Usefulness of Flow Cytometric Immunophenotyping for Bone Marrow Staging in Patients With Mantle Cell Lymphoma After Therapy

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Abstract

We evaluated the role of flow cytometric immunophenotyping (FCI) in the assessment of bone marrow (BM) specimens in 104 patients with mantle cell lymphoma (MCL) following treatment with aggressive combination chemotherapy. Of the patients, 77 had no morphologic or FCI evidence of MCL, 13 had morphologic and FCI evidence of MCL, and 14 patients were morphologically negative but FCI showed CD5+ clonal B-cells (M~/FCI+). Retrospective cyclin D1 immunostaining was positive in 3 of 12 M~/FCI+ cases. Clinical staging and follow-up showed that 4 of 12 patients had concurrent extramedullary involvement by MCL, 5 patients subsequently became M+ for MCL in BM (within 2-24 months), and 3 patients had no recurrent MCL on follow-up. We conclude that FCI is more sensitive than morphologic examination of BM at the time of restaging in patients with MCL and that positivity by only FCI in BM often correlates with concurrent disease or subsequent relapse.

Mantle cell lymphoma (MCL) is a distinctive B-cell neoplasm characteristically associated with t(11;14)(q13;q32) and overexpression of cyclin D1.1,2 Most patients have lymphadenopathy, and approximately 60% to 70% have bone marrow (BM) involvement at the time of diagnosis.3,4 In the past 20 years, dose-intensified regimens have demonstrated improved outcomes in patients with MCL. At our institution, rituximab plus fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone (hyper-CVAD) alternating with rituximab plus high-dose methotrexate-cytarabine has been used to treat patients with MCL, with excellent results.5,6 A trial conducted by the European MCL Network showed that myeloablative radiochemotherapy followed by autologous stem cell transplantation represents another therapeutic option in the first-line treatment of younger patients with MCL.2

BM aspiration and biopsy with morphologic examination are commonly used to assess response to therapy. Ancillary studies using flow cytometric immunophenotyping (FCI), immunohistochemical analysis, or molecular methods are commonly used to supplement morphologic examination, but the role of these ancillary studies in this setting is not well defined. In regard to FCI data, the significance of detecting a monoclonal B-cell population, usually small (<5%), in the absence of morphologic evidence of lymphoma in staging and posttherapy restaging BM specimens is controversial. For example, in 2007 Cheson and colleagues7 made recommendations for response criteria for patients with malignant lymphoma. They suggested that a small (<2%) monoclonal population detected by FC evaluation in a postchemotherapy...
BM specimen should not be considered clinically significant. However, Cheson and colleagues acknowledged that there were few studies available in the literature that assessed the clinical significance of FCI-detected evidence of lymphoma in BM specimens without morphologic evidence of disease.

The goal of this study was to evaluate the usefulness of FCI in the workup of BM specimens obtained from patients with MCL after aggressive chemotherapy.

Materials and Methods

The study group included 104 patients with MCL treated with the hyper-CVAD protocol at M.D. Anderson Cancer Center (Houston, TX) from January 1, 1999, to December 31, 2001, and who underwent BM examination after therapy. The diagnosis of MCL was supported by lymph node or extranodal site tissue biopsy that showed morphologic and immunophenotypic features of MCL. Fluorescence in situ hybridization or polymerase chain reaction methods were performed in a subset of cases and showed the presence of the CCND1-IGH fusion gene consistent with t(11;14)(q13;q32).

BM aspiration and biopsy, unilateral or bilateral, was routinely performed on each patient at the time of initial diagnosis of MCL. At our institution, FCI is usually done on BM aspirate material as part of the routine workup. For this study, we reviewed the clinical information, treatment history, pathology report, and immunophenotypic results for each BM specimen at the time of initial diagnosis of MCL. The procedures followed were in accord with the ethical standards established by our institution. The extent of involvement by lymphoma in relation to total BM cellularity was semiquantified as follows: less than 5%, 5% to less than 25%, 25% to 50%, and more than 50%. Patterns of lymphoma were classified as paratrabecular (PT), non-PT, interstitial, diffuse, or mixed.

As part of the clinical protocol for which these patients with MCL were participants, BM aspiration and biopsy are also routinely performed after therapy. The patients were regularly followed up with a median interval of 6 months (range, 1-6 months). Wright-Giemsa–stained BM aspirate smears and H&E-stained slides of the BM aspirate clot and core biopsy specimens were reviewed. A case of morphologic evidence of persistent MCL was classified as such if an infiltrate of lymphoma cells was identified on core biopsy or clot sections and/or if unequivocally atypical lymphocytes were seen on BM aspirate smears.

