Minimal residual disease detection in mantle cell lymphoma: methods and significance of four-color flow cytometry compared to consensus IGH-polymerase chain reaction at initial staging and for follow-up examinations

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ABSTRACT

Background

The increasing application of multi-color flow cytometry assays for staging and follow-up in mantle cell lymphoma necessitates that the specificity and sensitivity of this technique are evaluated. Data from prospective clinical trials comparing the clinical applicability of flow cytometry to routine diagnostic methods and to polymerase chain reaction are currently lacking.

Design and Methods

We applied a standardized four-color flow cytometry assay to 281 prospectively collected peripheral blood and bone marrow samples from 98 patients with mantle cell lymphoma participating in a multi-center clinical trial and compared the results to those obtained with conventional clinical staging and consensus primer IGH-polymerase chain reaction.

Results

The maximum sensitivity of flow cytometry using light chain restriction in $CD19^+CD5^+$ subpopulations was $8.0x10^{-4}$ while flow cytometry that relied on immunophenotypic aberrations was less sensitive ($2.4x10^{-3}$). Mantle cell lymphoma cells were detected in 87.3% of 110 pre-treatment samples from 84 patients by flow cytometry and in 94.5% by polymerase chain reaction. Eight out of 84 patients (9.5%) diagnosed clinically as having stage II or III disease showed peripheral blood or bone marrow involvement according to flow cytometry, thus documenting more advanced disease. At follow-up residual lymphoma cells were detected by flow cytometry and concordantly by polymerase chain reaction in 10/171 samples (5.8%); however, 31 follow-up samples (18.1%) were positive for minimal residual disease according only to polymerase chain reaction analysis.

Conclusions

The sensitivity of four-color flow cytometry is comparable to that of IGH-polymerase chain reaction at initial staging but is less sensitive at follow-up after immuno-chemotherapy. Both techniques are highly valuable methods for accurate initial staging.

Key words: mantle cell lymphoma, flow cytometry, PCR, staging, minimal residual disease.

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Introduction

Mantle cell lymphoma (MCL) is a mature B-cell neoplasm hallmarked by the t(11; 14)(q13;q32) translocation that juxtaposes the *CCND1* locus next to the immunoglobulin heavy chain (*IGH*) locus. The resulting cyclin D1 overexpression, the typical $CD19^{+}CD5^{+}CD23^{low}CD10^{-}CD20^{++}$ immunophenotype and an aggressive clinical course characterize this lymphoma.[1] With the advent of numerous new effective treatment options during the last decade,[2-5] the induction of complete clinical and even molecular remissions became feasible. Consequently, minimal residual disease (MRD) assessments by polymerase chain reaction (PCR) gained importance for predicting prognosis and monitoring the efficacy of treatment modalities.[3,5-11] The same PCR methods targeting either the characteristic t(11;14) or patient-specific clonal *IGH* rearrangements were also applied to obtain sensitive assessments of peripheral blood and bone marrow involvement at the time of initial staging of the disease.[8,9] The least labor-intensive, but not quantitative method for the detection of MRD is PCR using consensus *IGH*-primers (consensus *IGH*-PCR) or t(11;14) PCR followed by GeneScanning. The technique was reported to detect one monoclonal B cell in up to $1x10^{3}$ benign leukocytes.[12-15] In contrast, allele-specific real-time quantitative (RQ) PCR targeting the junctional region of the clonally rearranged *IGH* gene yields quantitative results and is sensitive enough to detect one malignant cell among 10^{5} normal cells.[6,16]

As a fast, cost-effective, broadly available and quantitative alternative to RQ-PCR, multi-color flow cytometric assays with sensitivities of about 10^{-4} have recently been applied to MRD monitoring in B-cell malignancies such as acute lymphoblastic leukemia[17-19] and B-lineage chronic lymphocytic leukemia.[13,20,21] Although flow cytometry was proposed for MRD monitoring in MCL as well,[22] and the method is used for staging in clinical practice, a comparative analysis with PCR is still lacking. In addition, it remained to be clarified whether light chain restriction[23,24] or immunophenotypic aberrations[22,24] are better suited for sensitive detection of MCL in the era of four-color flow cytometry.

Using peripheral blood and bone marrow samples from the current European MCL network trials we aimed to develop and evaluate standardized criteria for MCL detection by four-color flow cytometry. We, therefore, initially analyzed the lymphoma immunophenotype using three different four-color stainings and compared the applicability of light chain restriction to immunophenotypic aberrations to detect submicroscopic disease in MCL. The limit of detection of flow cytometry was calculated from measurements in control peripheral blood and bone marrow samples. We evaluated the specificity of the flow cytometric method by comparison with PCR and assessed its utility for detecting MRD in patients who received modern immunochemotherapy. Finally, a comparison with standardized conventional staging within this multicenter trial enabled us to assess the significance of flow cytometric detection of minimal disease during the initial work-up.

