

Review Article

Measurable residual disease in the treatment of chronic lymphocytic leukemia

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Treatment outcomes of chronic lymphocytic leukemia (CLL) have improved since chemoimmunotherapy and novel drugs became available for CLL treatment; therefore, more sensitive methods to evaluate residual CLL cells in patients are required. Measurable residual disease (MRD) has been assessed in several clinical trials on CLL using flow cytometry, real-time quantitative PCR (RQ-PCR) with allele-specific oligonucleotide (ASO) primers, and high-throughput sequencing. MRD assessment is useful to predict the treatment outcomes in the context of chemotherapy and treatment with novel drugs such as venetoclax. In this review, we discuss major techniques for MRD assessment, data from relevant clinical trials, and the future of MRD assessment in CLL treatment.

Keywords: measurable residual disease, chronic lymphocytic leukemia, multicolor flow cytometry, ibrutinib, venetoclax

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in Western countries and predominantly develops in the elderly.¹⁻³ In CLL, CD5⁺ small and mature B lymphocytes accumulate in the peripheral blood (PB), bone marrow (BM), spleen, and lymph nodes.^{2,4}

In CLL treatment, effective drugs and their combinations have recently become available, which has considerably improved the progression-free survival (PFS) and/or overall survival (OS) of most CLL patients.⁵ Therefore, CLL is generally known as an indolent disease, but the actual clinical outcomes are highly variable. It is difficult for CLL patients to be completely cured by chemotherapy; consequently, the major treatment goal has been to prolong their lives by controlling their conditions. It is essential to understand the patients' conditions because previous studies revealed that many prognostic factors play a role in the variable clinical outcomes of CLL.⁶

Measurable residual disease (MRD) is defined as a small number of malignant cells that is undetectable but clearly remains in patients after achieving complete response (CR). The accurate quantification of MRD during and after treatment is essential to predict clinical outcomes of CLL treatments. A negative MRD assessment with reliable criteria results in a significantly better PFS and OS than a positive MRD assessment.⁷ Several clinical trials demonstrated that

monitoring the MRD level is an independent predictor of CLL outcomes.⁸

In this review, we discuss currently available MRD detection methods, data on monitoring MRD from relevant clinical trials, and the future clinical role of MRD assessment in CLL patients outside of clinical trials.

DETECTION METHODS FOR MRD

MRD detection methods must be easy to perform and simple to interpret for daily use in clinical practice. CLL is a heterogeneous disease that is pathologically more complicated than any other leukemia. CLL cells spread throughout the body, requiring treatment even if the malignant cells cannot be found on morphological assessments. These undetected CLL cells present in patients cause relapse. Therefore, the current approaches in which only BM and/or PB are assessed to monitor MRD in CLL are not sufficient. It is clear, however, that improvements in the sensitivity in MRD detection methods provide highly reliable data to predict the prognosis of CLL.

The two major methods for MRD detection are flow cytometry (FCM)-based analyses⁸⁻¹⁰ and polymerase chain reaction (PCR)-based analyses.¹¹⁻¹⁴ A comparison between the FCM- and PCR-based approaches is summarized in Table 1.

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Table 1. The features of both FCM-based and PCR-based approaches for MRD assessment are compared

		FCM-based	PCR-based
Method	reported	multicolor FCM with different antigens (≥ 4 -color)	ASO-RQ-PCR, HTS
	advantage	easy, needs a shorter time	high sensitivity (10^{-5} ~ 10^{-6})
	disadvantage	relatively low sensitivity (10^{-4} ~ 10^{-5})	laborious, time-consuming
Requirement for materials		fresh, living cells, or possibly properly cryo-preserved mononuclear cells (<48 hours after collection)	do not need fresh samples
Application to clinical practice		easy	able to conduct with preserved materials
Cost		less expensive	difficult except for clinical trials
Course of action		Standardization with more sensitive combination of markers is required to improve the detection limit.	expensive
			Is application of ASO-RQ-PCR practical? (HTS is ideal.)

FCM-based analysis

In Western countries, MRD in CLL was conventionally monitored by three-color FCM, which measures the co-expression of CD5/CD19 on CLL cells and immunoglobulin (Ig) light chain κ/λ restriction.¹⁵ Three-color FCM has a sensitivity of only approximately 10^{-3} because it is difficult to distinguish CLL cells from normal CD5/CD19 cells.

