

Morphologic, flow cytometric and cytogenetic evaluation of bone marrow involvement in B-cell lymphoma

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Background and Objectives. Flow cytometric immunophenotypic analysis (FC) and cytogenetic analysis are essential techniques for the diagnosis and classification of many hematologic disorders. The roles of these analyses in B-cell lymphoma to detect bone marrow (BM) involvement in clinical staging and to evaluate the effectiveness of treatments have not yet been determined. The aim of this study was to evaluate the usefulness of FC and cytogenetic analysis in the assessment of BM involvement in B-cell lymphoma.

Design and Methods. We retrospectively analyzed the usefulness of three-color FC and cytogenetic analysis in detecting BM involvement by examining 104 BM specimens from patients with B-cell lymphoma.

Results. By morphologic evaluation of BM biopsy (BMB), the BM was involved in 11 specimens (10.6%), not involved in 92 specimens (88.5%) and involvement could not be determined in 1 specimen (0.9%). FC identified a monoclonal B-cell population in 24 samples (23.1%). FC detected BM involvement in all but one BMB positive sample, and showed negative results in a BMB undetermined sample. Conclusively, FC found a monoclonal B-cell population in 14 of 92 BMB negative samples (15.2%). In particular, FC detected smaller amounts of BM involvement than did morphologic evaluation. Cytogenetic analysis revealed clonal abnormalities in only 9 of 104 samples (8.7%). However, 2 of these 9 samples were from patients with aggressive lymphoma with complex structural chromosomal abnormalities detectable only by cytogenetic analysis.

Interpretation and Conclusions. Although morphologic evaluation of adequate amounts of BMB specimens remains essential for the evaluation of BM involvement, three-color FC is more sensitive in detecting BM disease than morphologic or cytogenetic analysis. Cytogenetic analysis seems to have low sensitivity and specificity, but this method may improve the detection of BM involvement in a small number of aggressive lymphomas that have many mitotic cells.

Key words: B-cell lymphoma, bone marrow involvement, bone marrow biopsy, flow cytometry, cytogenetic analysis.

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Bone marrow biopsies (BMB) are routinely performed in the clinical staging and post-therapy evaluation of non-Hodgkin's lymphoma (NHL). The clinical value of BMB in evaluating disease stage in patients with hematologic malignancies is well established, especially for patients with malignant lymphoma. The documentation of BM involvement results in a classification of clinical stage IV, upgrades the international prognostic index (IPI), and affects survival and therapeutic strategies for NHL.¹⁻³

Several groups have recommended the use of bilateral BM biopsies to assess BM involvement in patients with NHL. Bilateral trephine biopsy increases the positive yield of BM involvement by 10% to 22%.⁴⁻⁶ Moreover, there is a guideline that the total length of the biopsy in the aggregate should be at least 2.0 cm in order to evaluate BM involvement, because the yield may correlate with the size of the sample as well as the number of the samples.⁷ In spite of these recommendations, it is not always possible to obtain adequate amounts of specimens in clinical situations. Smaller amounts of BM might often reduce the accuracy of BM assessments, possibly leading to underevaluation of BM involvement. Moreover, it is often difficult to distinguish benign lymphocytic aggregates from focal lymphomatous involvement of the BM.⁸

To improve the sensitivity and the specificity of the BM evaluation, flow cytometric immunophenotypic analysis (FC) and cytogenetic analysis of BM aspirates are also used concomitantly with morphologic examination of BMB. However, the values of these examinations have not been established to date. The aim of this retrospective study was to evaluate the usefulness and the significance of FC and cytogenetic analysis in the detection of BM involvement in B-cell lymphoma.

Design and Methods

We retrospectively analyzed the results of 104 BM biopsies obtained from patients who were diagnosed as having B-cell lymphoma and whose BM aspirates underwent both FC and cytogenetic analysis from April 2000 to February 2003. The diagnosis of B-cell lymphoma was assessed in all patients according to REAL criteria by lymph node or other biopsy tissues.

