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Impact of occult bone marrow involvement on the outcome of R-CHOP therapy for diffuse large B-cell lymphoma

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Running title: Occult bone marrow involvement in DLBCL

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1 **Abstract**

2 We assessed the prognostic impact of occult bone marrow involvement,
3 determined by flow cytometry and/or polymerase chain reaction, in a population of 117
4 consecutive patients with newly-diagnosed DLBCL treated with R-CHOP. 24 (20.5%) had
5 morphologically-diagnosed and 16 (13.7%) had occult marrow involvement, and 77
6 (65.8%) had no marrow involvement. Although the pretreatment characteristics of the
7 negative or occult marrow involvement group were similar, severe hematological toxicity
8 after R-CHOP was more frequent in the occult marrow involvement group.
9 Progression-free survival (PFS; $p=0.015$) and overall survival (OS; $p=0.035$) for the occult
10 marrow involvement group were significantly shorter than those for the negative group,
11 and were comparable with those of the morphologic marrow involvement group,
12 independently of the International Prognostic Index score for PFS. Occult bone marrow
13 involvement predicts a severe hematological toxicity and negatively impacts on PFS and
14 OS of R-CHOP therapy.

1 **Introduction**

2 Evaluation of prognostic factors is important in the management of patients with
3 diffuse large B-cell lymphoma (DLBCL). Although the International Prognostic Index (IPI)
4 remains a useful prognostic tool even in the rituximab era [1], the IPI alone is no longer
5 sufficiently powerful to discriminate patients who will be cured by conventional therapy
6 from those who have refractory or relapsing disease [2,3]. While multiple biologic
7 predictors, such as germinal center or activated B-cell phenotype, appear promising [4-6],
8 none have been validated for routine clinical use [7]. On the other hand, several studies
9 demonstrate that the presence of morphological bone marrow involvement, particularly
10 concordant involvement, is associated with a poor outcome in patients with DLBCL,
11 independently of the IPI [8-10]. Previous reports show that approximately 10% to 25% of
12 patients with DLBCL exhibit bone marrow involvement at the time of diagnosis [8,9].

13 Recently, cytogenetic and molecular techniques, such as flow cytometry (FCM) and
14 polymerase chain reaction (PCR), have provided additional information for staging bone
15 marrow samples [11]. However, results of FCM and/or PCR are largely ignored or
16 underused because of uncertainty regarding their clinical role. At present, morphologically
17 normal bone marrow with a small (<2%) clonal B-cell population (detected by FCM) is
18 considered normal [12,13]. In many facilities, FCM and/or PCR are only used to examine
19 bone marrow in cases that are equivocal in terms of morphology [14].

1 Before rituximab was available, several studies identified a potential adverse
2 impact of occult bone marrow involvement which was detected by FCM [15,16], PCR [11],
3 or both [14,17] on outcome. Further studies are required to confirm that these results are
4 valid in rituximab treated patients.

5 Therefore, the aim of this study was to evaluate the prevalence of occult bone
6 marrow involvement and to examine its significance with respect to outcome in DLBCL
7 patients treated with R-CHOP (rituximab plus cyclophosphamide, doxorubicin, vincristine,
8 and prednisone [18]).

9

10 **Materials and methods**

11 **Patients**

12 The clinical charts of consecutive patients with DLBCL (newly-diagnosed at the
13 Kobe City Medical Center General Hospital from April, 2006 to March, 2011) were
14 reviewed. Patients were included in the study if they were ≥ 16 years-old, had a
15 biopsy-proven diagnosis of DLBCL, had received a pretreatment staging bone marrow
16 examination (including both morphological and immunological (FCM) and/or molecular
17 (PCR) studies), and had been treated with at least one cycle of R-CHOP. Patients were
18 excluded if they had a known prior history of an indolent lymphoproliferative disorder, or
19 showed composite lymphoma with an indolent B-cell lymphoma component. DLBCL

1 patients that were HIV-positive, or had intravascular lymphoma, primary mediastinal B-cell
2 lymphoma, or central nervous system (CNS) lymphoma, were also excluded, as most
3 were not treated with R-CHOP in our institution.

4 A total of 174 patients were diagnosed with *de novo* DLBCL who were not
5 HIV-positive and did not had intravascular lymphoma, primary mediastinal B-cell
6 lymphoma nor CNS lymphoma. Of these, 35 patients were excluded because their
7 pretreatment staging bone marrow examinations did not meet the study criteria. A further
8 22 patients were excluded because they initially received palliative chemotherapy other
9 than R-CHOP due to severe organ dysfunction or poor performance status. No patients
10 were initially treated with dose-dense or dose-intense therapies other than R-CHOP. Thus,
11 a total of 117 patients were included in the study.

12 The treatment strategy was as follows. Chemotherapy consisted of six to eight
13 cycles of R-CHOP, except for 16 patients who were elderly and had non-bulky limited
14 disease; these patients received three cycles of R-CHOP followed by involved field
15 irradiation). Patients with extranodal involvement of the adrenal gland, testis or breast
16 received prophylactic intrathecal chemotherapy. Eleven patients with advanced disease
17 received high-dose chemotherapy with autologous stem cell support after the completion
18 of six cycles of R-CHOP (at the discretion of the attending physicians). Hemophagocytic
19 syndrome at diagnosis was defined as bone marrow infiltration by activated, nonmalignant

1 macrophages, which phagocytose blood cells. Febrile neutropenia after chemotherapy
2 was defined as a body temperature $\geq 38.2^{\circ}\text{C}$ and a neutrophil count $< 0.5 \times 10^9/\text{L}$ (as
3 assessed on the same day, or the day after, the fever was diagnosed). The study was
4 approved by the local institutional ethics committee.