Design and Methods

Patients, treatment protocols, and normal controls

Between February 2, 2004 and November 17, 2005 four-color flow cytometry and PCR were applied in parallel to 281 samples (198 peripheral blood, 83 bone marrow) from 98 German patients who participated in the European MCL network trials (EU MCL trials). A detailed description of the treatment protocols is given in the Online Supplement. MCL was diagnosed by local pathologists according to the current World Health Organization classification. The histological diagnosis was additionally confirmed by members of the European MCL Pathology Panel in 91.8% of cases (reference pathology). *Online Supplementary Table S1* summarizes the baseline information of the patients from whom samples were used in this study. The characteristics of these patients, including the percentage with bone marrow infiltration, are comparable to those of previously reported MCL cohorts.[4,10,26-29] Peripheral blood samples from 25 healthy controls were collected after informed consent. Control bone marrow samples comprised left-over material from eight aspirates without malignant involvement collected for routine initial staging in patients with suspected hematologic malignancies.

Flow cytometry

The staining protocols for flow cytometry have been previously published by our group in detail.[21] Briefly, washed samples were adjusted to 10000 leukocytes/μL and incubated with rabbit serum (Sigma). Subsequently, samples were stained using a mixture of the pretitred directly conjugated monoclonal antibody combinations (fluorescein isothiocyanate[FITC]/phycoerythrin[PE]/peridin chlorophyll protein [PerCP] cyanine5.5/allophycocyanin[APC], κ/λ/CD5/CD19 (281 samples), CD23/CD22/CD5/CD19 (262 samples), and CD79b/CD22/CD5/CD19 (262 samples) for 15 min followed by incubation with BD FACS Lysing Solution and two washing steps. CD19 (clone SJ25C1), CD5 (L17F12), λ (TB 28-2), and κ (1-155-2) were obtained from Becton Dickinson (BD), Heidelberg, Germany. CD22 (RFB4) and CD79b (SN8) were from Caltag, Hamburg, Germany and CD23 (MHM6) from Dako, Hamburg, Germany. Samples were acquired using a BD FACSCalibur flow cytometer with CellQuest Pro v4.0.2 software. In addition to daily calibrations using Calibrite beads (BD) and FACSComp v4.2 software (BD), isotype controls (incubated with four isotype-matched irrelevant monoclonal antibodies labeled with FITC, PE, PerCP-Cy5.5, and APC, all from BD) were used to optimize light scatter, threshold, and amplifications.

Compensations were adjusted using four compensation controls (stained with single FITC, PE, PerCP-Cy5.5, and APC conjugated monoclonal antibodies) in each individual sample. We acquired data from a median of 123,244 leukocytes/tube (range, 5867 to 870,713).

Data were analyzed using CellQuest Pro v4.0.2 software. Figure 1 illustrates the standardized gating strategy. Light scatter characteristics of the B-cell population as a whole were used to define a lymphocyte gate as described.[13] The geometric mean fluorescence intensities within the lymphocyte gate of the isotype control tube ($MFI_{Isotype}$) were used to standardize mean fluorescence intensities of lymphocyte subpopulations (mean fluorescence intensity ratio [MFIR]=geometric mean of lymphocyte subpopulation/$MFI_{Isotype}$), as described elsewhere.[30,31] The upper limits of lymphocyte fluorescence intensity distributions for underexpressed antigens were standardized accordingly (e.g. 95% fluorescence intensity ratio [FIR95] = 95th percentile of fluorescence distribution/$MFI_{Isotype}$). To obtain a reproducible definition of $CD23^{low}$, $CD22^{low}$, and $CD79b^{low}$ in MCL we had calculated the following mean FIR95 values from a cohort of MCL samples: 30 for CD79b-FITC, 4.5 for CD23-FITC and 174 for CD22-PE. $CD19^{+}$ and $CD5^{+}$ were defined to encompass all lymphocytes brighter than 99% of the cells from the isotype control. $CD19^{+}CD5^{+}$ lymphocytes were classified as MCL as follows: in cases with a single $CD19^{+}CD5^{+}$ population, the population as a whole had to satisfy the MCL criteria, whereas in cases with distinguishable $CD19^{low}CD5^{++}$ and $CD19^{++}CD5^{low}$ subpopulations both were assessed separately for fulfilment of MCL criteria.

Table 1. Specificity of immunophenotypic approaches for detection of MCL. Twenty-five control peripheral blood samples and eight control bone marrow samples were assessed. The table lists the number of events that in addition to $CD19^{+}CD5^{+}$ satisfied the $CD22^{low}$,$CD23^{low}$ and $CD79b^{low+}$ gates as well as the $CD22^{low+}CD79b^{low+}$ and $CD22^{low+}CD23^{low}$ combined gates.