Multicolor FCM, particularly four-color FCM, is the most common approach to detect MRD and has recently been used in Europe. The first consensus method was reported by the European Research Initiative on CLL (ERIC) group.⁸ In this report, Rawstron *et al.* developed standardized four-color FCM with a sensitivity below 10^{-4} ; this assay was optimal for most clinical laboratories. Three combinations of monoclonal antibodies (mAbs), CD5/CD19 with CD20/CD38, CD81/CD22, and CD79b/CD43, made the smallest difference in the test results among different clinical laboratories and yielded the lowest false-positive rate in an assessment of 50 combinations of mAbs. The mAb panel separated CLL cells from normal leukocytes with an accuracy of 95.7%. In addition, the FCM assay with the panel had high concordance (above 95%) with real-time quantitative allele-specific oligonucleotide (RQ-ASO) PCR performed on the same samples used in the four-color FCM assay. There was no difference in sensitivity between four-color FCM and RQ-ASO PCR. As the result, this standardized and optimized four-color FCM was performed in most clinical trials. However, the conventional FCM method demonstrating co-expression of CD5/CD19 with the restriction of Ig light chains can be used for the evaluation of most CLL cases, although it has low sensitivity. Moreover, it is difficult to acquire a sufficient number of cells to monitor the presence of MRD in poor samples with the standardized four-color FCM method.

To improve the sensitivity in MRD detection with FCM, Rawstron *et al.* reported that the six-color FCM panel introduced in the ERIC & European Society for Clinical Cell Analysis Harmonisation Project was a reproducible approach with a higher sensitivity.⁹ The six-color panel consists of the combinations of CD19/CD5/CD20 and CD3/CD38/CD79b or CD81/CD22/CD43; this assay reached a sensitivity of $\sim 10^{-4}$ to $\sim 10^{-5}$ in the detection of MRD.

In the latest guidelines of the International Workshop on Chronic Lymphocytic Leukemia (iwCLL), the definition of

undetectable MRD (uMRD) is stated precisely as the presence of less than 1 CLL cell in 10,000 leukocytes ($<10^{-4}$) in BM and/or PB,¹⁶ which means that at least 500,000 events must be acquired to achieve uMRD in terms of sensitivity. Improvements have enabled FCM instruments to assess combinations of more than six parameters in a single tube, making it possible to detect MRD below 10^{-4} .^{17,18} The ERIC group assembled a new eight-color, single-tube FCM assay with the same combination of antibodies (CD19/CD20/CD5/CD43/CD79b/CD81/CD22/CD3) that was used in their six-color FCM panel.⁹ This single-tube eight-color assay was able to reliably detect MRD down to 10^{-5} .¹⁹ Dowling *et al.* optimized and validated the eight-color ERIC single-tube assay.²⁰ The 8-color assay was demonstrated to detect CLL cells below a level of 10^{-4} and had good correlation with the six-color method that they utilized for MRD detection ($R^2 = 0.991$). Furthermore, the eight-color ERIC assay costs less than the six-color FCM. Raponi *et al.* also conducted an eight-color FCM assay using mAb combinations of CD19/CD20/CD5/CD45/CD38/CD43/CD81/CD3; the result was comparable to the 4-color ERIC FCM assay.¹⁴ Moreover, a single-tube 10-color FCM assay was able to detect MRD to a level of 10^{-5} .¹⁸

Thus, FCM methods for MRD monitoring in CLL have been rapidly upgraded and the sensitivity has increased in the last decade. Several studies have been conducted to standardize FCM methods for MRD evaluation, leading to reliable and reproducible results across different laboratories.

PCR-based analysis

Using conventional PCR-based detection of MRD with a primer specific for the complementary determining region 3 (CDR3) adjacent to *Ig heavy chain variable region (IGHV)*, the qualitative visualization of MRD is impossible because the PCR product of residual CLL cells detected is the same size as its background normal counterparts, thus the sensitivity was only 10^{-2} .¹⁵ Improvements in sensitivity of up to 10^{-5} were reported in MRD evaluations using RQ-PCR with patient-specific primers and probes. In this method, the *IGHV*-CDR3 region of a CLL patient is sequenced, and ASO probes specific for the patient are designed to amplify the *IGHV* gene of CLL cells during PCR. This method can quantitatively evaluate MRD.²¹ Raponi *et al.* compared an MRD analysis of RQ-ASO-PCR with that of eight-color