5

6 **Bone marrow morphology**

7 Each patient underwent two bone marrow aspirations; the first sample was used to
8 prepare smear slides and clot sections, and the second was submitted for FCM and/or
9 PCR. Two experienced hematologists and two pathologists performed a blinded
10 examination of smear preparations and clot sections, respectively, for evidence of
11 morphologic lymphoma involvement. Bone marrow involvement was defined as the
12 presence of blast cells with a large nucleus, prominent multiple nucleoli and abundant
13 cytoplasm according to previously described criteria [19]. Immunohistochemical analysis
14 using anti-CD20 (Dako Denmark A/S, Glostrup, Denmark), anti-CD3, anti-CD10,
15 anti-CD56, anti- κ , and anti- λ (Nichirei, Tokyo, Japan) antibodies was performed for every
16 case in which lymphoma cell involvement was either confirmed or suspected. The level of
17 involvement was determined by counting 250 nucleated marrow cells. Discordant bone
18 marrow morphology was recorded for bone marrow samples that were positive for mixed
19 small- and large-cell lymphoma involvement. The original reports of bone marrow

1 morphology were utilized.

2

3 **Flow cytometry**

4 Four-color FCM was performed on bone marrow cells using monoclonal antibodies
5 conjugated to the following fluorochromes: fluorescein isothiocyanate (FITC),
6 phycoerythrin (PE), peridinin chlorophyll protein (PerCP), or allophycocyanin (APC).
7 Briefly, approximately 1×10^6 nucleated cells were stained with the following antibody
8 panels: anti- κ/λ /CD45/CD19; anti-CD20/CD25/CD45/CD19;
9 anti-CD43/CD10/CD45/CD19; and anti-CD23/CD5/CD45/CD19. After incubation for 15
10 minutes at room temperature, 1ml of BD Pharm Lyse lysing solution (BD Biosciences, San
11 Jose, CA, USA) was added to each tube. The cells were then washed twice in
12 phosphate-buffered saline (PBS) and re-suspended in 1 ml of PBS. At least 1×10^5 cells
13 were examined using a FACSCalibur flow cytometer (BD Biosciences) and the data were
14 analyzed using CellQuest Software (version 3.3; BD Biosciences). All antibodies were
15 purchased from BD Biosciences, with the exception of anti- κ and anti- λ (Dako Denmark
16 A/S). An isotype-matched IgG antibody was used to control for background non-specific
17 staining. Mature B-cells in the bone marrow were defined as CD45^{bright}, CD19-positive,
18 and with intermediate side scatter. Clonal B-cells within the gated mature B-cell population
19 were defined as follows: They showed light chain clonal restriction with a biased $\kappa:\lambda$ ratio

1 of >3:1 or <0.5:1, or showed obvious light chain deletion (>20% of mature B-cells); and the
2 restricted population representing κ , λ or light chain deleted one comprised $\geq 0.2\%$ of total
3 nucleated bone marrow cell population.

4

5 **Polymerase chain reaction**

6 DNA was extracted from nucleated bone marrow cells using a QIAamp DNA Mini
7 Kit (QIAGEN Sciences, Maryland, USA). The concentration and quality of the DNA were
8 determined using an ND1000 spectrophotometer (NanoDrop Technologies, Wilmington,
9 Delaware, USA). Multiplex PCR for the detection of clonal immunoglobulin heavy chain
10 (IGH) gene rearrangements was based on the BIOMED-2 Concerted Action
11 BMH4-CT98-3936 protocols [20]. All amplification reactions were performed using a T3
12 Thermocycler (Biometra, Göttingen, Germany). The PCR products were denatured and
13 subsequently renatured to induce duplex formation. The duplexes were then loaded onto
14 6% non-denaturing polyacrylamide gels and run in 0.5x Tris-boric acid-EDTA buffer to
15 resolve the different-sized amplicons. The GAPDH gene and water were used as positive
16 and negative controls for each PCR test, respectively. The presence of clonal B-cells was
17 defined as the presence of a distinct clonal band of the expected size. For example, the
18 sizes of the monoclonal PCR products representing the IGH VH-JH (FR1, FR2 and FR3)
19 regions ranged from 310–360, 250–295, and 100–170 base pairs, respectively. The size

1 of the IGH DH-JH (DH1-6) product was 110–290 (DH1/2/4/5/6-JH) or 390–420 (DH3-JH)
2 base pairs, and that of the IGH DH-JH (DH7) product was 100–130 base pairs [20]. PCR
3 products not corresponding to the expected sizes were considered nonspecific. As
4 previously reported, this protocol allowed the detection of clonal B-cells when present at
5 only 1% of the total nucleated bone marrow cell population [20].