	$CD22^{low}$	$CD23^{low}$	$CD79b^{low}$	$CD22^{low+}$ $CD79b^{low}$	$CD22^{low+}$ $CD23^{low}$
	% false positive events				
Of total leukocytes					
Peripheral blood					
Mean	0.12	0.31	0.39	0.06	0.10
Mean±2*SD	0.30	0.84	1.04	0.16	0.24
Bone marrow					
Mean	0.05	0.12	0.09	0.02	0.05
Mean±2*SD	0.13	0.32	0.27	0.04	0.11
Of $CD19^{+}CD5^{+}$ lymphocytes					
Peripheral blood					
Mean	24.0	54.7	66.4	12.2	18.2
Mean±2*SD	39.4	89.0	87.3	22.4	33.2
Bone marrow					
Mean	37.6	71.7	58.8	20.4	32.8
Mean±2*SD	82.6	124.9	100.3	50.8	73.5

The results are expressed as percentages of total leukocytes and as percentages of the $CD19^{+}CD5^{+}$ lymphocytes.

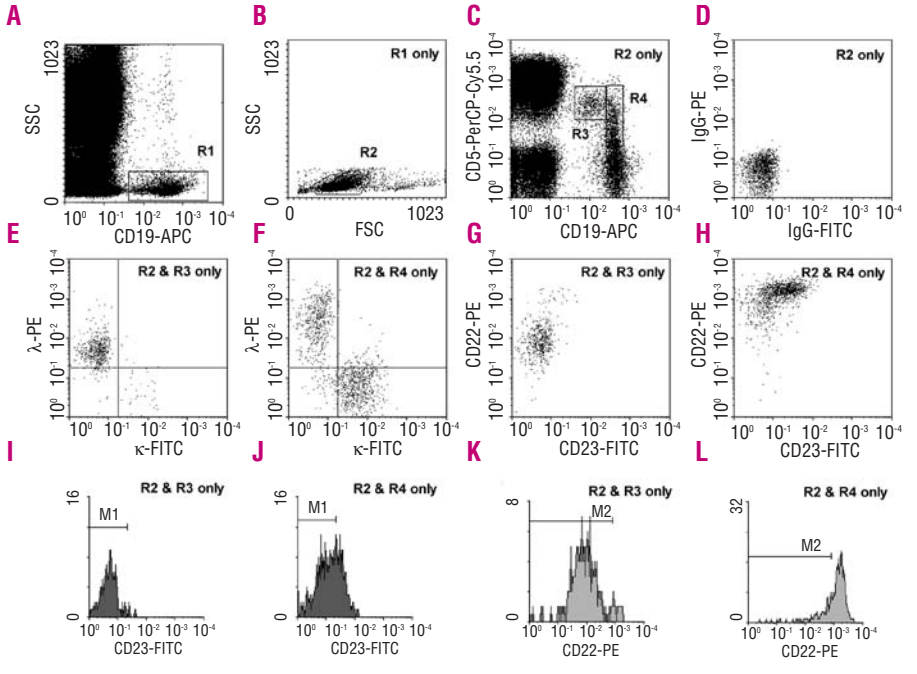

Figure 1. Typical example for gating to assess MCL involvement. (A) Of all leukocytes defined by light scatter, B cells are grossly identified by R1. (B) Lymphocytes are designated by R2, thus excluding debris (to the left) and doublets (to the right). (C) Two distinct B-cell subpopulations are assessed separately ($CD19^{++}CD5^{low}$ [R4] and $CD19^{low}CD5^{+}$ [R3]). (D) Isotype control staining: $MFI_{Isotype}$-FITC=5.2; $MFI_{Isotype}$-PE=4.9. (E-F) The quadrant marker for light chain restriction was set to include more than 99% of all T cells ($CD19^{-}CD5^{+}$ population in C) in the lower left quadrant. (E) Light chain restriction of the MCL cells (λ/κ=32/445=0.07). (F) Lack of light chain restriction in the $CD19^{+}CD5^{+}$ B cells (κ/λ=648/448=1.4). (G) CD23 and CD22 expression of the MCL population. (H) CD23 and CD22 expression of the $CD19^{low}CD5^{+}$ population.

(I-L) Standardized gating for MCL immunophenotypes: right boundaries (FIR95) are calculated from FITC and PE $MFI_{Isotype}$ using empirically determined constants. Right boundary M1: $FIR95_{CD23\text{-}FITC}$=4.5 x $MFI_{Isotype\text{-}FITC}$ (4.5x5.2 = 23.4), Right boundary M2: $FIR95_{CD22\text{-}PE}$ = 174 x $MFI_{Isotype\text{-}PE}$ (174x4.9 = 853). Percentages of populations included: (I) 99.5 (K) 65.7 (L) 98.5% (M) 22.7%. The combined regions of M1 and M2 were satisfied by 21.7% of all R4/R2 events and by 98.1% of all R3/R2 events. MCL level in this sample: $1.0x10^{-3}$ (R3 events/total leukocytes). *IGH*-PCR showed a monoclonal signal and a polyclonal background.