FCM in 243 cases.¹⁴ This study reported that RQ-ASO-PCR was consistent with eight-color FCM in 199 out of 243 cases (81.9%) and that 42 cases (22.7%) were FCM MRD-negative but PCR MRD-positive; these 42 patients had a poor PFS, similar to patients who were FCM MRD-positive. Although the RQ-ASO-PCR approach has a high sensitivity and specificity in MRD detection for CLL, it is a less popular method for assessing MRD because it is laborious, time-consuming, and costly. In addition, the most important limitation of RQ-ASO-PCR is that the standard curve must be generated by using each patient's specific primers derived from sequencing *IGHV* before starting treatments. However, compared with FCM, RQ-ASO-PCR has the advantages of requiring less materials and can be performed using cryo-preserved specimens.

A novel PCR-based approach, high-throughput sequencing (HTS), was applied to MRD monitoring.^{10,22,23} This method does not require patient-specific primers. The HTS assay is widely applicable not only to cells from PB or BM, but it can also be used with cell-free DNA such as plasma circulating tumor DNA (ctDNA).^{24,25} Moreover, it has a higher sensitivity ($\sim 10^{-5}$ to $\sim 10^{-6}$) than any other approach for MRD detection. Logan *et al.* utilized consensus primers for the V and J segments of *IGH* genes followed by HTS quantification of *IGH* sequences, which were specific for patients and obtained from blood samples before treatment.^{22,23} This method demonstrated a good correlation with RQ-ASO-PCR and achieved the detection limit of 10^{-6} .

Thompson *et al.* used HTS to assess MRD in all of the patients who achieved uMRD ($<10^{-4}$) in BM according to FCM at the end of fludarabine and cyclophosphamide (FC) plus rituximab (FCR) treatment.²⁶ In this study, BM, PB mononuclear cells (PBMcs), and plasma samples were used for the MRD assessment. In total, 27.4% of the patients reached uMRD according to HTS ($<10^{-6}$). The MRD rates in the BM, PBMcs, and plasma were 25%, 55%, and 75%, respectively. Patients who had uMRD according to HTS at the end of therapy had a better PFS than those who were positive for MRD for all sample types.

MRD DETECTION IN CLINICAL TRIALS

FCM-based detection

The German CLL study group (GCLLSG) gave a brief report on the significance of MRD assessment for prognosis.²⁷ This phase III randomized study, CLL8, confirmed the superiority of FCR compared with FC in 493 treatment-naïve (TN) patients with CLL, in which the MRD level was prospectively tested using the ERIC four-color FCM method. Based on the MRD assessment results, the levels of MRD were classified into three groups: low ($<10^{-4}$), intermediate ($\geq 10^{-4}$ up to $<10^{-2}$), and high ($\geq 10^{-2}$). The median PFS for the low, intermediate, and high MRD groups was 68.7, 40.5, and 15.4 months, respectively. The median PFS for the low MRD group was significantly better than that for the other groups ($p < 0.0001$). Both the univariate and multivariate

analyses of that study revealed that MRD levels are essential for the prediction of PFS and OS. A low MRD did not indicate that CLL cells were eliminated from patients and instead indicated that the MRD level was just below the detection limit, and a small number of CLL cells remained in the patients. This study emphasized MRD as an independent prognostic factor to predict the PFS and OS in CLL.

HTS in MRD assessment

Allogeneic stem cell transplantation (allo-HCT) has a curative potential in CLL and one study reported applying HTS to obtain MRD measurements after allo-HCT.²³ Of 40 CLL patients who underwent reduced-intensity allo-HCT, 31 were examined for disease-free survival (DFS) at 12 months after HCT. The DFS of patients who were evaluated as MRD-negative ($<10^{-4}$) was 86%, whereas that of those who were evaluated as MRD-positive ($\geq 10^{-4}$) was 20% ($p < 0.0001$). Compared with the detection limit of HTS, the DFS at 12 months post-HCT was 37.5% for 16 patients with an MRD $\geq 10^{-6}$ and 93.3% for 15 patients with an MRD $<10^{-6}$ ($p = 0.0002$).