Bone marrow specimens and morphologic evaluation

Unilateral trephine biopsies were obtained from the posterior iliac crest. Prior to biopsy, a BM aspirate was collected for clot section, FC and cytogenetic analysis. BM trephine and clot biopsy specimens were fixed in

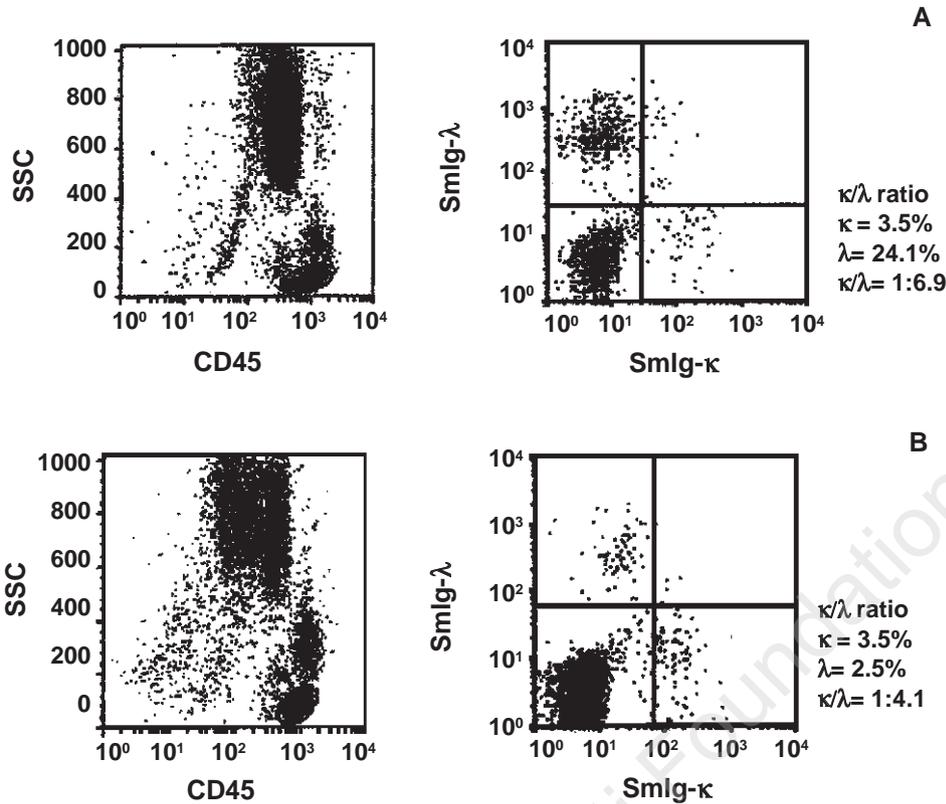


Figure 1. Three-color flow cytometric analysis of bone marrow aspirates. **A:** examples of bone marrow involvement in B-cell lymphoma. Abnormal κ/λ ratio (1:6.9). **B:** examples of negative bone marrow involvement. Normal κ/λ ratio (1.4:1).

10% neutral buffered formalin. The trephine biopsy specimens were then decalcified in EDTA for two days before being routinely processed, embedded in paraffin, and cut into 3-5 μm sections.

The morphologic evaluation was carried out on hematoxylin-eosin- (H-E) and reticulin-stained sections. A trephine or clot biopsy specimen was considered positive only if it could be unequivocally diagnosed as containing neoplastic cells. Bone marrow involvement was classified as positive, negative, or undetermined. Immunohistochemical studies were performed for the morphologically undetermined cases or the cases that showed discrepant results from morphologic evaluation of H-E- and reticulin-stained sections and FC or cytogenetic analysis. Antibodies employed for the immunohistochemical studies were: anti- κ light chain, anti- λ light chain and anti-CD20 (L26). To visualize the antibody binding, we used a peroxidase-based system (ENVISION kit/HRP[®], Dako, Carpinteria, CA, USA). The lengths of the BM biopsy sections on the slides were measured and registered.