In accordance with the literature on MRD in acute leukemias[32-35] and our experience in chronic lymphocytic leukemia[21] we defined that at least 20 positive events forming a population were required as evidence for MRD. Samples were regarded as MRD-positive by light chain restriction if the κ/λ ratio of the putative subpopulation fell below 0.38 or exceeded 6, requiring a 4-fold excess of either light chain for a positive result. The definition was derived from the published normal κ/λ ratio of 1.5,[36-38] published ranges in normal B-cell subpopulations,[37,38] and the variability in the use of that criterion in clinical flow cytometry.[39,23,40,24] A $CD5^+$ B-cell subpopulation was defined as MCL MRD-positive by immunophenotypic aberration if the percentage of this population in relation to total leukocytes and to total $CD19^+CD5^+$ lymphocytes exceeded the mean percentage in controls by more than two standard deviations (SD), as detailed in the results section and in Table 1. A sample was designated MRD-positive if at least either the light chain restriction or the immunophenotypic criterion was positive. MRD levels were obtained by dividing the numbers of MCL cells by the numbers of leukocytes. The MRD level was multiplied by the number of leukocytes/μL peripheral blood or bone marrow (assessed using a Sysmex KX 21 cell counter, Norderstedt, Germany) to give the number of MCL cells/μL.

Consensus IGH-PCR and GeneScanning

Consensus *IGH*-PCR as well as interphase-FISH and PCR methods for the detection of the t(11;14)(q13;q32) translocation have been published before[12,15,41-43] and are detailed in the Online Supplement.

Statistical analysis

The two-tailed non-parametric Mann-Whitney and Fisher's exact tests were used to compare quantitative and qualitative parameters, respectively. The non-parametric correlation coefficient r (Spearman's correlation coefficient) was calculated to compare the levels of MCL in peripheral blood and bone marrow. Deviation from a Gaussian distribution was assessed by the Kolmogorov-Smirnov test. The level of statistical significance was set at 0.05. Calculations were done using GraphPad Prism software (release 4.00; San Diego, CA, USA).

Results

Immunophenotype of MCL cells

The MFIR characterizes the average expression of a given antigen on a cell population. $CD5^+$ B cells are generally regarded as the benign counterpart of MCL, so we compared the MFIR of the lymphoma cells to that subpopulation of normal lymphocytes. For unequivocal evidence of MCL involvement, the analysis of immunophenotypes was restricted to 85 light chain restriction positive samples from 62 patients simultaneously stained for κ/λ/CD5/CD19, CD23/CD22/CD5/CD19, and CD79b/CD22/CD5/CD19. Five samples from four patients (6%) exhibited CD5 on a minority of MCL cells only. Since normal $CD19^+CD5^+$ cells served as the reference population, those samples were excluded from further analysis of immunophenotype. Of note, all four patients showed an *IGH-CCND1* fusion indicating t(11;14) by FISH. *Online Supplementary Figure S1* depicts the MFIR of CD19, CD5, CD23, CD79b, and CD22 on MCL cells in the remaining 80 samples in comparison to that of benign $CD19^+CD5^+$ lymphocytes. MCL cells significantly underexpressed CD19, CD23, CD79b, and CD22 but showed an increased expression of CD5. The MFIR of CD19, CD5, CD22, and CD23 on MCL populations had a Gaussian distribution. CD79b was unique in being underexpressed in the majority of cases but clearly overexpressed in a sizable proportion of patients (bimodal expression). The MFIR of all studied antigens varied considerably between MCL patients, thus leading to overlapping levels of expression with those of normal controls. The greatest difference of median MFIR between MCL cases and normal cells was observed for CD22 (MCL *vs.* peripheral blood: 61 *vs.* 252, $p< 0.0001$), followed by CD79b (6.4 *vs.* 16.3, $p<0.0001$), CD19 (64 *vs.* 142, $p<0.0001$), CD5 (75 *vs.* 35, $p<0.0001$), and CD23 (1.3 *vs.* 2.5, $p<0.0001$). Compared to their putative normal counterpart, we demonstrated immunophenotypic aberrations of MCL cells in most patients for all investigated antigens, but also found surprisingly large immunophenotypic variations between cases.

Specificity and applicability of immunophenotypic aberrations for MCL detection

Having studied the immunophenotypic aberrations of MCL we investigated their usefulness for detecting minimal disease. To assess the specificity of marker combinations, standardized gating strategies were applied to benign $CD19^+CD5^+$ lymphocytes (Table 1). The criterion $CD22^{low}$ more effectively excluded background from analysis than the criteria $CD23^{low}$ and $CD79b^{low}$. The four-color-combinations $CD19^+CD5^+CD22^{low}CD79b^{low}$ and $CD19^+CD5^+CD22^{low}CD23^{low}$ further improved the specificities. Our measurements demonstrate that MCL cells can be specifically detected by a $CD19^+CD5^+CD22^{low}CD23^{low}$

Table 2. Comparison of flow cytometry to bone marrow histology and to consensus *IGH*-PCR for the detection of mantle cell lymphoma.