Immunophenotypic Studies

Immunohistochemical analysis using fixed, paraffin-embedded tissue sections of BM aspirate, clot, or biopsy specimens was performed on a subset of cases, at the time of initial diagnosis of MCL or after therapy, using a variable panel of antibodies that included 1 or more reagents specific for CD3, CD5, CD20, Pax-5, and cyclin D1. Cyclin D1 expression was assessed at the time of initial diagnosis in most cases by using the mouse monoclonal antibody AM29 (Zymed Laboratories, South San Francisco, CA). For this study, restaging BM specimens that were reported to lack morphologic evidence of MCL but were positive by FCI were assessed for cyclin D1 using the rabbit monoclonal antibody SP4 (Neomarkers, Fremont, CA). In our validation studies, a series of lymph node specimens positive for MCL showed cyclin D1 positivity with the mouse AM29 and rabbit SP4 antibodies, but staining was frequently stronger and easier to interpret with SP4 (unpublished data).

FCI studies were performed at the time of initial diagnosis of MCL at our institution on BM, peripheral blood, or lymph node or extranodal site biopsy specimens. BM aspirates obtained after chemotherapy were also submitted routinely for FCI studies. If bilateral BM examination was performed, FCI was routinely performed on only 1 side.

BM aspirate samples were assessed using 3- or 4-color FCI analysis and a FACSscan or FACSCalibur instrument (Becton Dickinson Biosciences [BDB], San Jose, CA). Lymphocytes were gated for analysis using CD45 expression and right-angle light scatter (side scatter; SSC) as described previously. Fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated IgG1 and IgG2 antibodies with irrelevant specificities were used as negative controls, and cursors were set to include more than 95% of events as negative. The panel of antibodies, conjugated to FITC, PE, peridinin-chlorophyll protein (PerCP), or allophycocyanin (APC), included reagents specific for CD3, CD5, CD19, CD20, CD23, FMC-7, and immunoglobulin κ and λ light chains (BDB).

For all initial staging specimens and the follow-up specimens from 1999 through 2003, a 3-color panel for light chain analysis included the combinations κ-FITC or λ-FITC/CD19-PE/CD45-PerCP, with collection of 10,000 cells per tube and gating on lymphocytes on CD45 vs SSC plots Image 1. In some cases with few B cells, no light chain assessment was performed. For follow-up specimens from June 2004 onward, the 4-color tubes for light chain analysis included CD19 and CD5 in the same tube (eg, κ-FITC/CD19-PE/CD45-PerCP/CD5-APC). From September 2004 onward, collection was increased to 100,000 cells per tube, and from May 2005 onward, the combination κ-FITC/λ-PE/CD19-PerCP/CD5-APC was included, with gating on total CD19+ lymphocytes and the specific CD19+/CD5+ subset Image 2.

We required a minimum of 20 events for a diagnosis of disease involvement, so the theoretical maximum sensitivity of a 10,000 event collection is 0.2% and for 100,000 events, 0.02%. The 3-color flow panels performed at the beginning
Results

BM Staging Specimens at Time of Initial Diagnosis of MCL

BM staging specimens were available for review for 99 patients at the time of initial diagnosis of MCL. These included 74 men and 25 women, with a median age of 60 years (range, 42-79 years). In total, 49 unilateral BM aspirations and biopsies and 50 bilateral BM biopsies with unilateral aspiration were performed, resulting in a total of 149 BM specimens. Of the patients, 84 had at least 1 BM specimen positive for MCL using morphologic criteria alone: 88% (44/50) of patients with bilateral and 86% (42/49) of patients with unilateral BM biopsies. Bilateral BM biopsy increased the sensitivity of detection of MCL by only 6%, or 3% if one side would have been chosen by chance.