NOVEL MARKERS FOR MRD DETECTION BY FCM

CD160

CD160 is an Ig-like activating natural killer (NK) cell receptor found on normal NK cells and T cells, but not on normal B cells.^{28,29} However, it was reported that CD160 is expressed on malignant B cells.³⁰ Farren *et al.* demonstrated that the utilization of CD160 in FCM improved the detection limit of MRD.³¹ A CD160 flow cytometry assay (CD160FCA) with a single tube of CD2/CD5/CD19/CD23/CD45/CD160 had an improved sensitivity of $\sim 10^{-4}$ to $\sim 10^{-5}$ for MRD monitoring in CLL. In a validation cohort, CD160FCA was compared and had a strong correlation with the ERIC FCM method. Moreover, patients in CR who were MRD-negative according to CD160FCA had a significantly longer event-free survival (EFS) following the first-line treatment than those in CR who were MRD-positive (median EFS of 63 vs. 16 months, respectively, $p < 0.001$). In a multivariate analysis, MRD monitoring by CD160FCA was identified as an independent predictor of EFS.

CD200

CD200 is a member of the Ig superfamily; it is expressed on T and B cells, but not on NK cells. CD200 suppresses the immune response mediated by T cells through interaction with its receptor.³² CD200 is overexpressed particularly in CLL and hairy cell leukemia, but not in mantle cell lymphoma (MCL).³³⁻³⁶ Mora *et al.* reported that CD200 is a valuable diagnostic marker for CLL.³⁷ Ting *et al.* found that CD200 was a powerful tool in distinguishing CLL from MCL by FCM, particularly when distinguishing between atypical CLL and MCL.³⁸ In this study, all 63 CLL samples, including 7 atypical CLL samples, expressed CD200, whereas the

CD200 expression of 6 MCL samples was dim or negative. Matutes scores of atypical CLL samples were ≤ 3 , and all cases were negative for t(11:14) translocation and characteristics of MCL. Atypical CLL samples tested in this study overexpressed CD200 strongly on the surface.

Our group investigated CD200 expression on CLL and MCL cells using six-color FCM (Fig. 1). As shown in Fig. 1A, CD19⁺CD20⁺CD5⁺ and Ig light chain λ -restricted CLL cells had CD200 expression on their surfaces. Conversely, CD200 was not expressed on the surface of CD19⁺CD20⁺CD5⁺ and Ig light chain λ -restricted MCL cells (Fig. 1B). All CLL cells we tested were positive for CD200, whereas MCL cells were not. Our observation supports CD200 being a useful marker for MRD detection.

MRD ASSESSMENT IN THE DEVELOPMENT OF NOVEL TREATMENTS

Several clinical trials using MRD assessment suggested it to be an essential factor in the course of developing novel treatments. Novel inhibitors for signal transduction were recently developed. These inhibitors are small molecules that can be orally administered. An overview of the clinical trials of novel inhibitors is summarized in Table 2.

Ibrutinib

Ibrutinib is an irreversible inhibitor of bruton tyrosine kinase (BTK); it inhibits B cell receptor signaling. Ibrutinib is highly effective for both TN and relapsed/refractory (r/r) CLL patients,³⁹⁻⁴² but ibrutinib monotherapy rarely achieves uMRD. However, the addition of rituximab (R) and chemotherapy (CIT) to ibrutinib achieved higher uMRD rates. Fraser *et al.* reported updated results from the HELIOS trial of ibrutinib+bendamustine and R (IBR) vs. BR for previously treated CLL without deletion of 17p.^{43,44} In total, 26.3% of patients receiving IBR treatment achieved uMRD, whereas only 6.2% of patients in the BR arm achieved uMRD ($p < 0.0001$). The best responses for patients who achieved uMRD in the IBR arm were CR/CRi (CR with incomplete BM recovery) and partial response (PR) (67.1% and 32.9%, respectively). In the BR arm, 44.4% of patients who achieved uMRD had a best response of PR. In a multicenter, randomized, open-label, phase III trial, the iLLUMINATE study, Moreno *et al.* reported that 20% and 34% of the patients treated with ibrutinib and obinutuzumab achieved uMRD in the BM and PB, respectively.⁴⁵ In the chlorambucil and obinutuzumab arm, 17% and 20% of patients achieved uMRD in the BM and PB, respectively. Overall, 35% of patients in the ibrutinib and obinutuzumab group achieved uMRD in the BM or PB, whereas 25% of patients in the chlorambucil and obinutuzumab group achieved uMRD.