Flow cytometric analysis

BM aspirates were immunophenotypically evaluated by three-color flow cytometric analysis with

CD45 gating,⁹ using FACS Calibur flow cytometers (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The following antibody combinations were used: peridinin chlorophyll protein (PerCP)/phycoerythrin (PE)/fluorescein isothiocyanate (FITC): CD45/CD10/CD7, CD45/CD33/CD2, CD45/CD13/CD19, CD45/CD14/CD5, CD45/CD20/CD3, CD45/CD4/CD8, CD45/CD34/HLA-DR, CD45/CD56/CD16, CD45/KORSA3544¹⁰/CD41a, CD45/ λ/κ . CD 2, 5, 7, 20, 33 and 56 were purchased from Becton Dickinson/Pharmingen (San Diego, CA, USA); CD 3, 4, 8, 13, 14, 16, 19, 34, 45, HLA-DR, k and I were from Becton Dickinson; CD10 was from Beckman Coulter (Hialeah, Florida, USA); CD41a was from Beckman Coulter/Immunotech (Marseille, France); and KOR-SA3544 was from BML (Nagoya, Aichi, Japan). Monoclonality was determined by the percentage of cells stained singly with κ vs λ . The results of FC were considered positive if the $\kappa:\lambda$ ratio was greater than 3:1 or less than 1:2 (Figure 1A, 1B).

Cytogenetic analysis

Chromosomes were analyzed using Giemsa-banding of short-term cultures of cells from the BM aspirates. At least 20 metaphases were examined from

each sample except for one case (only 18 metaphases could be analyzed). Karyotypes and clonal definitions are described according to the International System for Human Cytogenetic Nomenclature.¹¹ Normal male or female karyotypes and the sole abnormality of monosomy Y were considered negative for clonal abnormalities associated with lymphoma.

Analysis of immunoglobulin heavy chain gene rearrangements by polymerase chain reaction

Immunoglobulin heavy chain (IgH) gene rearrangements were analyzed by polymerase chain reaction (PCR) in 14 samples in which morphologic evaluation did not detect BM involvement but FC gave a positive result. DNA was extracted from formalin-fixed, paraffin-embedded bone marrow biopsy tissues¹² using TaKaRa DEXPAT™ (TaKaRa BIO, Otsu, Shiga, Japan). PCR studies were performed as previously described,^{13,14} with minor modifications. Briefly, the IgH gene was amplified by a semi-nested PCR using a Fr3 V-region primer in conjunction with downstream primers directed to the J region. The first round of amplification used primer Fr3A (5'-ACACGGC[C/T] [G/C]TGTACTGT-3') plus a downstream consensus primer directed to the joining region (LJH: 5'-TGAGGAGACGGTGACC-3'). The second round used the same upstream primer (Fr3A) in conjunction with an inner downstream primer (VLJH: 5'-GTGACCAGGGTNCCTTGGCCCCAG-3'). The first round of amplification consisted of 30 cycles of 95°C for 15 sec, 45°C for 30 sec and 72°C for 60 sec were performed. In the second round of amplification, 20 cycles of 95°C for 15 sec, 50°C for 30 sec and 72°C for 60 sec were performed. In each round, an initial denaturation step at 95°C for 7 min preceded the addition of enzyme, and a primer extension step at 72°C for 5 min concluded the reaction. The reaction products were then run on 6% polyacrylamide gels, which were stained with ethidium bromide and viewed under UV light.

Results

Clinicopathologic characteristics of patients

One hundred and four BM samples from eighty-nine patients with B-cell lymphoma were evaluated. All samples were submitted to morphologic, FC and cytogenetic evaluation of lymphoma.

The patients' mean age was 59 years (range 15-86). Fifty-two patients were male and thirty-seven were female. The pathologic diagnosis was diffuse large B-cell lymphoma (DLBCL) in 57 samples (54.8%), follicular center lymphoma (FL) in 23 samples (22.1%), extranodal marginal zone B-cell lymphoma in 21 samples (20.2%), Burkitt's lymphoma (BL) in 2 samples (1.9%) and mantle cell lymphoma (MCL) in 1 sample (1.0%). The clinical indication for

BM examination included initial staging of lymphoma (n=72), restaging for recurrence (n=18) and follow-up of BM involvement (n=14).