6

7 **Definition of bone marrow involvement**

8 Morphologic bone marrow involvement was defined as morphologically-diagnosed
9 marrow involvement with lymphoma, enabling the identification of positive cases if
10 involvement was detected in at least one of the smear preparations, clot sections or
11 immunohistochemical stains. Occult bone marrow involvement was defined as the
12 presence of a clonal B-cell population (detected by either FCM or PCR) in the absence of
13 any morphological detection. For Ann Arbor clinical staging, occult bone marrow
14 involvement *per se* was not considered as lymphoma involvement.

15

16 **Statistical analysis**

17 Differences in the clinical characteristics between the cohorts were compared using
18 an independent samples *t*-test, with a logarithmic transformation of each individual value
19 for continuous variables, and Fisher's exact test for categorical variables. The incidence of

1 hematological toxicity after R-CHOP therapy (assessed according to the lowest white
2 blood cell [WBC] counts, neutrophil counts, hemoglobin [HGB] levels, and platelet [PLT]
3 counts, and according to the incidence of febrile neutropenia within the first six rounds of
4 R-CHOP therapy) was compared between those patients in each cohort who were initially
5 scheduled to receive six to eight cycles of R-CHOP (excluding those who received initial
6 therapy comprising three cycles of R-CHOP followed by involved field irradiation). Toxicity
7 due to high-dose chemotherapy with autologous stem cell support, which was
8 occasionally performed after the completion of six cycles of R-CHOP, was not included in
9 the comparison. Progression-free survival (PFS) was calculated as the time from the date
10 of diagnosis to documented disease progression, relapse, or death from any cause.
11 Overall survival (OS) was calculated as the time from the date of diagnosis until death
12 from any cause. Patients were censored at the time they were last known to be alive. PFS
13 and OS were assessed using the Kaplan-Meier method and the log-rank test was used to
14 compare them between groups [21,22]. Weighted log-rank test with Fleming-Harrington
15 weights of $\rho=0$ and $\gamma=1$ was used to shift the weighting toward late survival differences
16 when Kaplan-Meier curves crossed [23]. Post-hoc power analyses of the log-rank tests
17 used to analyze PFS and OS were performed to determine the adequacy of the sample
18 size [24]. The cause-specific probabilities of disease progression or relapse were
19 estimated on the basis of cumulative incidence rates (CIR) to accommodate death in

1 remission, the competing event for disease progression or relapse. The groups were
2 compared using Gray's test [25]. Multivariate analysis was performed for PFS and OS
3 using a Cox proportional hazards model [26], whereas Fine and Gray's proportional
4 subdistribution hazard model was used for CIR of disease progression or relapse [27], to
5 assess the independent effect of bone marrow status on outcome after controlling for the
6 IPI score. The final multivariate model was built using a backward stepwise model
7 selection approach. Finally, the power of the IPI score for predicting the PFS and OS in the
8 Cox proportional hazards model were compared in the following three situations using
9 Harrell's *c* [28]: 1) when positive marrow involvement was identified according to
10 morphological staging alone and discordant cases were not counted as positive); 2) when
11 positive involvement was identified according to morphological staging alone and
12 discordant cases were counted as positive); and 3) when occult bone marrow involvement
13 *per se* was considered as lymphoma involvement summative to morphological staging. All
14 tests were two-sided and $p < 0.05$ was considered significant. Data were analyzed using
15 STATA (version 11; Stata Corp., College Station, TX) and R (version 2.13.0; The R
16 Foundation for Statistical Computing, Vienna, Austria) software.

17

18 **Results**

19 **Frequency and pattern of bone marrow involvement**

1 A total of 117 patients were included in the study. FCM and PCR analysis was
2 performed for 96 (82.1%) and 101 patients (86.3%), respectively. Morphologic bone
3 marrow involvement was identified in 24 patients (20.5%). In five patients, morphologic
4 bone marrow involvement was suspected from the smear preparations and/or clot
5 sections, and was confirmed by immunohistochemistry. The level of morphologic marrow
6 involvement ranged from 1.2% to 32.4% (median, 3.2%). Six cases of morphologic
7 marrow involvement were documented as “discordant involvement”. In patients with
8 morphologic marrow involvement, with the exception of one patient, FCM or PCR clearly
9 showed clonal B-cells in the bone marrow samples. Of the 93 patients without
10 morphological evidence of lymphoma in the bone marrow, clonal B-cells were detected by
11 FCM- or PCR-based analysis in 16 (13.7%). In this group (with occult marrow
12 involvement), FCM showed that the clonal B-cell population ranged from 0.2% to 2.5%
13 (median, 0.4%) of the overall cell population. No evidence of bone marrow involvement
14 was detected in the remaining 77 patients (negative group) (Table I).

15

16 **Pretreatment characteristics according to bone marrow status**

17 Clinical characteristics according to bone marrow status are listed in Table II. In
18 addition to the anticipated higher stage and greater extranodal involvement, patients with
19 morphologic bone marrow involvement were more likely to have increased LDH levels and

1 poorer performance status than patients with negative bone marrow, thereby making them
2 more likely to have a higher IPI score. By contrast, there were no significant differences in
3 the pretreatment characteristics between patients with occult marrow involvement and
4 those with negative bone marrow, except for the increased rate of complicating
5 hemophagocytic syndrome at diagnosis observed in the occult marrow involvement group.
6 When the characteristics of the patients with morphologic marrow involvement were
7 compared with those with occult involvement, the morphologic marrow involvement group
8 was more likely to have a higher stage, more extranodal sites, poorer performance status
9 and, therefore, a higher IPI score.