	FC-positive	*FC-negative*
Initial diagnosis (n = 110)		
Bone marrow histology-positive	84 (76.4%)	4 (3.6 %)
Bone marrow histology-negative	12 (10.9 %)	10 (9.1 %)
PCR-positive	95 (86.4 %)	9 (8.2 %)
PCR-negative	1 (0.9 %)	5 (4.5 %)
Follow-up (n = 171)		
PCR-positive	10 (5.8 %)	31 (18.1 %)
PCR-negative	0 (0.0 %)	130 (76.0 %)

Fc, flow cytometry.

immunophenotype if they account for more than 0.24% ($2.4x10^{-3}$) of all leukocytes and for more than 73.5% of all $CD19^+CD5^+$ lymphocytes. With a background of 0.16% of all leukocytes the specificity of the four-color-immunophenotype $CD19^+CD5^+CD22^{low}CD79b^{low}$is even higher.

The clinical utility of standardized gating strategies not only depends on their specificity but also on their broad applicability. As we had found a great inter-individual variability of immunophenotypes in MCL, we next assessed the percentage of $CD19^+CD5^+$ MCL cells in individual samples that displayed a $CD22^{low}$, $CD23^{low}$, and $CD79b^{low}$ immunophenotype (*Online Supplementary Table S2*). The majority of MCL cells satisfied the standardized gates that described underexpression; however, there were samples that were not included by those markers. This was particularly evident for $CD79b^{low}$ and the combination $CD22^{low}CD79b^{low}$, while the criterion $CD23^{low}$ was met in almost all samples. Assuming that the detection of at least 75% of all MCL cells in a sample is clinically adequate, the marker combination $CD19^+CD5^+CD22^{low}CD79b^{low}$ was applicable to only 52/80 (65%) of all $CD5^+$ MCL samples, whereas the marker combination $CD19^+CD5^+CD22^{low}$ $CD23^{low}$ was applicable to 68/80 samples (85%) ($p<0.01$). In summary, due to its broader applicability, the $CD19^+CD5^+CD22^{low}$ $CD23^{low}$ immunophenotype is more suitable than $CD19^+CD5^+CD22^{low}$ $CD79b^{low}$ for MRD monitoring in MCL by immunophenotypic aberration.

Flow cytometry, bone marrow histology, and consensus IGH-PCR at initial diagnosis

To assess the clinical significance of four-color flow cytometry for initial staging, we compared the method to conventional bone marrow histology and to consensus *IGH*-PCR in 110 samples (80 peripheral blood, 30 bone marrow) from 84 patients (Table 2). Eighty-eight of 110 pre-treatment samples (80%) were collected from patients with histological bone marrow involvement. A total of 96/110 specimens (87.3%) showed MCL cells in flow cytometric analysis (86.7% of bone marrow and 87.5% of peripheral blood samples). Within the group of 96 samples positive for MCL by flow cytometry, the diagnosis relied exclusively on light chain restriction in 24 samples, exclusively on immunophenotypic aberrations in 5 samples and on both criteria in 67 samples (15 samples tested for light chain restriction only). One hundred and four of 110 samples (94.5%) showed monoclonal rearrangements on *IGH* GeneScanning analysis. In 12/110 samples (10.9%; 11 peripheral blood, 1 bone marrow) from 12 patients flow cytometry identified MCL cells while bone marrow histology results were negative. The median infiltration level was $1.7x10^{-2}$ in those patients (range, $1.0x10^{-1}$ to $1.0x10^{-3}$). Four of the cases were diagnosed as stage II, four as stage III, and four as stage IV disease by conventional methods. Thus, an additional examination by flow cytometry would have led to clinical upstaging in 8/84 patients (9.5%). The level of infiltration as measured by four-color flow cytometry was clearly related to the results of conventional bone marrow histolgy (Figure 2). The median MCL infiltration in the 84 samples positive by flow cytometry from patients with histological bone marrow involvement was $9.3x10^{-2}$ (range, $8.0x10^{-4}$ – $8.3x10^{-1}$), but significantly lower in the 12 samples from patients with negative histology (median: $1.7x10^{-2}$, $p<0.001$). If only peripheral blood samples were considered, the same relation was observed: a median level of 9.4 $x10^{-2}$ in patients with histological bone marrow involvement compared to a median level of $1.6x10^{-2}$ in patients without bone marrow involvement ($p<0.005$). The results show that four-color flow cytometry improves staging accuracy, particularly in patients with a low level peripheral blood or bone marrow involvement.

Flow cytometry was negative in two patients with positive bone marrow histology. For one of these two patients only peripheral blood was available while in the other patient both bone marrow and peripheral blood were analyzed by flow cytometry. Both cases showed small B-cell populations with typical $CD19^+CD5^+CD22^{low}CD23^{low}$ MCL immunophenotype, but, due to low immunoglobulin-expression, no unequivocal light chain restriction. They were judged as negative since the levels of involvement ($1.2x10^{-4}$, 3.9 $x10^{-4}$, $6.9x10^{-4}$) were below the threshold of sensitivity of the immunophenotypic assay. All three samples were positive by consensus *IGH*-PCR. There was one peripheral blood sample from a patient with positive bone marrow histology that was regarded as negative by flow cytometry while the corresponding bone marrow sample showed flow cytometric evidence of MCL.