Morphologic evaluation of bone marrow biopsy

Morphologic evaluation of BMB was positive for involvement in 11 (10.6%) of the specimens, was unable to determine involvement in 1 (0.9%) case and was negative for involvement in 92 (88.5%) of the specimens (Figure 2). The length of BM biopsy specimens ranged from 0.2 cm to 2.2 cm (average 0.87 cm).

Flow cytometric analysis of bone marrow aspirates

FC was positive in 10 of 11 BMB positive samples. Monoclonal B-cell populations ranged from 0.62% to 53.9% (average 18.4%) in the samples that were both BMB and FC positive. Seven of 10 BMB negative but FC positive samples had definite detectable monoclonal B-cell populations constituting more than 5% of all nucleated cells. On the other hand, a BMB sample in which BM involvement was undetermined morphologically showed no evidence of BM involvement by FC. Of the 92 BMB negative samples, 14 (15.2%) were FC positive and 78 (84.8%) were FC negative (Figure 2). The concurrence rate of morphologic evaluation and FC was 84.6% (88 of 104 samples). In the samples that were morphologically negative but FC positive, monoclonal B-cell populations ranged from 0.25% to 7.35% (average 2.92%), and 10 of 14 (71.4%) BMB negative but FC positive samples had minimal detectable monoclonal B-cell populations constituting less than 5% of all nucleated cells analyzed.

Cytogenetic analysis of bone marrow aspirates

Nine out of 104 samples (8.7%) showed clonal cytogenetic abnormalities. Four of 10 samples that were both BMB and FC positive had clonal cytogenetic abnormalities. Two of four cases were DLBCL, and the remaining two were FL. Cytogenetic analysis detected clonal abnormalities in 66.7% of BMB positive cases of DLBCL and 28.5% of FL. Among 14 BMB negative but FC positive samples, only one DLBCL showed an abnormal karyotype.

It is noteworthy that 4 of 78 samples that were concordantly negative by both BMB and FC for BM involvement showed clonal cytogenetic abnormalities. Three of these four cases were DLBCL, the other was a FL (Figure 2).

Immunoglobulin heavy chain gene rearrangement

Clonal rearrangement of the IgH gene was confirmed by PCR in 9 of 14 BMB negative but FC positive samples (64.3%).