10

11 **Adverse events post R-CHOP chemotherapy according to bone marrow status**

12 With respect to adverse events after R-CHOP, patients with occult and morphologic
13 bone marrow involvement were significantly more likely to develop febrile neutropenia
14 than patients with negative bone marrow. In addition, patients with occult and morphologic
15 marrow involvement were more likely to show leucocytopenia, neutropenia, anemia, and
16 thrombocytopenia, even though the difference between the occult involvement and
17 negative marrow groups did not reach statistical significance (except for the lowest platelet
18 count) after chemotherapy (Table II). In summary, the pretreatment characteristics of the
19 patients with occult marrow involvement were more similar to those of patients with

1 negative bone marrow. However, patients with occult marrow involvement experienced a
2 more severe hematological toxicity profile after R-CHOP.

3

4 **Effect of bone marrow status on outcome**

5 At the time of analysis, the median follow-up time for living patients was 35.5
6 months (range, 1.4–72.0). Overall, 20 patients died; 14 (12.0%) due to lymphoma, three
7 (2.6%) due to treatment-related toxicity, and three (2.6%) due to unrelated causes.
8 Mortalities due to treatment-related toxicity or unrelated causes other than lymphoma
9 were documented in morphologic marrow involvement or negative marrow group, and
10 were not documented in the occult marrow involvement group in our cohort.

11 Kaplan-Meier estimates, according to bone marrow status, for PFS and OS are
12 shown in Figure 1. Compared with the PFS for patients with negative bone marrow, the
13 PFS for patients with either occult (3-year PFS 43.6% vs 80.9%; $p=0.015$, log-rank test;
14 [the calculated post-hoc power for the log-rank test was 0.946]) or morphologic (3-year
15 PFS 41.3% vs 80.9%; $p<0.001$, log-rank test; [the calculated post-hoc power for the
16 log-rank test was 0.985]) bone marrow involvement was significantly worse. Mortality rates,
17 especially late deaths, for patients with both occult (3-year OS 84.6% vs 90.1%; $p=0.035$,
18 weighted log-rank test; [the calculated post-hoc power for log-rank test was 0.096]) and
19 morphologic (3-year OS 70.6% vs 90.1%; $p=0.003$, weighted log-rank test; [the calculated

1 post-hoc power for the log-rank test was 0.689)) marrow involvement were significantly
2 greater than for those with negative bone marrow. There was no significant difference in
3 PFS and OS between the occult and morphologic marrow involvement groups. The power
4 of the log-rank test was thought to be sufficient for predicting PFS; however, it may not
5 have been sufficient for predicting OS.

6 To evaluate the influence of bone marrow status on the cause-specific probabilities
7 of disease progression or relapse, the CIRs of disease progression or relapse were
8 compared between the groups. Compared with that for the negative bone marrow group,
9 the CIR of disease progression or relapse was significantly higher for patients with either
10 occult (3-year CIR 56.4% vs 18.6%; $p=0.016$, Gray's test) or morphologic (3-year CIR
11 58.8% vs 18.6%; $p<0.001$, Gray's test) marrow involvement (Figure 2). The same trend
12 was observed for PFS.

13 As patients with morphologic bone marrow involvement were more likely to have a
14 higher IPI score than patients without, multivariate analysis was performed to assess the
15 independent prognostic significance of bone marrow status after controlling for the IPI
16 (score 0 to 5). Both occult (hazard ratio [HR] 2.756, 95% confidence interval [CI]
17 1.17–6.51; $p=0.021$) and morphologic (HR 2.42, 95% CI 1.11–5.29; $p=0.026$) bone
18 marrow involvement were significant negative prognostic factors for PFS independently of
19 the IPI. At the same time, both occult (subhazard ratio [SHR] 2.84, 95% CI 1.23–6.54;

1 $p=0.014$) and morphologic (SHR 2.48, 95% CI 1.10–5.58; $p=0.028$) marrow involvement
2 were significant predictors of a high CIR of disease progression or relapse independently
3 of the IPI. Neither occult nor morphologic bone marrow involvement was a significant
4 factor for OS after controlling for the IPI (Table III).

5

6 **Power of the IPI score for predicting PFS and OS**

7 In our cohort, there was no difference in the IPI scores between 1) patients in whom
8 positive marrow involvement was identified according to morphological staging alone, with
9 discordant cases not included as positive; and 2) patients in whom positive involvement
10 was identified according to morphological staging alone, with discordant cases included as
11 positive. However, when occult marrow involvement *per se* was considered as lymphoma
12 involvement summative to morphological staging, 11 (9.4%) cases were upgraded to
13 stage IV and a change in IPI was noted in 10 (8.5%) cases. The power of the IPI score for
14 predicting PFS and OS in the Cox proportional hazards model when occult marrow
15 involvement *per se* was considered as lymphoma involvement was higher than the power
16 of the IPI score for predicting PFS and OS when positive marrow involvement was
17 identified according to morphological staging alone (Harrell's c , 0.711 vs 0.693 for PFS,
18 and 0.663 vs 0.648 for OS).