We next compared flow cytometric results to *IGH*-PCR in order to assess the specificity and detection rate of flow cytometry. Ninety-five samples (69 peripheral blood, 26 bone marrow) from 74 patients were positive for MCL according to both flow cytometry and *IGH*-PCR. The median levels of involvement were $7.5x10^{-2}$ (range, $7.9x10^{-4}$–$8.3x10^{-1}$) in those samples. One sample was polyclonal in FR1, FR2, and FR3 PCR, but showed evidence of MCL by flow cytometry (MCL involvement $4.5x10^{-2}$). The sample was, however, positive for the t(11;14) translocation by

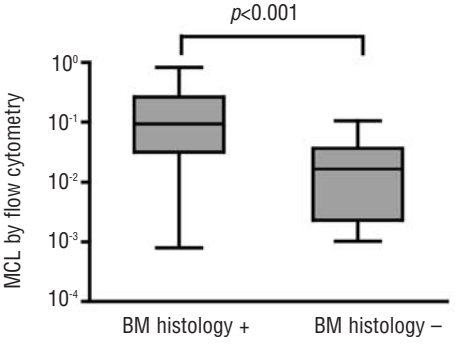

Figure 2. Four-color flow cytometry at initial diagnosis. The median involvement of peripheral blood and bone marrow was significantly lower in patients with negative bone marrow histology ($1.7x10^{-2}$) than in patients with positive bone marrow histology ($9.3x10^{-2}$; $p<0.001$). The figure shows the median involvement, interquartile range (boxes) and range (whiskers).

PCR. Five samples from four patients were concordantly negative by PCR and flow cytometry, whereas in nine samples from eight patients monoclonal PCR results were obtained without evidence of MCL cells by four-color flow cytometry.

In summary, our data show that at initial staging, MCL cells are most sensitively detected by PCR, followed by flow cytometry. Flow cytometry is more sensitive than conventional bone marrow histology in cases with low-level involvement. Our flow cytometric assay is 100% specific, as confirmed by PCR.

Flow cytometry and consensus IGH-PCR for minimal residual disease assessment during follow–up examinations

One hundred and seventy-one follow-up samples (53 bone marrow, 118 peripheral blood) from 67 patients were simultaneously assessed for residual MCL cells by flow cytometry and PCR (Table 2). These included 134 samples taken during or immediately after induction therapy, 23 samples from patients during observation after therapy (maximum follow-up period 8 months), and 14 samples from patients on maintenance therapy. The majority of all samples during or after therapy were negative by flow cytometry and by consensus *IGH*-PCR (76.0%). Ten samples from ten patients were positive by flow cytometry (8 samples positive by light chain restriction and immunophenotype, 1 sample each positive only by light chain restriction or by immunophenotype) and PCR. In these samples a median MRD level of 1 $x10^{-2}$ was detected (range, 2.6×10^{-3}–6.2×10^{-1}), corresponding to a median MCL cell content of 41 cells/μL (range, 14 - 8,200 cells/μL). A sizable subgroup of all follow-up samples (31/171; 18.1%) comprised specimens in which a monoclonal signal was evident by PCR but the criteria for flow cytometric MRD positivity were not met. This subgroup of samples included 9 bone marrow and 22 peripheral blood samples from a total of 22 patients. In these 31 PCR-positive, flow cytometry-negative samples we acquired data from a median of 88,563 leukocytes / tube. In 16/31 samples (51.6%) a B-cell population was discernible, while no B cells were detectable in the other 15 samples. A median B-cell content of only 6.5×10^{-4} (range, 1.3×10^{-4} to 1.4×10^{-1}) was calculated even in samples with a detectable B-cell population.

Our data show that rituximab-containing immunochemotherapy led to a profound depletion of benign and malignant B cells in the majority of the MCL patients. In this situation consensus *IGH*-PCR was clearly more sensitive than four-color flow cytometry.

Comparison of paired peripheral blood and bone marrow samples

Seventy-four bone marrow samples (26 taken at initial diagnosis, 48 taken during follow-up) with the corresponding peripheral blood samples from 45 patients were available to assess the influence of the source of material on MRD results. Twenty-two paired samples of peripheral blood and bone marrow were concordantly positive, 5 bone marrow samples were positive with corresponding negative peripheral blood samples, while in 47 paired samples neither bone marrow nor peripheral blood showed evidence of MCL. Comparable results were obtained when only initial diagnostic samples were considered: 3/26 (11.5%) peripheral blood and bone marrow samples were concordantly negative, 21/26 (80.8 %) were concordantly positive while in 2/26 (7.7%) cases, the peripheral blood sample was negative but the corresponding bone marrow sample positive. There were no cases of leukemic MCL without bone marrow involvement. In the bone marrow samples with corresponding positive peripheral blood samples we found a positive quantitative correlation of disease levels ($r=0.77$; $p<0.0001$). Bone marrow samples for which the corresponding peripheral blood samples did not show flow cytometric evidence of MCL had low-level involvement ranging from $7.9x10^{-4}$ to $9.8x10^{-2}$.