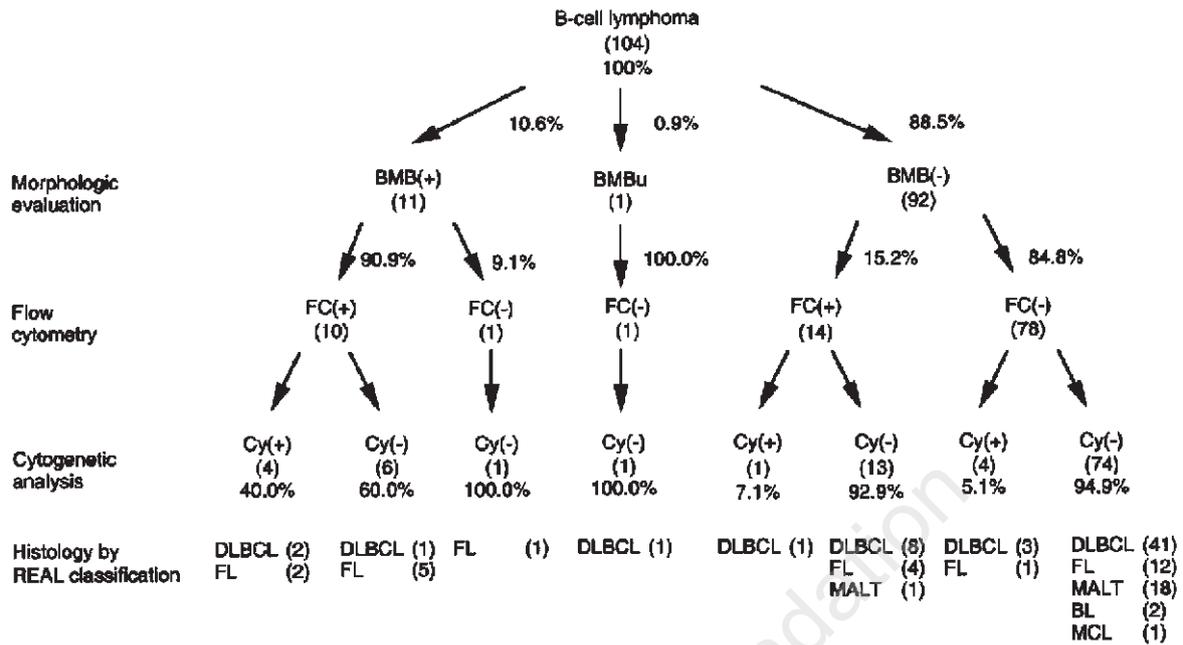


Figure 2. Summary of the results of morphologic, flow cytometric and cytogenetic studies. BMB(+): positive for bone marrow involvement by morphologic evaluation; BMBu: BM involvement undetermined by morphologic evaluation; BMB(-): negative for involvement by morphologic evaluation; FC(+): positive for involvement by flow cytometric analysis; FC(-): negative for involvement by flow cytometric analysis; Cy(+): positive for clonal cytogenetic abnormalities by chromosomal analysis; Cy(-): negative for clonal cytogenetic abnormalities by chromosomal analysis; DLBCL: diffuse large B-cell lymphoma; FL: follicular center lymphoma; MALT: extranodal marginal zone B-cell lymphoma; BL: Burkitt's lymphoma; MCL: mantle cell lymphoma.

In the 5 samples with discrepant FC and PCR results, the percentage of monoclonal B-cell populations detected by FC ranged from 0.34% to 7.35% (average 3.55%); two of these samples had more than 7% monoclonal B cells detectable by FC. Four of the samples were DLBCL, and one was FL.

Flow cytometric evaluation related to stage and International Prognostic Index

We focused on the cases for which BM examination was performed for initial staging (Table 1) because the clinical staging affects the prognosis as well as the standard therapy. Of 72 cases evaluated as part of the initial staging, there were 41 cases of DLBCL, 15 cases of MALT lymphoma, 15 cases of FL and 1 case of BL. There were 17 stage I, 18 stage II, 13 stage III cases, and 24 cases were categorized as stage IV based on extranodal disease or BM involvement evaluated morphologically by BMB. In 12 of 72 cases (16.7%), FC indicated BM involvement but morphologic evaluation was negative. Three stage I cases (one DLBCL and two FL) and one stage II case (DLBCL) were positive by FC but negative by morphologic evaluation. Thus, a total of 17.6% of stage I cases and 5.6% of stage II cases

Table 1. The clinical stage of patients whose bone marrow examination was done at initial diagnosis and the histologic subtype. In brackets, the number with positive flow cytometric results.

| | DLBCL | FL | MALT | BL | Total |
|--------------|----------------|---------------|---------------|--------------|----------------|
| Stage I | 6 (1) | 3 (1) | 7 (1) | 1 (0) | 17 (3) |
| Stage II | 12 (1) | 2 (0) | 4 (0) | 0 (0) | 18 (1) |
| Stage III | 7 (2) | 3 (1) | 3 (0) | 0 (0) | 13 (3) |
| Stage IV | 16 (7) | 7 (6) | 1 (0) | 0 (0) | 24 (13) |
| Total | 41 (11) | 15 (8) | 15 (1) | 1 (0) | 72 (20) |

DLBCL: diffuse large B-cell lymphoma; FL: follicular center lymphoma; MALT: extranodal marginal zone B-cell lymphoma; BL: Burkitt's lymphoma.

could be considered as stage IV disease based on FC results. The IPI was upgraded from low and low/intermediate groups to intermediate/high and high groups in 5 cases based on the FC results.