Venetoclax

Venetoclax is an orally administered and highly selective BCL2 inhibitor. It yielded promising results in CLL treatment for patients with or without adverse features such as

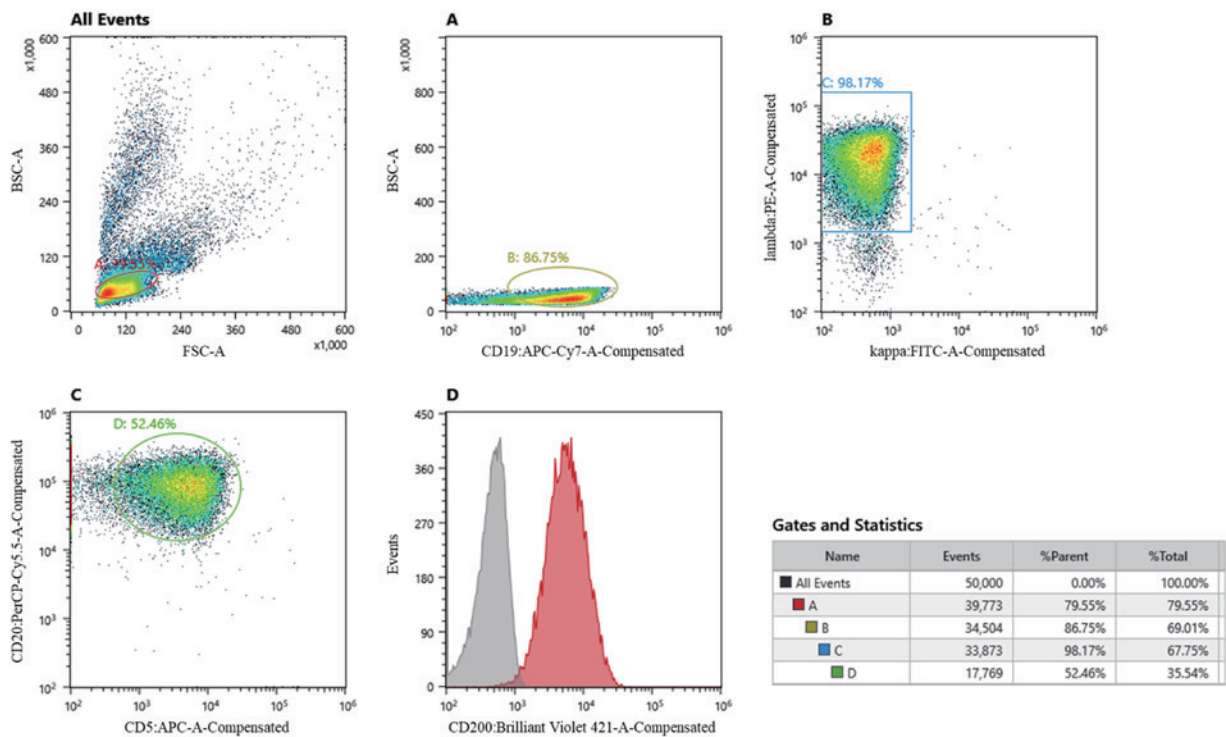
chromosome 17p deletion.^{46,47} Stilgenbauer *et al.* provided the results of MRD assessment for 158 r/r or TN CLL patients with del(17p) who received venetoclax monotherapy.⁴⁷ Of the 158 patients, 48 (30%) achieved uMRD in the PB according to the intent-to-treat analysis. A higher rate of uMRD after treatment with venetoclax in combination with rituximab (R) was reported in the randomized, open-label, phase III MURANO trial.^{48,49} In the MURANO trial, Seymour *et al.* compared venetoclax + R (VR) treatment with bendamustine + R (BR) treatment.⁴⁸ MRD assessments of the PB by RQ-ASO-PCR and BM by flow cytometry were performed. In the PB assessment for MRD, 62.4% of patients achieved uMRD in the VR arm, whereas 13.3% achieved uMRD in the BR arm. In the BM assessment for MRD, the uMRD rate of the VR arm was higher than that of the BR arm (27.3% and 1.5%, respectively). In addition, 83.5% of patients in the VR arm achieved uMRD at any time during the study even though 23.1% of patients in the BR arm were below the uMRD threshold. Two phase II trials revealed that treatment with venetoclax in combination with ibrutinib was well tolerated in both TN and r/r patients. Jain *et al.* examined TN CLL patients and reported that 61% of those who had CR or CRi achieved uMRD.⁵⁰ The other phase II trial, the CLARITY trial, in which r/r CLL patients were treated with venetoclax and ibrutinib, reported that the achievement of uMRD was higher in both the PB and BM of the patients at 53% and 36%, respectively.⁵¹