In summary, the data suggest that most of the samples with bone marrow involvement show leukemic MCL cells when assessed by sensitive four-color flow cytometry. There are cases with low-level involvement of the bone marrow without detectable disease in the peripheral blood. Nevertheless, it appears that for the majority of patients (i.e. those with a positive result in peripheral blood) a four-color flow cytometry assessment of peripheral blood is a sufficient first test for initial staging.

Discussion

Using an analysis in 98 consecutive patients from the European MCL network trials, this study established methods and significance of four-color flow cytometry in MCL. Previously, bone marrow and peripheral blood involvement had been studied in small series only[22-24,39,27] applying two-[40] or three-color flow cytometry[22-24,39] Our study was the first to evaluate a flow cytometric assay in comparison to PCR, thus allowing us to assess the sensitivity and specificity of flow cytometry by an independent and sensitive reference method. Furthermore, the contribution of four-color flow cytometry and consensus *IGH*-PCR to standardized conventional staging within a large prospective trial was investigated for the first time.

We demonstrate that four-color flow cytometry and consensus *IGH*-PCR are more sensitive than bone marrow histology for initial staging in MCL. Disease levels measured by flow cytometry were significantly lower in patients without histological evidence of bone marrow involvement compared to in patients with a positive histology (Figure 2). This observation suggests that flow cytometry is more sensitive than bone marrow histology. However, it should be taken into consideration that, according to the study protocol, bone marrow histology for staging could be performed by local or reference pathologists. The bone marrow histology results used in this study represent the standard of care in Germany, but it cannot be excluded that mandatory applica-

tion of standardized immunohistochemical staining and central review of all bone marrow samples would have led to higher detection rates. The routine application of flow cytometry in initial staging would lead to an approximately 10% increase in stage IV patients among all MCL patients. An additional 6% of patients would have been diagnosed with bone marrow or peripheral blood involvement if *IGH*-PCR monoclonality had formed the basis of assessments. Since clinical stage has prognostic significance,[29,26,28] prospective studies need to re-evaluate this parameter after the inclusion of more sensitive methods such as flow cytometry or *IGH*-PCR. It might be advantageous for further risk stratification that flow cytometry (but not consensus PCR) yields quantitative results. The use of flow cytometry and PCR in addition to bone marrow histology may be recommended in patients who are considered candidates for radiation therapy to rule out low-level generalization of the disease.

Four-color flow cytometry was sensitive enough to detect leukemic MCL cells in peripheral blood in 87.3% of cases. This paper reports the highest proportion of leukemic MCL at initial diagnosis described so far, most likely reflecting the increased sensitivity of four-color flow cytometry assays compared to two-[27] and three-color[23,44] flow cytometric approaches and to morphologic assessments.[45,28,27] A leukemic course of the disease was always associated with detection of bone marrow involvement by flow cytometry. Thus a flow cytometric assessment of peripheral blood might replace bone marrow histology for staging in the majority of MCL patients whereas this method (plus an aspirate for flow cytometry and PCR) remains necessary in patients with a negative peripheral blood result.

While immunophenotypic MRD detection was found to be superior to light chain restriction analysis in chronic lymphocytic leukemia,[13] this study was the first to compare both diagnostic approaches in MCL. Published studies described the MCL immunophenotype as $CD19^{low}$,[46,47], usually $CD5^{++}$,[46,48], $CD23^{low}$,[22,46,47], $CD22^{low}$,[22,46,47], $CD79b^{high}$,[47] and LCR^{+}. Sanchez *et al*. proposed $CD19^{+}$ $CD22^{low}CD23^{low}$ as an immunophenotype suitable for MRD detection in MCL and predicted an achievable sensitivity of $1x10^{-5}$ from dilutional experiments.[22] Our study therefore compared the assessment of immunophenotypic aberrations (CD19/CD5/CD22/CD23, CD19/CD5/CD22/ CD79b) to light chain restriction assessments (CD19/ CD5/λ/κ) in B-cell subpopulations. We confirmed that $CD19^{low+}CD5^{++}$ $CD23^{low}$ $CD22^{low}$ is the most prevalent MCL immunophenotype as well as the existence of rare, but well-characterized $CD5^{-}$ cases of MCL.[48] In contrast to observations of D'Arena *et al.*[47] only a small proportion of our MCL cases overexpressed CD79b, whereas most cases underexpressed this antigen. Of note, we demonstrated that the mean expression levels of CD22, CD19, CD79b, and CD23 on MCL cells from some patients overlapped with those of normal controls. Since the detected immunophenotypic aberrations were subtle, the combination of multiple markers and a well-standardized definition for antigen underexpression became mandatory. Applying a standardized gating approach to normal controls we calculated a maximum sensitivity of $2.4x10^{-3}$ for $CD19^{+}CD5^{+}CD22^{low}CD23^{low}$ to detect MCL cells. Furthermore, we demonstrated that the $CD19^{+}CD5^{+}CD22^{low}CD23^{low}$ immunophenotype was applicable to only 79% of all MCL cases. However, the approach was specific, since all MCL cases diagnosed as positive by the presence of that immunophenotype were at the same time monoclonal by PCR. The proposed gating strategy crucially depends on the definition of low level expression of the antigens. Lower constants to describe underexpression would have increased the specificity and detection limit of our approach, but at the same time further reduced its applicability. Since our current gating strategy is not applicable to all MCL patients, it can be used to rule out bone marrow and peripheral blood involvement with a specified detection limit only in follow-up samples with known presenting immunophenotype. Consequently, the immunophenotypic detection of MCL cells can currently only supplement light chain restriction-based approaches in cases without detectable light chain expression.

