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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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Abstract

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

Introduction

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

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

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

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

Materials and methods

Patients

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

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

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

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

macrophages, which phagocytose blood cells. Febrile neutropenia after chemotherapy was defined as a body temperature ≥38.2°C and a neutrophil count $<0.5\times10^9$/L (as assessed on the same day, or the day after, the fever was diagnosed). The study was approved by the local institutional ethics committee.

Bone marrow morphology

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

morphology were utilized.

Flow cytometry

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

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

Polymerase chain reaction

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

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

Definition of bone marrow involvement

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

Statistical analysis

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

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

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

Results

Frequency and pattern of bone marrow involvement

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

Pretreatment characteristics according to bone marrow status

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

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

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

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

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

Effect of bone marrow status on outcome

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

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

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

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

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

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

Power of the IPI score for predicting PFS and OS

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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Potential conflict of interest

The authors declare no conflict of interest.

Author contributions

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

References

1. Ziepert M, Hasenclever D, Kuhnt E, et al. Standard International prognostic index remains a valid predictor of outcome for patients with aggressive CD20+ B-cell lymphoma in the rituximab era. J Clin Oncol 2010;28:2373-2380.
2. Sehn LH, Berry B, Chhanabhai M, et al. The revised International Prognostic Index (R-IPI) is a better predictor of outcome than the standard IPI for patients with diffuse large B-cell lymphoma treated with R-CHOP. Blood 2007;109:1857-1861.
3. Ngo L, Hee SW, Lim LC, et al. Prognostic factors in patients with diffuse large B cell lymphoma: before and after the introduction of rituximab. Leuk Lymphoma 2008;49:462-469.

4. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 2000;403:503-511.

5. Shipp MA, Ross KN, Tamayo P, et al. Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. Nat Med 2002;8:68-74.

6. Fu K, Weisenburger DD, Choi WWL, et al. Addition of rituximab to standard chemotherapy improves the survival of both the germinal center B-cell–like and non–germinal center B-cell–like subtypes of diffuse large B-cell lymphoma. J Clin Oncol 2008;26:4587-4594.

7. Sehn LH. Early detection of patients with poor risk diffuse large B-cell lymphoma. Leuk Lymphoma 2009;50:1744-1747.

8. Campbell J, Seymour JF, Matthews J, Wolf M, Stone J, Juneja S. The prognostic impact of bone marrow involvement in patients with diffuse large cell lymphoma varies according to the degree of infiltration and presence of discordant marrow involvement. Eur J Haematol 2006;76:473-480.

9. Chung R, Lai R, Wei P, et al. Concordant but not discordant bone marrow involvement in diffuse large B-cell lymphoma predicts a poor clinical outcome independent of the International Prognostic Index. Blood 2007;110:1278-1282.

10. Sehn LH, Scott DW, Chhanabhai M, et al. Impact of concordant and discordant

bone marrow involvement on outcome in diffuse large B-cell lymphoma treated with R-CHOP. J Clin Oncol 2011;29:1452-1457.

11. Mitterbauer-Hohendanner G, Mannhalter C, Winkler K, et al. Prognostic significance of molecular staging by PCR-amplification of immunoglobulin gene rearrangements in diffuse large B-cell lymphoma (DLBCL). Leukemia 2004;18:1102-1107.

12. Cheson BD, Horning SJ, Coiffier B, et al. Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. J Clin Oncol 1999;17:1244-1257.

13. Cheson BD, Pfistner B, Juweid ME, et al. Revised response criteria for malignant lymphoma. J Clin Oncol 2007;25:579-586.

14. Talaulikar D, Dahlstrom JE, Shadbolt B, McNiven M, Broomfield A, Pidcock M. Occult bone marrow involvement in patients with diffuse large B-cell lymphoma: results of a pilot study. Pathology 2007;39:580-585.

15. Yamamoto-Kawano C, Muroi K, Izumi T, et al. Clinical significance of flow cytometric evaluation of bone marrow involvement in B-cell lymphoma. Jichi Medical University journal 2006;29:105-113.

16. Talaulikar D, Shadbolt B, Bell J, et al. Clinical role of flow cytometry in redefining bone marrow involvement in diffuse large B-cell lymphoma (DLBCL)–a new

perspective. Histopathology 2008;52:340-347.