Table 2. Studies reporting evaluation of bone marrow involvement by flow cytometry and morphology.

| N. of cases | Disease | Method | Concurrence rate of BMB and FC | BMB+/FC- | BMB-/FC+ | Reference |
|-------------|------------------|--------------|--------------------------------|----------|----------|-----------|
| 188 | B cell neoplasms | 2 color | 81.4% | 11.7% | 2.6% | 15 |
| 175 | B cell lymphoma | 2 or 3 color | 91.4% | 5.7% | 2.9% | 16 |
| 273 | NHL | 1 or 2 color | 82.1% | 9.2% | 0.4% | 17 |
| 227 | NHL | 3 or 4 color | 78.4% | 7.0% | 9.7% | 18 |
| 79 | FL, DLBCL | 3 color | 82.3% | 0% | 3.8% | 19 |
| 39 | B-cell lymphoma | 2 color | 69% | 12.8% | 2.6% | 20 |
| 104 | B-cell lymphoma | 3 color | 84.6% | 1.0% | 13.5% | our study |

NHL: non Hodgkin's lymphoma; FL: follicular center lymphoma; DLBCL: diffuse large B-cell lymphoma; concurrence rate of BMB and FC; the concurrence rate of morphologic evaluation and flow cytometric analysis in evaluating bone marrow involvement; BMB+/FC-: morphology positive but flow cytometric analysis negative; BMB-/FC+: morphology negative but flow cytometric analysis positive.

Discussion

We performed three-color FC to evaluate BM involvement in B-cell lymphoma. FC detected BM involvement in all but one BMB sample that was morphologically positive, but gave a negative result in a BMB in which lymphoma involvement was undetermined. In addition, FC improved the detection of BM involvement in 14 out of 104 samples (13.5%), and in 12 of 72 samples (16.7%) examined at initial staging. Compared with the previous studies,¹⁵⁻²⁰ our FC showed good sensitivity in detecting BM involvement (Table 2). Dunphy *et al.* reported 5 BMB negative but FC positive cases (2.6%) and 22 BMB positive but FC negative cases (11.7%) in 188 B-cell malignancies including mature B-cell leukemia.¹⁵ However, they employed less sensitive single or dual color FC, whereas we used three-color analysis. Hanson *et al.* reported 5 BMB negative but FC positive cases (2.9%), and also found 10 BMB positive but FC negative cases (5.7%) in a series of 175 B-cell malignant lymphomas.¹⁶ However, they employed two-color as well as three-color flow cytometric analysis and thus, concluded that FC is not a cost-effective replacement for good morphologic evaluation. Naughton *et al.* reported that FC improved the detection of BM involvement in only 3 of 273 samples (1.1%). Two of the three cases were morphologically suspicious for involvement. Moreover, FC could not detect BM involvement in 25 of 62 samples which were morphologically positive

(40.3%). Therefore, they also concluded that FC had a limited role in detecting BM involvement in their series of 273 NHL cases including non-B-cell lymphomas.¹⁷ On the other hand, Duggan *et al.* reported that FC detected BM involvement in 27 BMB negative or morphologically suspicious cases (12%). However, FC did not detect the BM involvement in 16 of 65 BMB positive cases (24.6%) even using three- or four-multicolor analysis in their series of 227 cases of NHL. Thus, they concluded that neither morphologic evaluation of BM nor FC alone is adequate to detect all cases of NHL with BM involvement.¹⁸ Palacio *et al.* reported that FC, using three-color analysis, identified a monoclonal B-cell population in not only all BMB positive, but also 3 BMB negative cases (3.8%) among 79 FL or DLBCL. They concluded that FC is just as sensitive or perhaps slightly more sensitive than histology for detecting BM involvement.¹⁹ Sah *et al.* very recently reported that FC might be slightly more sensitive than BMB for detecting minimal residual disease in B-cell chronic lymphocytic leukemia, whereas BMB may be slightly better than FC in B-NHL.²⁰ The relatively low sensitivity of FC in B-NHL might be partly related to the high sensitivity of detecting BM involvement afforded by their very careful immunohistochemical evaluation on the BMB sections and the use of less sensitive two-color FC. Taken together with these studies, our results suggest that the better sensitivity of FC over morphologic evaluation might be partly due to the use of three-color FC