19

1 **Discussion**

2 The pretreatment characteristics of patients with occult bone marrow involvement
3 were more similar to those with negative bone marrow than to those with morphologic
4 marrow involvement. After R-CHOP, however, the hematological toxicity profile of the
5 occult marrow involvement group was more severe than that of the negative group, and
6 was comparable with that of the morphologic marrow involvement group. In addition, both
7 the PFS and OS of patients in the occult and morphologic marrow involvement groups
8 were significantly lower than those in the negative group. The main reason for the reduced
9 survival in the occult and morphologic involvement groups was thought to be a significant
10 increase in the rate of disease progression or relapse. Moreover, the risk associated with
11 occult and morphologic marrow involvement in term of PFS reduction was greater than
12 that encompassed by the IPI score.

13 The use of different ancillary investigations to detect occult DLBCL involvement in
14 the bone marrow may result in heterogeneity between positive cases [14]. Four-color FCM
15 in conjunction with side scatter and CD45 gating has increased the specificity and
16 sensitivity of the investigation avoiding the inclusion of normal B-cell precursors
17 (hematogones) in the analyzed bone marrow specimens [29]. In the present study, FCM
18 was helpful in identifying cases of occult marrow involvement, with clonal populations
19 ranging from 0.2% to 2.5% of the overall cell population analyzed. Similarly, a study by

1 Hanson et al. reported five DLBCL cases that showed FCM-positive, but
2 morphologically-negative, bone marrow, containing a small clonal population ranging from
3 0.09% to 3% of the overall cell population [30]. However, the false-negative results could
4 be obtained by FCM when examining samples with morphologically-detectable disease,
5 which could be attributed to a number of factors including the sampling method or the
6 presence of residual populations of non-neoplastic lymphocytes along with the partial
7 involvement of lymphoma cells [14,31]. At the same time, somatic hypermutations are one
8 of possible causes of false-negative molecular results, even though the BIOMED-2
9 protocol for detecting immunoglobulin gene clonality is highly specific and sensitive, and
10 has been validated in numerous studies of B-cell malignancies [20,32]. Overall, as argued
11 by Talaulikar et al., these two ancillary staging tests should be regarded as a summative
12 model rather than as a concordance model because, although each is highly specific, they
13 both have inherent limitations in sensitivity [14].

14 In our cohort of 117 consecutive patients, the incidence rates of morphologic
15 (20.5%) and occult bone marrow involvement (13.7%) were similar to those reported
16 previously [11,14-16]. Recently, The Canberra Hospital and Australian National University
17 Medical School group published comprehensive data on the incidence of morphologic and
18 occult bone marrow involvement. They found that 30 (19.2%) out of 156 cases of DLBCL
19 were positive on routine bone marrow histology plus consensual review of H&E stains,

1 and that immunohistochemistry and four-color FCM (using similar diagnostic parameters
2 to those used in the present study) each detected histologically-inapparent involvement in
3 17 out of the evaluable 154 and 152 cases, respectively [17]. They also found that 41
4 (26.5%) out of 155 evaluable cases were positive upon immunoglobulin heavy and light
5 chain gene analysis; however, routine histological analyses showed that 12 of these cases
6 were positive and 29 were negative [17]. Bone marrow trephine biopsies were not
7 performed routinely in the present study, which may have reduced both the time required
8 for bone marrow examination and the amount of distress caused to the patient; however,
9 this may also have reduced the sensitivity of the morphologic studies used to detect
10 marrow involvement [33,34]. However, the summative definition of morphologic marrow
11 involvement utilized in the present study (enabling the identification of positive cases if
12 involvement was detected in at least one of the smear preparations, clot sections or
13 immunohistochemical stains) may have increased the detection of marrow involvement to
14 a level similar to that expected after examination of bone marrow trephine biopsies.

15 Several studies have addressed the clinical role of occult bone marrow involvement
16 in DLBCL patients treated with CHOP, or with CHOP-like regimens without rituximab
17 [11,14-17]. Mitterbauer-Hohendanner et al. reported a significantly lower CR rate for cases
18 with clonal immunoglobulin gene rearrangements in the bone marrow than for
19 PCR-negative cases, and significant differences in the estimated OS at 5 years among

1 patients with morphologically-negative bone marrow with negative PCR results, those with
2 morphologically-negative but positive PCR results and those with positive bone marrow
3 morphology [11]. Talaulikar et al. reported that cases with and without marrow involvement
4 (according to conventional morphological staging alone) showed no significant difference
5 in survival in their cohort; however, when positive marrow involvement was identified by
6 morphology and/or FCM, the median survival of patients with involvement was
7 significantly worse than that of patients without involvement [16]. More recently, when
8 using an immunophenotyping method (FCM and immunohistochemistry), Talaulikar et al.
9 noted a change in IPI in 18 (18.5%) out of 156 cases [17]. They also showed that this
10 revised IPI model allowed better differentiation among the IPI categories than the
11 conventional IPI model based on morphology alone [17]. To the best of our knowledge, the
12 present study is the first to validate the negative clinical impact of occult marrow
13 involvement on PFS and OS in DLBCL patients treated with rituximab. Moreover, the
14 adverse prognostic impact of occult marrow involvement was indicated to be comprised of
15 a high risk of disease progression or relapse, but not of treatment-related mortality. Indeed,
16 mortalities due to treatment-related toxicity or unrelated causes other than the refractory
17 lymphoma were not documented in the occult marrow involvement group in our cohort
18 despite the severe hematological toxicity profile after R-CHOP for the group. Previous
19 reports showed high frequency of the presence of bone marrow involvement in cases with