17. Talaulikar D, Shadbolt B, Dahlstrom JE, McDonald A. Routine use of ancillary investigations in staging diffuse large B-cell lymphoma improves the International Prognostic Index (IPI). J Hematol Oncol 2009;22:49-57.

18. Coiffier B, Lepage E, Brière J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med 2002;346:235-242.

19. Wilkins BS. Lymphoma. In: Wickramasinghe SN and McCullough J, editors. Blood and Bone Marrow Pathology; Churchill Livingstone, Philadelphia, PA, USA; 2002. p 405-436.

20. Van Dongen J, Langerak A, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003;17:2257-2317.

21. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958:457-481.

22. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer chemotherapy reports. Part 1 1966;50:163-170.

23. Fleming TR, Harrington DP. Weighted Logrank Statistics. In: Fleming TR,

Harrington DP, editors. Counting Processes and Survival Analysis; John Wiley & Sons, Inc., Hoboken, NJ, USA; 1991. p 255-286.

24. Machin D, Cambel M, Fayers P, Pinol A. Comparing Two Survival Curves. In: Machin D, Cambel M, Fayers P, Pinol A, editors. Sample Size Tables for Clinical Studies, 2nd edition; Blackwell Science, Oxford, UK; 1997. p 174-253.

25. Gray RJ. A class of K-sample tests for comparing the cumulative incidence of a competing risk. Ann Stat 1988;16:1141-1154.

26. Cox DR. Regression models and life-tables. J Roy Stat Soc B Stat Meth 1972;34:187-220.

27. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. J Am Stat Assoc 1999;94:496-509.

28. Harrell Jr FE, Califf RM, Pryor DB, Lee KL, Rosati RA. Evaluating the yield of medical tests. J Am Med Assoc 1982;247:2543-2546.

29. Morice WG, Kurtin PJ, Hodnefield JM, et al. Predictive value of blood and bone marrow flow cytometry in B-cell lymphoma classification: comparative analysis of flow cytometry and tissue biopsy in 252 patients. Mayo Clin Proc 2008:776-785.

30. Hanson CA, Kurtin PJ, Katzmann JA, et al. Immunophenotypic analysis of peripheral blood and bone marrow in the staging of B-cell malignant lymphoma. Blood 1999;94:3889-3896.

31. El-Sayed AM, El-Borai MH, Bahnassy AA, El-Gerzawi S. Flow cytometric immunophenotyping (FCI) of lymphoma: correlation with histopathology and immunohistochemistry. Diagn Pathol 2008;3:43-55.

32. Morales AV, Arber DA, Seo K, Kohler S, Kim YH, Sundram UN. Evaluation of B-cell clonality using the BIOMED-2 PCR method effectively distinguishes cutaneous B-cell lymphoma from benign lymphoid infiltrates. Am J Dermatopathol 2008;30:425-430.

33. Dee J, Valdivieso M, Drewinko B. Comparison of the efficacies of closed trephine needle biopsy, aspirated paraffin-embedded clot section, and smear preparation in the diagnosis of bone-marrow involvement by lymphoma. Am J Pathol 1976;65:183-194.

34. Coller B, Chabner B, Gralnick H. Frequencies and patterns of bone marrow involvement in non-Hodgkin lymphomas: observations on the value of bilateral biopsies. Am J Hematol 1977;3:105-119.

35. Shimazaki C, Naba T, Nakagawa M. B-cell lymphoma-associated hemophagocytic syndrome. Leuk Lymphoma 2000;38:121-130.

36. Shimazaki C, Inaba T, Shimura K, et al. B-cell lymphoma associated with haemophagocytic syndrome: a clinical, immunological and cytogenetic study. Brit J Haematol 2005;104:672-679.

37. Miyahara M, Sano M, Shibata K, et al. B-cell lymphoma-associated hemophagocytic syndrome: clinicopathological characteristics. Ann Hematol 2000;79:378-388.

38. Selleri C, Maciejewski JP, Sato T, Young N. Interferon-gamma constitutively expressed in the stromal microenvironment of human marrow cultures mediates potent hematopoietic inhibition. Blood 1996;87:4149-4157.

39. Kewalramani T, Zelenetz AD, Nimer SD, et al. Rituximab and ICE as second-line therapy before autologous stem cell transplantation for relapsed or primary refractory diffuse large B-cell lymphoma. Blood 2004;103:3684-3688.