including anti-CD45, anti- κ and anti- λ monoclonal antibodies. In general, BM involvement has been reported in 5-33.9% of DLBCL,²¹ 30-55% of FL^{22,23} and 14.5-44% of marginal zone lymphomas.²⁴⁻²⁷ In our study, the incidence of BM involvement at initial diagnosis by morphologic evaluation was 4.9% in DLBCL, 40.0% in FL and 0% in MALT lymphoma. These incidences seem to be relatively low especially in DLBCL and MALT lymphoma. However, FC detected BM involvement in 26.8% of DLBCL, 53.3% of FL and 6.7% of MALT lymphoma cases. The relatively low incidence of BM involvement in MALT lymphoma may in part reflect the greater proportion of cases with limited stage MALT lymphoma of ocular adnexa (11 of 15 cases; 73.3%). The length of BM trephine biopsies in our study ranged from 0.2 cm to 2.2 cm (average 0.87 cm) which was relatively shorter than those in other studies (2 to 6.5 cm,¹⁶ at least 1 cm of subcortical bone marrow,^{18,20} 0.2cm to 4 cm (average 1.5 cm).¹⁹ Focal BM involvement is more common than diffuse involvement (70% as opposed to 30%) in NHL.^{22,28} Thus, small BM biopsy specimens might cause underdiagnosis of BM involvement, especially when focal. We, therefore, recommend that when BM specimens are limited for any reason, FC (at least three-color analysis combining anti-CD45, anti- κ and anti- λ monoclonal antibodies) should be employed to evaluate BM involvement as a complement to morphologic examination.

One of the important questions is whether the criteria we used to determine monoclonality are appropriate. We considered the FC results as positive if they showed that the κ : λ ratio was either greater than 3:1 or less than 1:2.

Various authors have reported different normal values of the κ : λ ratios according to the respective normal values obtained in their laboratories.^{16,17,19} We established the normal range of the κ / λ ratio to be between 0.53 and 2.31 (mean 1.54) based on studies of normal peripheral blood samples. We then set the normal range of κ / λ as 0.5 to 3, compromising the sensitivity in favor of a higher accurate specificity. Many other investigators have used the same range.²⁹⁻³² Immunoglobulin light chain restriction analysis is convenient and one of the most useful immunologic procedures to determine the clonality of B-cells.^{33,34} However, it is not omnipotent. Loss of surface immunoglobulin light chains, loss of certain pan-B antigens and positivity of CD5 are also useful for diagnosing B-lineage malignancy.³³ Therefore, we employed many antibody combinations including anti-CD45, anti- λ and anti- κ monoclonal antibodies.

We also examined IgH gene rearrangement of all 14 morphologically negative but FC positive samples by PCR. PCR detected the monoclonal gene rearrangement in only 64.3% of these samples. Our results were consistent with a report that FC was

more sensitive (a sensitivity of 97.5%) than PCR (a sensitivity of 67.5%) in detecting BM involvement of B-cell neoplasia.²³ Moreover, it has been reported that PCR-based clonality studies of BM do not significantly add to the sensitivity of diagnosing involvement of the BM by B-lineage lymphoma.³³ Failure to detect all clonal populations by PCR might be due to low quality DNA extracted from formalin-fixed, paraffin-embedded bone marrow biopsy tissues, insufficient primer homology for amplification due to abnormal IgH VDJ gene rearrangement or high somatic hypermutations in target primer sequences of transformed B-cell clones.^{13,14,23,35,36} Lymphoma subtypes might also influence the efficacy of PCR.^{23,35}

