monoclonality on the frozen part. The histological detection rate of infiltrated or suspicious infiltrates (95%) on the fixed part was not altered by saving a frozen part.

The evaluation of bone marrow biopsy specimens has become a standard procedure in the staging and monitoring of patients with B cell lymphoma. Histological examination easily recognises paratrabeular and diffuse malignant infiltrates, whereas interstitial or focal small B cell lymphoma are difficult to differentiate from benign lymphoid aggregates and may be classified as suspicious for lymphoma.^{1–3}

Therefore, polymerase chain reaction (PCR) amplification of the immunoglobulin heavy chain (*IgH*) gene is increasingly used to assess B cell monoclonality in a wide variety of bone marrow sources, including smears or touch preparations, fresh aspirates, formalin-fixed paraffin-wax-embedded (FFPE) decalcified trephine biopsy specimens and clot sections.^{1 2 4 5}

Reproducible monoclonal *IgH* rearrangements are highly specific for the presence of malignant B cells in the context of suspected lymphoid infiltrates.² Conversely, PCR may yield false-negative results for several reasons. The lack of FR3

region amplification may be due to primer mismatch or somatic hypermutation of *IgH*.^{1 4} Moreover, lymphoma cells may be trapped in the bone marrow but absent from the aspirate or smear from which the DNA is extracted.^{1 4} Combining morphological and molecular analysis on the same FFPE bone marrow fragment would avoid such pitfalls, but fixation or decalcification considerably reduces the scope of amplifying *IgH* rearrangements.⁶

We evaluated the performance of molecular testing (DNA quality and FR3-JH amplification) in a pilot series of 12 bone marrow specimens split into frozen and fixed parts without the decalcification step. Thereafter, we examined histological data in 134 split bone marrow specimens with B cell monoclonality on the frozen part to determine whether splitting had led to false-negative histological results on FFPE sections.

MATERIALS AND METHODS

The first series included 12 trephine bone marrow biopsy specimens with obvious B-cell lymphoma infiltration. The frozen part was obtained by cutting a fragment (≤ 4 mm long) of a bone marrow biopsy specimen at least 15 mm long, taken from beneath the cortical bone. The remaining part was fixed in formalin containing 2% acetic acid (48 h) and embedded in paraffin wax without decalcification (Dako, Trappes, France).

Secondly, 134 cases were retrieved from our files (2000–2005) on the basis of the presence of *IgH* monoclonality on the frozen part in bone marrow specimens. The other part was fixed in Bouin's liquid, decalcified and embedded. Histological analysis included sequential staining and immunohistochemistry for CD20 and CD3 antigens (Dako). Specimens were classified and the results reported as positive, suspect or negative for lymphoma.^{1–3}

Finally, a consecutive series of 33 split bone marrow specimens were collected at the initial staging of patients

Abbreviations: FFPE, formalin-fixed paraffin-wax-embedded; *IgH*, immunoglobulin heavy chain; PCR, polymerase chain reaction

Table 1 Comparison of morphological and molecular analysis on separate bone marrow parts

	<i>IgH</i> gene monoclonality	Infiltrated (%)	Suspected (%)	Negative (%)
CLL	53	52 (98)	1 (2)	0 (0)
FL	29	25 (86)	2 (7)	2 (7)
MCL	31	25 (81)	1 (3)	5 (16)
SMZL	21	20 (95)	1 (5)	0 (0)
	134	122 (91)	5 (4)	7 (5)

CLL, chronic lymphocytic lymphoma; FL, follicular lymphoma; *IgH*, immunoglobulin heavy chain; MCL, mantle cell lymphoma; SMZL, splenic marginal zone lymphoma.

Figure 1 (A) Three examples with parallel amplification of two control gene amplicons in frozen (F) and fixed (f) bone marrow parts. (B) Same examples with parallel amplification of immunoglobulin heavy chain (*IgH*) gene FR3-JH rearrangement in frozen (F) and fixed (f) bone marrow parts. The numbers below indicate cases with monoclonality in F and polyclonality in f ($n = 2$), monoclonality of the same intensity in F and f ($n = 7$) and monoclonal bands of stronger intensity in F than in f ($n = 3$). C+, positive control; C-, negative control.

with follicular lymphoma, irrespective of their molecular profile.