The extent of BM involvement by MCL was as follows in the 114 positive biopsies: 28 (24.6%), less than 5%; 48 (42.1%), 5% to less than 25%; 16 (14.0%), 25% to 50%; and 22 (19.3%), 50% or more. In 103 evaluable samples, BM involvement by MCL was characterized by a number of patterns, including 15 (14.6%) PT, 8 (7.8%) non-PT, 24 (23.3%) mixed PT and non-PT, 23 (22.3%) interstitial, 18 (17.5%), interstitial and non-PT, and 15 (14.6%) diffuse.
In 9 (9%) of 99 patients reviewed at diagnosis, morphologic analysis (M) and FCI results were discordant. In 4 patients, M was positive for lymphoma but FCI was negative (M+/FCI−), and in 5 patients, FCI was positive but M was negative (M−/FCI+) for lymphoma. In the M+/FCI− subgroup, there was no predominant pattern, and the extent of disease by morphologic assessment ranged from less than 5% to 30%. One case was reportedly CD5− by FCI performed at a referring institution, and, thus, a small MCL population, if present, would be more difficult to detect in a background of polyclonal benign CD5− B cells. On retrospective review of the 3-color FCI data, the other 3 FCI− specimens contained a small population of CD5+ B cells with atypically dim CD19 expression, involving 0.2% to 0.7% of total analyzed events. These findings were highly suggestive of involvement by MCL. However, there were relatively low numbers of B cells in these FCI specimens, and no light chain staining was performed, so a definitive diagnosis was not possible. In the M−/FCI+ subgroup, the tumor load detected by FCI was less than 1% of total analyzed events in all cases.

FCI showed a higher rate of detection of MCL at initial staging than FISH or polymerase chain reaction (PCR) studies. Of the patients, 72 were tested by FCI and FISH, with 59 (82%) positive by FCI and 49 (68%) by FISH. In 48 cases, there were concordant results, whereas 18 cases were positive by FCI and negative by FISH and 6 cases were positive by FISH and negative by FCI. Of 83 patients tested by FCI and PCR, 67 (81%) were positive by FCI and 18 (22%) were positive by PCR. In 30 cases, results were concordant; the majority of cases (n = 51) were positive by FCI and negative by PCR, and 2 were positive by PCR but negative by FCI.

BM Restaging Specimens After Chemotherapy

The median survival time (calculated from the initial treatment date to death or most recent follow-up date) was 90 months. All patients were regularly followed up by routine restaging unilateral BM aspiration and bilateral BM biopsies. The median interval between initial diagnosis and restaging was 6 months. All cases with immunophenotypic evidence of lymphoma by FCI were positive for CD5 and CD19 and demonstrated immunoglobulin κ or λ light chain restriction (Images 1 and 2).

In 77 patients, there was no morphologic or FCI evidence of lymphoma in follow-up BM specimens, and this subgroup was not further analyzed. Of interest to this study was the subgroup of 27 patients in whom evidence of MCL was present in the restaging BM specimens. In 13 patients, M and FCI were positive for lymphoma. By contrast, in 14 patients, only FCI was positive for lymphoma. Most of these biopsy specimens appeared technically adequate, with a mean aggregate core length of 2.1 cm, and 7 had an aggregate length of 1.8 cm or greater. Four were judged suboptimal in quality for restaging purposes. There were no patients with BM specimens at restaging that were M+/FCI−. The percentage of monoclonal B cells in BM aspirates identified by FCI was higher in patients with M+ BM specimens (mean, 8.7%) compared with patients with M− BM specimens (mean, 1.2%). This finding was statistically significant (P < .01; Student t test). Of the 27 cases with evidence of relapse in the restaging BM, 26 had a positive marrow at initial staging. For the remaining patient, the initial staging marrow was FCI−/M− and the restaging marrow was FCI+/M−.

Clinical Follow-up

To determine the significance of BM involvement in restaging biopsy specimens that were M−/FCI+, the results were correlated with clinical follow-up, which was available for 12 (86%) of 14 patients. At the time of the M−/FCI+ restaging BM, 4 (33%) of 12 patients had concurrent morphologic evidence of MCL involving lymph nodes or other non-BM sites. An additional 5 (42%) of 12 patients subsequently developed morphologic evidence of MCL involving the BM. Lymphoma was first detected by morphology in biopsies performed a median of 3 months later (range, 2-24 months). Of 12 patients, 3 (25%) had no morphologic evidence of lymphoma involving the BM and also had no clinical or radiologic evidence of relapsed MCL during the follow-up interval. Of note, no specific clinical intervention was based on an M−/FCI+ restaging marrow. However, restaging studies were often performed after only a partial course of therapy, so that additional rounds of chemotherapy may have eradicated residual disease in the M−/FCI+ cases.