Cytogenetic analysis showed clonal abnormalities in only 4 of 10 samples that were both morphologically and FC positive and 1 of 14 BMB negative but FC positive samples. It is noteworthy that 4 of 92 samples that were both morphologically and FC negative nevertheless showed clonal cytogenetic abnormalities. One of them had an abnormal karyotype including t(3;14)(q27;q32) in 3 of 20 metaphases analyzed. This aberration is associated with BCL6 (3q27) and immunoglobulin heavy chain (14q32), and it is frequently seen in NHL.³⁷ Another case showed complex abnormalities including add(1)(q32), -6, add(9)(p11), +12, add(15)(q22), add(16)(p11), -17, -19, add(19)(p13), -22, -22, +mar1, +mar2, +mar3, +mar4, +mar5 in 6 of 20 metaphases analyzed. Some of these breakpoints have been reported in NHL, but are not specific for NHL.³⁷ This patient had clinical stage IV disease and disseminated involvement of the peripheral nerves and central nervous system. Unfortunately, the patient died from disease progression; BM involvement could not be identified even during the autopsy. These two cases were classified as having DLBCL. The last two cases (FL and DLBCL) had 46, X, add(X)(q13) in all 20 metaphases analyzed, and 47, XX, +mar in 19 of 20 metaphases analyzed. BM examination revealed no morphologic abnormalities associated with myelodysplastic syndrome in any of these four cases. The pathologic significance of the karyotypic abnormalities seen in the last three cases was unknown. Kang *et al.* reported that 19 of 40 BMB positive cases (47.5%) had abnormal karyotypes and one BMB negative case showed abnormal karyotypes involving 14q32 among 88 cases that underwent cytogenetic analysis.³⁵ Wang *et al.* reported that only 1 of 6 morphologically positive specimens had an abnormal karyotype.³⁸ Taken together with these reports, our study suggests that cytogenetic analysis has low sensitivity and specificity. However, cytogenetic analysis may improve the detection of BM involvement in a small number of aggressive lymphomas that have many mitotic cells.

Fluorescence *in situ* hybridization (FISH) is a sen-

sitive method for analyzing numerical and structural chromosomal aberrations by using interphase cells. Juenger *et al.* reported that FISH could detect BM involvement in about 50% of cytogenetically normal samples that had been pathologically identified as containing lymphoma.³⁹ Indeed, FISH is more sensitive in detecting BM involvement of B-cell lymphoma with specific chromosomal abnormalities than is conventional cytogenetic analysis of 20 cells. However, B-cell lymphomas are a varied group of lymphoid neoplasms with heterogeneous features including a number of different chromosomal translocations. Moreover, our study included many cases whose BM examination had been done for initial staging and whose chromosomal abnormalities of the lymphoma cells had not been identified before BM examination. Probably, FISH analyses using multiple probes can improve the sensitivity. However, our study did not address the role of FISH, because this still seems unclear and not cost-effective as a screening test.

FC potentially upgrades the clinical stage and IPI of some patients. The minimal involvement detectable by FC but not by morphologic evaluation does not qualify as a change in the clinical stage, under the current guidelines.⁷ However, not only FC, but also PCR confirmed the presence of BM disease in some morphologically negative cases. Thus, it is of interest to clarify the clinical significance of minimal BM disease detectable by FC. Unfortunately, the number of BMB negative but FC positive cases is too small, and the follow-up too short to clarify the statistical differences between BMB negative but FC positive and FC negative cases at present. Further clinical studies are needed to understand the clinical significance of bone marrow disease detectable only by FC.

Morphologic evaluation of adequately sized BM samples remains the standard method to evaluate BM involvement at present. Three-color FC including anti-CD45, anti- κ and anti- λ is also helpful in the routine staging and follow-up of patients with B-cell lymphoma to investigate minimal BM disease, especially in those patients whose BM specimens are small.

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HG and TM designed the study. HG, MS, KM, KYak, NU, AO, KYam, MI performed bone marrow examination. All the authors except YH were responsible for patients' clinical management and involved in clinical assessment of the patients. YH was responsible for pathological diagnoses. KC and TM revised the paper.

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