Genomic DNA was extracted by a standard phenol-chloroform procedure. DNA quality of 12 bone marrow specimens split into frozen and FFPE fragments was checked in parallel by duplex-PCR of albumin (200 bp) and ribosomal protein (400 bp) gene amplicons. Clonality analysis of the FR3-JH *IgH* gene was carried out as described elsewhere.⁷

RESULTS

Parallel analysis of frozen and FFPE bone marrow pairs ($n = 12$)

The histological quality of undecalcified specimens was found to be equivalent to that of Bouin's liquid-fixed specimens (data not shown). DNA quality control showed amplification of only the 200-bp amplicons in 7 of 12 (58%) FFPE parts, whereas both 200-bp and 400-bp amplicons were detected in all frozen parts (fig 1A). Amplification of monoclonal FR3-JH *IgH* amplicons was achieved in 10 of the 12 FFPE parts of DNA, whereas a monoclonal band was detected in all frozen parts (fig 1B). The monoclonal amplicon was always of similar size in FFPE and frozen parts, but its intensity was weaker in three FFPE samples. Two specimens with monoclonal rearrangement on frozen DNA exhibited a false polyclonal profile on FFPE DNA.

Histological evaluation of the 134 bone marrow specimens with *IgH* monoclonality

The 134 bone marrow specimens were obtained from 90 patients either at staging ($n = 90$) or during follow-up ($n = 44$) of patients with mantle ($n = 31$), follicular ($n = 29$), lymphocytic ($n = 53$) or splenic marginal zone ($n = 21$) B cell lymphoma. The frozen DNA exhibited a reproducible dominant monoclonal FR3-JH rearrangement. Lymphoma infiltration was seen on fixed sections in 91% of the samples. Histologically, samples suspected of lymphoma (4%) corresponded to bone marrow biopsy carried out either at staging ($n = 2$) or during follow-up ($n = 3$). The negative samples (5%) corresponded to post-therapeutic samples of seven patients with either follicular or mantle cell lymphoma, followed by recurrence in two patients (table 1).

Splitting bone marrow trephines at staging in 33 patients with follicular lymphoma

In all, 27 (82%) patients exhibited nodular and paratrabeular lymphoma infiltration. Five patients showed both negative histological results and *IgH* gene polyclonality. Only one patient had a negative histological result, *IgH* monoclonality in the frozen part.

DISCUSSION

It is unusual to analyse frozen bone marrow tissue, as saving a frozen fragment could theoretically lead to false-negative histological results for focal or nodular infiltrates. Biases, such as failing to aspirate sufficient lymphoma cells owing to their entrapment or patchy distribution in the bone marrow, may be avoided by combining histological and molecular testing on the same FFPE sample.¹ Unreproducible bands may be observed by using several molecular protocols, including semi-nested PCR, which is a sensitive technique developed to improve the detection of monoclonal B cells in FFPE specimens.^{1-4, 8} Both the performance and the inter-laboratory reproducibility of *IgH* gene analysis are poor on FFPE tissues compared with fresh or frozen tissue.^{6, 9} Our study extends these findings to FFPE bone marrow specimens, even without the decalcification step. Such fixation greatly improved the detection of several antigens such as cyclin D1, CD10 and MIB-1-Ki-67 (our personal results). However, only the frozen part proved to be a sufficiently reliable source of optimal DNA to allow the amplification of larger fragments used to detect FR1-JH rearrangement or translocation genomic breakpoints.¹⁰ In comparison with other series,¹⁻³ the saving of a small frozen part did not distort our rates of histological detection on the FFPE part of either malignant (91%) or suspicious (4%) lymphoid infiltrates. This was especially checked in a consecutive series of 33 follicular lymphomas, a lymphoma subtype with focal bone marrow infiltration. Finally, splitting bone marrow trephines

permits optimal parallel histological and molecular examination, provided the biopsy is of sufficient length.

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