1 DLBCL-associated hemophagocytic syndrome [35,36]. In addition, the percentages of
2 lymphoma cells in the bone marrow in patients with DLBCL-associated hemophagocytic
3 syndrome were reported to be sometimes less than 1% of total bone marrow cell
4 population [36]. Therefore, we can infer that both occult marrow involvement and
5 morphologic involvement contribute to the pathogenesis of hemophagocytic syndrome.
6 Another study reported hypercytokinemia (increased levels of interferon- γ and
7 macrophage colony-stimulating factor) and a low CD4/CD8 ratio in the peripheral blood
8 and bone marrow of patients with DLBCL-associated hemophagocytic syndrome [37]. We
9 also infer that the potential hematopoietic inhibition caused by hypercytokinemia [38],
10 coupled with the phagocytosis of hematopoietic cells, may have caused the severe
11 hematological toxicity profiles observed in the marrow involvement group.

12 The finding that the prognostic impact of occult marrow involvement on a poor PFS
13 was independent of the IPI score is consistent with the pretreatment individual IPI factors,
14 and with the finding that the IPI scores of patients with occult marrow involvement were
15 similar to those of patients with no marrow involvement. At the same time, the significant
16 relationship between occult marrow involvement and a higher rate of late mortality also
17 appears to be rational, because these patients are more likely to relapse and not all would
18 be salvaged by second-line therapy and high-dose therapy supported by hematopoietic
19 stem cell transplantation [39,40]. In fact, two cases of late mortality were observed in the

1 occult marrow involvement group more than 3 years after diagnosis, and were due to
2 repeated episodes of lymphoma relapse (data not shown). Therefore, as with the
3 independent negative prognostic impact of occult marrow involvement on PFS, its impact
4 on OS may potentially be independent of the IPI after a longer follow-up period. As FCM
5 and PCR analyses were not able to differentiate between concordant and discordant
6 marrow involvement, we included the discordant marrow involvement among the
7 morphologic marrow involvement group in comparison of survival, which may have led to
8 an underestimation of the relapse or mortality rates for patients with occult and
9 morphologic marrow involvement [9,10]. Nevertheless, the finding that the final summative
10 model (for diagnosing positive marrow involvement if either morphologic or ancillary
11 investigations yielded a positive result) led to the best predictive value for the IPI is
12 noteworthy.

13 On the other hand, Tierens et al. recently reported that DLBCL with an activated
14 B-cell immunophenotype, which was previously reported to be associated with worse
15 overall survival and failure free survival rates [4-6], showed a much higher frequency of
16 bone marrow infiltration with monoclonal small B-cells (28.2%) than DLBCL with a
17 germinal center B-cell immunophenotype (3.7%) [41]. Whether these monoclonal small
18 B-cells are precursors of DLBCL, or arise from a common background that favors clonal
19 B-cell expansion remains to be seen [41]. Further studies are also required to investigate

1 how closely the occult and morphologic marrow involvement groups are associated with
2 the activated B-cell subgroup and its poor outcome.

3 In conclusion, this retrospective study represents the first comprehensive analysis
4 of the significance of occult bone marrow involvement in patients with DLBCL receiving the
5 current standard of care, R-CHOP. Occult bone marrow involvement predicts a severe
6 hematological toxicity profile after R-CHOP and negatively impacts on PFS and OS,
7 possibly by increasing the risk of disease progression or relapse, although this is
8 independent of the IPI for PFS. Proper detection of occult marrow involvement, and
9 practical use of the information to determine the most appropriate treatment plan, would
10 substantially improve the outcome of patients with DLBCL.

11

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18

19 **Potential conflict of interest**

1 The authors declare no conflict of interest.

2

3 **Author contributions**

4 HA was the principal investigator and takes primary responsibility for the paper. HM
5 and KN performed the laboratory analysis. YI performed the histological studies. ST, MK,
6 and AM recruited the patients and reviewed the manuscript. TT, TI, HA, and HM designed
7 and co-ordinated the research. HA and HM wrote the paper. All authors approved the final
8 draft of the paper.

9

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Table Legends

Table I

Detection of lymphoma involvement in morphologically positive and negative bone marrow samples by flow cytometry and/or PCR

		PCR+	PCR-	PCR not done
Negative bone marrow involvement n=77 (65.8%)	FCM+	0	0	0
	FCM-	0	51 (66.2%)	13 (16.9%)
	FCM not done	0	13 (16.9%)	-
Occult bone marrow involvement n=16 (13.7%)	FCM+	5 (31.3%)	5 (31.3%)	0
	FCM-	3 (18.7%)	0	0
	FCM not done	3 (18.7%)	0	-
Morphologic bone marrow involvement n=24 (20.5%)	FCM+	11 (45.8%)	3 (12.5%)	3 (12.5%)
	FCM-	1 (4.2%)	1 (4.2%)	0
	FCM not done	5 (20.8%)	0	-

FCM, flow cytometry; PCR, polymerase chain reaction.

PCR +, clonal B-cells detected by PCR.