Role of Cyclin D1 Immunostain in Assessing Restaging BM Specimens

At our institution, cyclin D1 immunostaining was not routinely performed on restaging BM specimens when there was no morphologic evidence of lymphoma involvement. In the situation in which morphologic evaluation was negative but a small monoclonal B-cell population was detected by FCI, various strategies were adopted. Some pathologists cut deeper levels of the BM biopsy specimen and performed cyclin D1 immunostaining, often paired with a pan B-cell marker, usually Pax-5 (rather than CD20, as patients had been treated with rituximab). Other pathologists chose to report the BM as morphologically negative for lymphoma and stated in the pathology report that a small monoclonal B-cell population was detected by FCI.

For this study, BM specimens of 12 patients (in 2 patients BM blocks were not available) that were M−/FCI+ were reassessed by using immunohistochemical staining to assess for cyclin D1 and Pax-5. (If already performed, these immunostains were reviewed.) In 3 (25%) of 12 patients, aberrant cyclin D1+ lymphocytes were identified. In these cases, the
antibodies specific for cyclin D1 and Pax-5 both highlighted subtle involvement by lymphoma cells in loose small aggregates or present as scattered single cells. In 2 of these patients, morphologic evidence of MCL in the BM subsequently developed.

Discussion

Standardized staging and response assessment are critical in managing patients with lymphoma, evaluating therapeutic strategies, and in clinical trials. For these reasons, BM examination is routinely used to stage the disease in patients at initial diagnosis and to restage after therapy. In 1999, an international workshop to standardize response criteria for non-Hodgkin lymphomas was held, and standard guidelines were proposed. Bilateral BM biopsies were recommended to initially stage and assess therapeutic response in patients with non-Hodgkin lymphomas. As a part of these consensus guidelines, it was recommended that a minimum of 2.0 cm of BM be examined histologically, either as a single biopsy specimen or an aggregate of bilateral BM specimens. The authors also recommended that BM biopsy specimens should be scored as follows: positive, unequivocal cytologic or architectural evidence of lymphoma; negative, no aggregates or only a few well-circumscribed lymphoid aggregates; or indeterminate, an increased number or size of aggregates without cytologic or architectural atypia. For the scenario in which FCI detected a monoclonal B-cell population, usually small, but there was no morphologic evidence of lymphoma, it was recommended that BM staging be considered negative for clinical management. The rationale for this decision at that time was that there was no evidence supporting a different clinical outcome for this subgroup of patients. These guidelines were revised in 2007, but the role of FCI data was not changed substantially.

The group of 104 patients with MCL in this study was entered into a clinical protocol in which BM was examined every 6 months after therapy. Fourteen patients in this study, approximately 10%, had restaging BM specimens in which FCI was positive with negative morphologic findings. For 12 of these patients, clinical follow-up data were available. Therefore, this subgroup has value because the data can be used to comment on the consensus guidelines by Cheson and colleagues. Of 12 patients, 5 (42%) developed subsequent morphologic evidence of MCL in the BM. The median time to morphologic detection of MCL in BM was 3 months, but in some patients, it took up to 2 years for MCL to be detected morphologically. In addition, 4 additional patients (33%) at the time of BM restaging had concurrent evidence of MCL involving lymph nodes and other non-BM sites. Therefore, 9 of 12 patients with only FCI evidence of disease had clinical evidence of MCL progression. These data suggest that FCI evidence of MCL in restaging BM specimens should not be considered as negative for clinical management, contrary to the recommendation of the 1999 and 2007 consensus guidelines.

In the 2007 revised response criteria, a role for immunohistochemical studies was proposed, at time of initial staging and for restaging after therapy. With antibodies useful for detecting CD20 and CD3 expression or other pan B-cell or T-cell antibodies, morphologically negative BM biopsy
and perhaps our patients have a greater burden of disease. The patient selection as our institution is a major referral center frequency of BM involvement in this study may be related to others using anti–cyclin D1 antibodies in BM specimens of specific for cyclin D1. Similar results have been reported by FCI+. In this study, the sensitivity of detecting MCL in BM staining on the subgroup of BM specimens that were M–/FCI+ cases. Follow-up data on the subset of patients in this study who had M–/FCI+ restaging BM specimens showed that approximately 75% of the patients had concurrent evidence of MCL elsewhere (lymph nodes or other non-BM sites) or developed morphologic evidence of MCL in BM within 2 years. We believe these data show the clinical significance of positive FCI data in patients with MCL in the postchemotherapy setting. Our data also may have greater usefulness in the light of the consensus guidelines for assessing the response of non-Hodgkin lymphomas to therapy proposed by Cheson and colleagues.7 We suggest that FCI may be helpful in the workup of other types of non-Hodgkin lymphoma in BM and that a positive FCI result may justify clinical action.

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