THE FUTURE OF MRD ASSESSMENT IN CLL

The efficacy of MRD assessment has been extensively studied in several clinical trials. These studies clarified that MRD monitoring is a powerful tool to predict treatment outcomes. In these studies, three major techniques, multicolor FCM, RQ-ASO-PCR, and HTS, were established for MRD evaluation and achieved a cutoff below $<10^{-4}$ for uMRD. Although the sensitivity of multicolor FCM is lower than that of the other two methods, multicolor FCM is a highly standardized and optimized assay. In addition, it requires less time to obtain results, is able to directly quantify residual cells, and is relatively cheaper than the other methods. Thus, multicolor FCM has been widely utilized in clinical trials. Both RQ-ASO-PCR and HTS have the disadvantage of being time-consuming to obtain results. RQ-ASO-PCR requires that specific primers be designed; moreover, it costs relatively more than multicolor FCM, although RQ-ASO-PCR is more sensitive. HTS solves the technical problems in RQ-ASO-PCR and is the most sensitive of the three methods currently available. HTS is, therefore, expected to be the primary method for MRD evaluation. As to whether multicolor FCM or HTS is better, the answer depends on whether MRD is regarded as a sensitive prognostic factor or as a deeper remission than CR such as deep molecular remission in chronic myelogenous leukemia.

Rawstron *et al.* compared multicolor FCM with a six-color core panel (CD19/CD20/CD5/CD43/CD79b/CD81) with HTS.¹⁰ Although both multicolor FCM and HTS

(A)



(B)