PCR -, clonal B-cells undetectable by PCR.

FCM +, clonal B-cells ($\geq 0.2\%$ of total nucleated bone marrow cell population) detected by FCM.

FCM -, clonal B-cells undetectable by FCM.

Table II

Patient and disease characteristics according to bone marrow status

Characteristic	Bone Marrow Involvement									
	Negative (n=77)		Occult (n=16)		Morphologic (n=24)		<i>p-value</i>			
	No. of patients	%	No. of patients	%	No. of patients	%	Negative vs Occult*	Negative vs Morphologic**	Occult vs Morphologic†	
Age, years, Median (Range)	69 (22–89)		66 (48–89)		71.5 (48–88)					
Sex	Male	46	59.7	9	56.3	15	62.5	0.788	1.000	0.750
	Female	31	40.3	7	43.8	9	37.5			
IPI factors	Age>60 years	57	74.0	11	68.8	19	79.2	0.758	0.788	0.482
	LDH>ULN	36	46.8	10	62.5	20	83.3	0.284	0.002	0.159
	ECOG PS≥2	29	37.7	4	25.0	18	75.0	0.401	0.002	0.003
	Stage III/IV	38	49.4	9	56.3	24	100.0	0.785	<0.001	<0.001
	Extranodal sites≥2	20	26.0	4	25.0	17	70.8	1.000	<0.001	0.009
IPI score	Low/Low-intermediate (0–2)	43	55.8	11	68.8	2	8.3	0.412	<0.001	<0.001
	High/High-intermediate (3–5)	34	44.2	5	31.3	22	91.7			
B symptoms		27	35.1	6	37.5	18	75.0	1.000	<0.001	0.025
Hemophagocytic syndrome at diagnosis		0	0.0	2	12.5	6	25.0	0.028	<0.001	0.439
<i>After R-CHOP chemotherapy^{††}:</i>										
		n=61		n=16		n=24				
Lowest WBC count, × 10 ⁹ /L, Median (Range)		1.1 (0.4–4.1)		1.1 (0.1–2.8)		0.75 (0.1–3.0)		0.130	0.001	0.313
Lowest neutrophil count, ×10 ⁹ /L, Median (Range)		0.34 (0.04–2.02)		0.30 (0.01–0.77)		0.19 (0.01–0.99)		0.062	0.020	0.871
Lowest HGB level, g/dL, Median (Range)		10.3 (6.2–13.4)		9.45 (6.1–12.9)		7.95 (5.1–14.1)		0.150	<0.001	0.075
Lowest PLT count, ×10 ⁹ /L, Median (Range)		137 (39–362)		106 (16–174)		93.5 (10–212)		0.003	<0.001	0.222
Febrile neutropenia		12	19.7	8	50.0	14	58.3	0.023	0.001	0.748

IPI, International Prognostic Index; LDH, lactate dehydrogenase; ULN, upper limit of normal; ECOG, Eastern Cooperative Oncology Group; PS, performance status; R-CHOP, rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone; WBC, white blood cell; HGB, hemoglobin; PLT, platelets.

*Comparison of patients with no bone marrow involvement with those with occult bone marrow involvement.

**Comparison of patients with no bone marrow involvement with those with morphologic bone marrow involvement.

†Comparison of patients with occult with those with morphologic bone marrow involvement.

††Toxicity after R-CHOP therapy was compared between patients in each cohort that were initially scheduled to receive six to eight cycles of R-CHOP (excluding those that received initial treatment comprising three cycles of R-CHOP followed by involved field irradiation). Toxicity induced by high-dose therapies other than R-CHOP was not included in the comparison.

Table III

Multivariate regression model of bone marrow involvement and IPI score for PFS, OS, and the CIR of disease progression or relapse

Variable	PFS			OS			CIR of progression/relapse		
	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>	SHR	95% CI	<i>p</i>
Negative bone marrow involvement	1.00	-	-	1.00	-	-	1.00	-	-
Occult bone marrow involvement	2.76	1.17–6.51	0.021	-	-	-	2.84	1.23–6.54	0.014
Morphologic bone marrow involvement	2.42	1.11–5.29	0.026	-	-	-	2.48	1.10–5.58	0.028
IPI (0–5)	1.38	1.09–1.75	0.008	1.44	1.08–1.92	0.013	1.39	1.10–1.74	0.005

IPI, International Prognostic Index; PFS, progression-free survival; OS, overall survival; CIR, cumulative incidence rate; HR, hazard ratio; SHR, subhazard ratio; CI, confidence interval.