40. Thomson KJ, Morris EC, Bloor A, et al. Favorable long-term survival after reduced-intensity allogeneic transplantation for multiple-relapse aggressive non-Hodgkin's lymphoma. J Clin Oncol 2009;27:426-432.

41. Tierens AM, Holte H, Warsame A, et al. Low levels of monoclonal small B cells in the bone marrow of patients with diffuse large B-cell lymphoma of activated B-cell type but not of germinal center B-cell type. Haematologica 2010;95:1334-1341.

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 (≥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						*p-value*		
		Negative (n=77)		Occult (n=16)		Morphologic (n=24)				
		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
After R-CHOP chemotherapy††:		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

	PFS			OS			CIR of progression/relapse		
Variable	HR	95% CI	*p*	HR	95% CI	*p*	SHR	95% CI	*p*
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 ρ=0 and γ=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

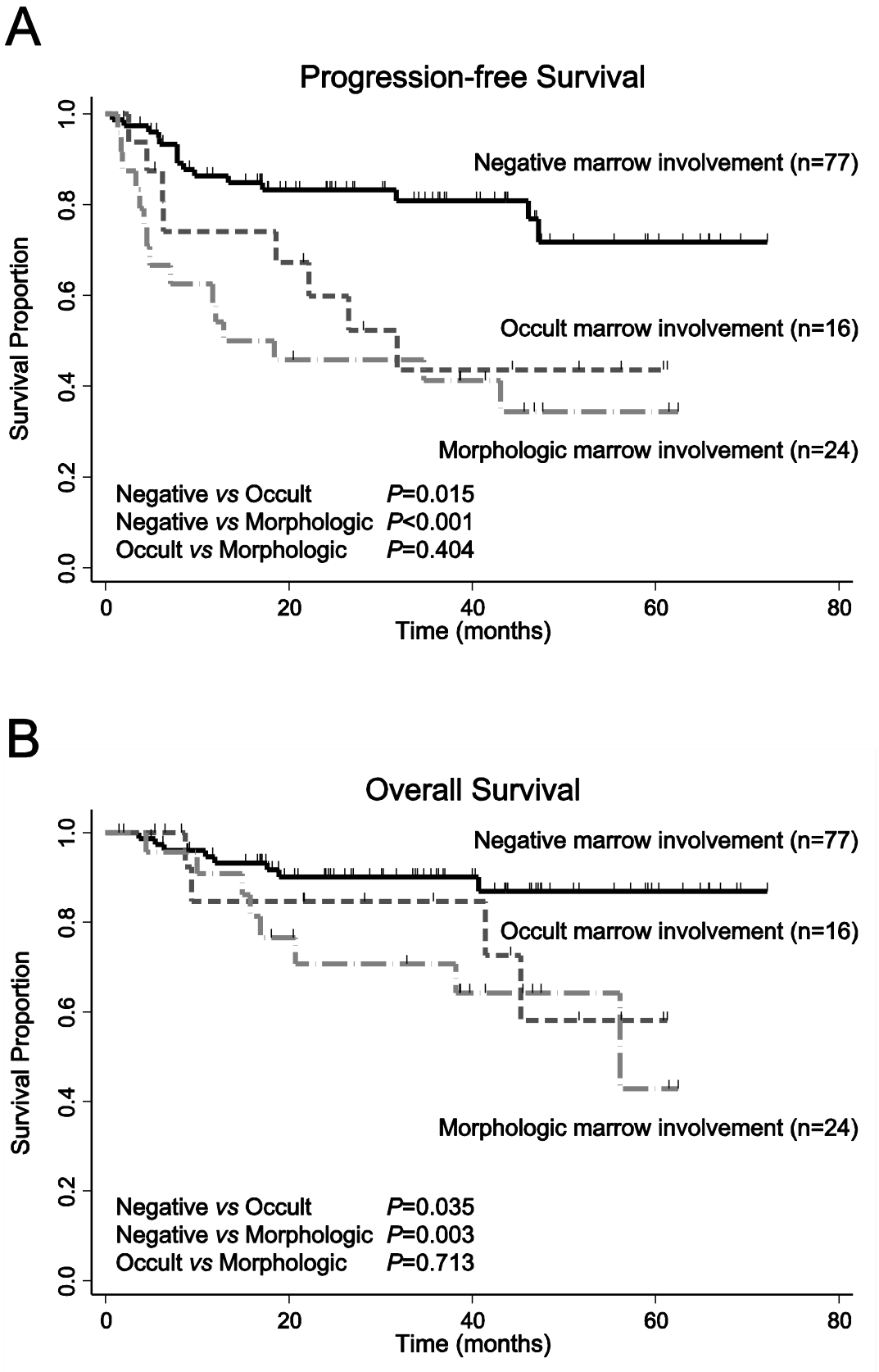
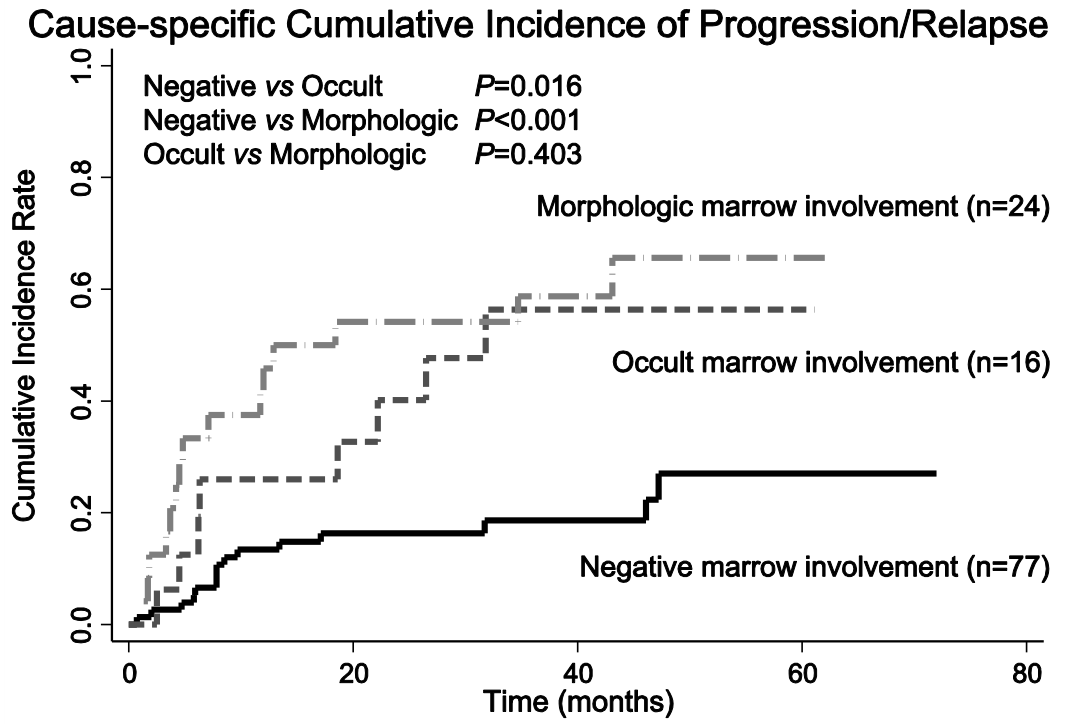

A
Progression-free Survival
Survival Proportion
0.0
0.2
0.4
0.6
0.8
1.0
Negative marrow involvement (n=77)
Occult marrow involvement (n=16)
Morphologic marrow involvement (n=24)
Negative vs Occult P=0.015
Negative vs Morphologic P<0.001
Occult vs Morphologic P=0.404
0
20
40
60
80
Time (months)
B
Overall Survival
Survival Proportion
0.0
0.2
0.4
0.6
0.8
1.0
Negative marrow involvement (n=77)
Occult marrow involvement (n=16)
Morphologic marrow involvement (n=24)
Negative vs Occult P=0.035
Negative vs Morphologic P=0.003
Occult vs Morphologic P=0.713
0
20
40
60
80
Time (months)

Figure 2


Cause-specific Cumulative Incidence of Progression/Relapse
Negative *vs* Occult *P*=0.016
Negative *vs* Morphologic *P*<0.001
Occult *vs* Morphologic *P*=0.403
Morphologic marrow involvement (n=24)
Occult marrow involvement (n=16)
Negative marrow involvement (n=77)
Cumulative Incidence Rate
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
Time (months)