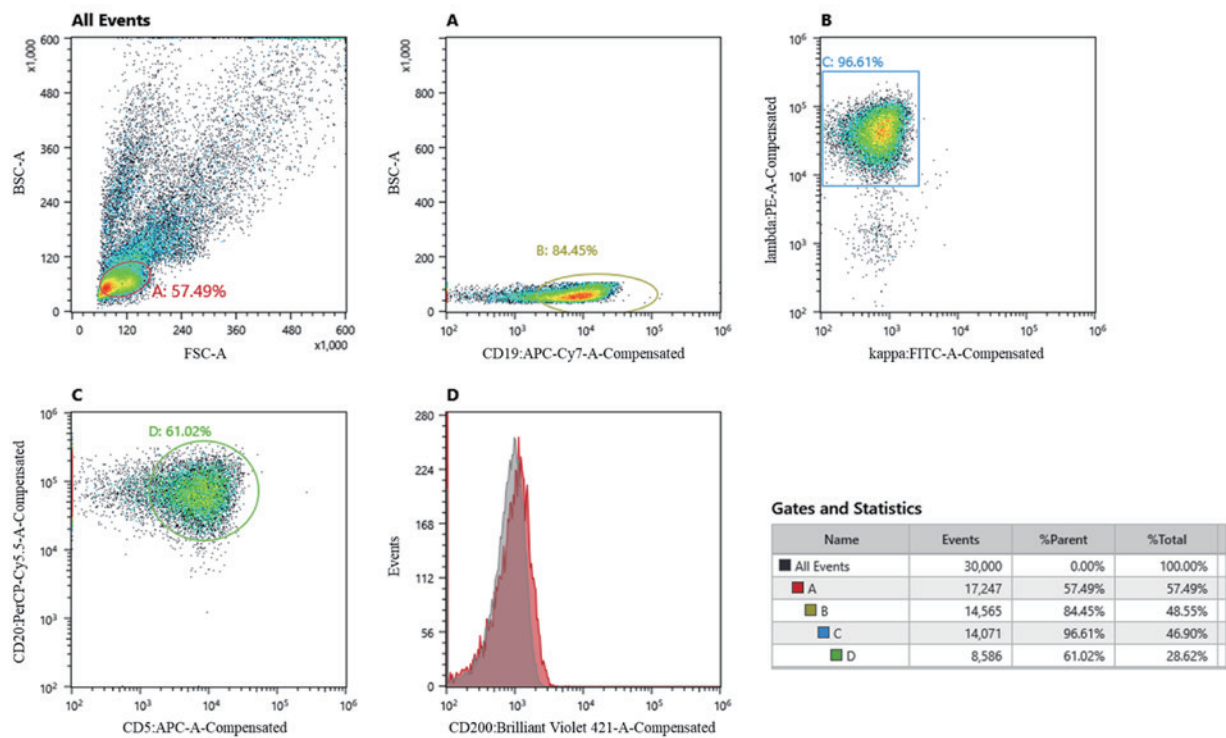


Fig. 1. A representative analysis of CD20 expression on CLL and MCL cells. CD20 expression was analyzed using six-color FCM. A sequential gating strategy was used to identify CD20 expression on both CLL (A) and MCL (B) cells. The lymphocytes were determined by their FSC and BSC. Then, the B cell population was gated by their expression of CD19 and Ig light chain restriction of the B cells was determined. The Ig light chain-restricted B cells were then analyzed for the expression of CD5 and CD20. Lastly, both CD5⁺ and CD20⁺ B cells were examined for CD20 expression. In the histograms, red is CD20 and gray is the isotype control.

Table 2. The relevant clinical trials of ibrutinib and venetoclax are summarized

Treatment	Disease status	Number	Age	Method / threshold	uMRD rate (%)	ORR (%)	CR (%)	PFS (%)	OS (%)	References
Ibrutinib+BR/BR	TN	289/289	64	FCM/10 ⁻⁴	26/6.0	87/66	38/8.0	68/14 (36M)	82/71 (36M)	43, 44
Ibrutinib+obinutuzumab/ chlorambucil+obinutuzumab	TN	113/116	70/72	FCM/10 ⁻⁴	35/25	88/73	19/8.0	79/31 (30M)	86/85 (30M)	45
Venetoclax	r/r or TN with del (17P)	158	67	FCM/10 ⁻⁴	30	77	20	54 (24M)	73 (24M)	47
Venetoclax+rituximab/ bendamustine+rituximab	r/r	194/195	65/65	FCM, RQ-ASO- PCR/10 ⁻⁴	62/13	92/72	27/8.0	85/36 (24M)	92/87 (24M)	48
Ibrutinib+venetoclax	TN	80	65	FCM/10 ⁻⁴	61	88	74	98 (12M)	99 (12M)	50
Ibrutinib+venetoclax	r/r	54	64	FCM/10 ⁻⁴	53 (PB) / 36 (BM)	89	51	NA	NA	51

reached the conventional uMRD level (10⁻⁴), it was clear that HTS is more sensitive than multicolor FCM. Therefore, they concluded that HTS in combination with multicolor FCM may be a powerful tool in improving MRD evaluation.

A novel PCR-based approach, droplet digital PCR (ddPCR), also solved the limitations of RQ-ASO-PCR as it does not require individual standard curves for each patient. The ddPCR approach for MRD assessment was intensively studied in other hematological malignancies, such as acute lymphoblastic leukemia, Ph-negative myeloproliferative neoplasms, multiple myeloma, mantle cell lymphoma, and follicular lymphoma, and was reported to be highly correlated with RQ-PCR.⁵²⁻⁵⁶ Moreover, in MRD detection, ddPCR has the advantage of enabling the accurate and precise detection and quantification of MRD even when a very small number of residual cells remains in samples.¹⁹

As novel drugs, such as venetoclax, and the addition of rituximab to chemotherapy have considerably improved the treatment outcomes of CLL patients, the need for new treatment endpoints has been considered. CLL patients who achieve uMRD throughout the treatment course exhibit a prolonged PFS; therefore, uMRD achievement may be a surrogate endpoint of CLL treatment. Anti-CD19 chimeric antigen receptor (CAR) T cell therapies in combination with ibrutinib for CLL treatment have been evaluated and yielded high uMRD rates.⁵⁷

CONCLUSION

As mentioned above, three methods are currently available to evaluate MRD. As they are costly, time-consuming, and difficult to standardize and centralize, limited facilities are able to utilize them in daily clinical practice for CLL treatment.

The achievement of uMRD is not equivalent to the eradication of CLL cells from patients. According to the MURANO trial and CLL14 trial, MRD evaluations are useful for venetoclax-based regimens.^{48,58} However, MRD assessment was valuable for predicting treatment outcomes in several clinical trials with different treatment regimens. MRD assessment may be surrogate end point of clinical

practice, and an MRD-based treatment strategy may improve the outcomes of the treatment and lead to the cessation of therapy.

CONFLICT OF INTEREST

SA received speaking fees from AbbVie GK and Janssen Pharmaceutical K.K.

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