Light chain restriction, when combined with gating on a $CD19^{low}CD5^{++}$ lymphocyte subpopulation, represents a sensitive method for the detection of MCL. Since light chain restriction is a unique feature of lymphoma cells, the sensitivity limits of light chain restriction assessments are constrained only by the number of leukocytes acquired, the ability to distinguish lymphoma from normal B cells by $CD19^{low}CD5^{++}$ and the number of cells required for a positive result. With a maximum sensitivity of $8.0x10^{-4}$ as demonstrated in this paper the method is slightly more sensitive in MCL than previously reported in CLL,[13] an observation probably related to the unique $CD19^{low}CD5^{++}$ immunophenotype in MCL and the higher levels of expression of surface immunoglobulin in many MCL cases.[46] Due to its broader applicability and superior sensitivity MRD assessment by light chain restriction remains advantageous compared to that based on immunophenotyping. In this study we proved the specificity of low-level MCL detection by flow cytometry using simultaneous PCR assessments. However, flow cytometric MCL detection in follow-up samples after rituximab-containing induction therapy is clearly less sensitive than *IGH*-ASO-primer RQ-PCR, which routinely achieves a sensitivity between $1x10^{-4}$ and $1x10^{-5}$.[6]

The detection limit of consensus IGH-PCR as used in this study depends on the fragment size of the individual amplified PCR product and on the number of polyclonal B cells in the sample. Moreover, mutations within the primer binding sites or inhibitors can interfere with the method. It is, therefore, conceivable that the single flow cytometry-positive, PCR-negative sample observed in this study by chance was the consequence of unfavorable conditions for PCR. We had previously shown that polyclonal PCR results could be observed in chronic lymphocytic leukemia simultaneously with MRD-levels ranging from $2.1x10^{-4}$ to $5.1x10^{-3}$, while monoclonal results occurred with minimal MRD levels of $2.2x10^{-4}$.[21] These data are in keeping with sensitivities calculated herein for flow cytometry and con-

sensus *IGH*-PCR in MCL. The high proportion of follow-up cases (18.1%) with flow cytometry-negative, PCR-positive results was particularly striking. Flow cytometric analysis in these cases demonstrated a profound depletion of benign B cells due to the application of the anti-CD20 monoclonal antibody rituximab, concomitant with the reduction of MCL cells. This resulted in an easier identification of the MCL-related monoclonal *IGH*-genes by PCR while the total B-cell numbers in the samples rarely exceeded the detection limit of our flow cytometry assay. The high detection rate of PCR in this situation might be explained by the efficient reduction of polyclonal background cells by rituximab. In brief, our results show that four-color flow cytometry is less sensitive than consensus IGH-PCR for detecting MRD and cannot, therefore, at present replace the quantitative and even more sensitive RQ-PCR assays for follow-up evaluation.

In summary, flow cytometry is a sensitive method for initial staging in MCL patients, which might obviate the need for bone marrow biopsies in a substantial proportion of patients, and improves staging accuracy. Currently, its clinical value for follow-up examinations during and shortly after treatment with rituximab-containing regimens is limited. The introduction of six- to eight-color flow cytometry as well as the inclusion of novel markers is expected to improve the sensitivity of the method in the near future.

Authorship and Disclosures

SB developed and performed the FACS assays and drafted the manuscript.; CP optimized and performed the PCR analysis and was responsible for data collection and interpretation; MR and SBu were responsible for sample collection for flow cytometry and participated in the development of the FACS assays. WK was responsible for the pathologic review. SG and RS were responsible for cytogenetic and molecular cytogenetic data collection and interpretation; EH and MU are the statisticians of the European MCL network and together with MD and WH provided clinical data from study patients and assisted in data interpretation; MD and WH are the German co-ordinators of the European MCL network and responsible for its clinical trial design, conduct and data interpretation; MK was responsible for the overall conduct of the MRD study and participated in manuscript preparation and data interpretation.

The authors reported no potential conflicts of interest.

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