Figure Legends

Figure 1

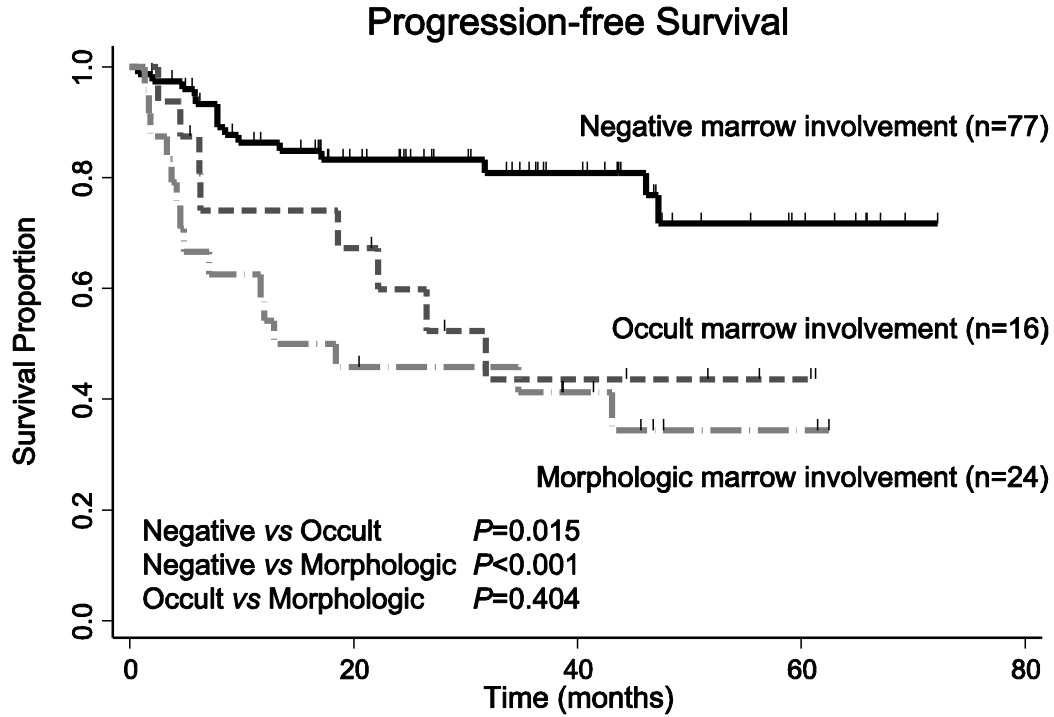
Kaplan-Meier curves showing progression-free survival (PFS; A) and overall survival (OS; B) according to presence and type of bone marrow involvement. The statistical significance between groups was calculated using the log-rank test or the weighted log-rank test with Fleming-Harrington weights of $\rho=0$ and $\gamma=1$ for PFS and OS, respectively. Negative vs Occult, comparison of survival between the negative and occult marrow involvement groups; Negative vs Morphologic, comparison of survival between the negative and morphologic marrow involvement groups; Occult vs Morphologic, comparison of survival between the occult and morphologic marrow involvement groups.

Figure 2

Cause-specific cumulative incidence rate (CIR) curves for disease progression or relapse according to presence and type of bone marrow involvement. The statistical significance between groups was calculated using Gray's test. Negative vs Occult, comparison of CIRs between the negative and occult marrow involvement groups; Negative vs Morphologic, comparison of CIRs between the negative and morphologic marrow involvement groups; Occult vs Morphologic, comparison of CIRs between the occult and morphologic marrow involvement groups.

Figure 1

A



B

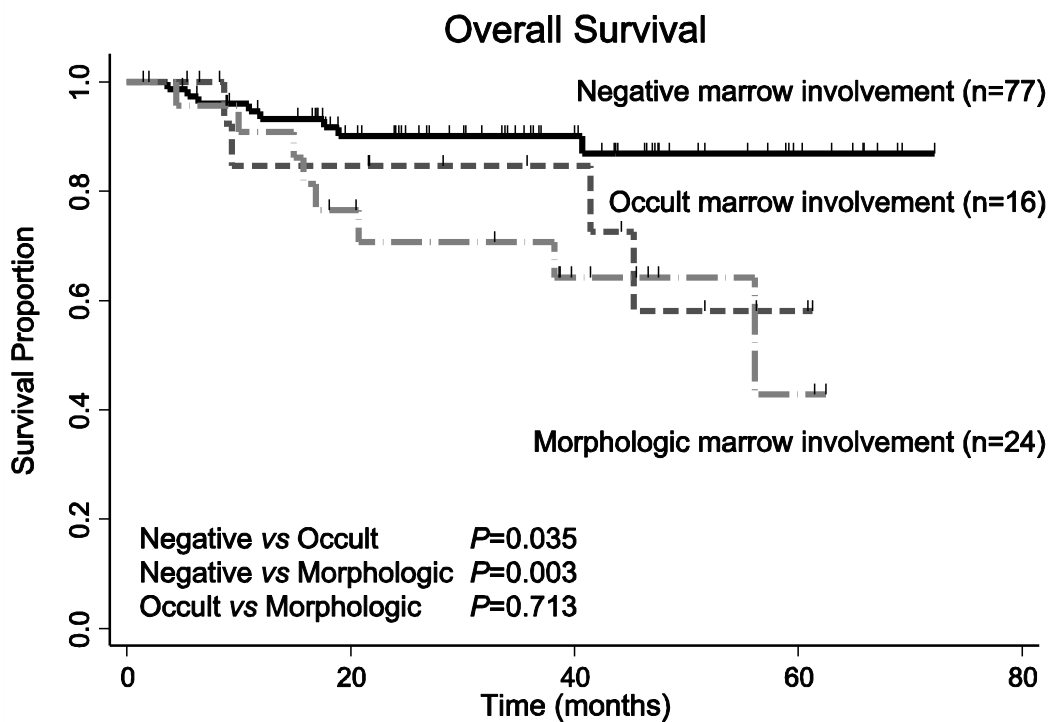


Figure 2

