

Flow cytometric detection of peripheral blood involvement by mycosis fungoides and Sézary syndrome using T-cell receptor V β chain antibodies and its application in blood staging

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Peripheral blood involvement has been recognized as an adverse prognostic factor in patients with mycosis fungoides and Sézary syndrome. However, accurate identification and enumeration of the neoplastic cells in these diseases can be challenging. We assessed the clinical utility of flow cytometric immunophenotypic analysis of T-cell receptor V β expression in 82 mycosis fungoides and 6 Sézary syndrome patients, with an atypical T-cell immunophenotype, or abnormal CD4:CD8 ratio, identified from peripheral blood specimens of 723 patients submitted for routine mycosis fungoides/Sézary syndrome blood staging. To improve detection sensitivity, V β expression was analyzed on gated CD3+CD4+ T cells or T cells with an aberrant immunophenotype, if present. The flow cytometric results were compared with traditional morphologic assessment ($n=88$) and molecular methods to assess the T-cell receptor γ or β genes ($n=41$ tested in parallel). Flow cytometric immunophenotyping yielded a clonal V β pattern in 60/82 mycosis fungoides and 6/6 Sézary syndrome patients. By contrast, flow cytometric V β was negative in all 10 healthy donors and 18 control patients, showing a specificity of 100% and concordance with molecular testing of 86%. Using flow cytometric V β results instead of morphologic assessment, 12 patients were upstaged from B1 to B2, and 20 patients from B0 to B1 ($P<0.0001$). The 12 upstaged B2 patients had no morphologic evidence of involvement, but had an aggressive clinical course similar to those staged by traditional morphologic assessment (median survival 27 vs 41 months, log-rank $P=0.701$). In 30/44 patients with a tumor-associated V β expression, a single V β tube was used to monitor treatment response. In conclusion, flow cytometric V β analysis is rapid and convenient, can assess T-cell clonality and tumor quantity simultaneously, and is useful both in initial blood staging and monitoring tumor burden during therapy in patients with mycosis fungoides or Sézary syndrome.

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Mycosis fungoides is a clinically indolent neoplastic proliferation of T cells that typically involves skin and is almost always of T-helper cell lineage. Patients with Sézary syndrome present with peripheral blood involvement by erythrodermic cutaneous T-cell lymphoma and frequently with generalized lymphadenopathy.¹ Mycosis fungoides and Sézary

syndrome are thought to be closely related, together representing ~65% of all lymphomas involving skin, and historically have been staged using the same TNM staging system as for other non-mycosis fungoides/Sézary syndrome cutaneous T-cell lymphomas.² However, in recent years, lymphoma cells in the peripheral blood, especially at high levels, has been recognized as an independent adverse prognostic indicator in patients with mycosis fungoides and Sézary syndrome.^{3–5} Therefore, the International Society for Cutaneous Lymphomas and the Cutaneous Lymphoma Task Force of European Organization for Research and Treatment of Cancer revised the mycosis fungoides/Sézary syndrome

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staging criteria in 2007 to incorporate blood (B) involvement as a major prognostic factor, and defined the criteria for B-staging.⁶

When assessing B-stage, one critical issue is the optimal method used to identify and quantify lymphoma cells. The assessment of peripheral blood involvement by mycosis fungoides or Sézary syndrome using morphologic criteria alone is neither sensitive nor specific, because interpretation is subject to interobserver variability, and in a subset of cases the circulating neoplastic cells are small and difficult to identify reliably.^{7,8} Moreover, low numbers of circulating atypical lymphocytes, some with cerebriform features, can be observed in healthy donors⁹ and in patients with benign skin conditions.^{7,10} In the past decade, flow cytometric immunophenotyping has proven to be more reliable than morphologic examination in assessing for lymphoma (also known as Sézary) cells in peripheral blood of mycosis fungoides and Sézary syndrome patients, as the neoplastic cells often have an aberrant immunophenotype.^{11–16} However, it has been recognized that T-cell antigenic alterations are not entirely specific for mycosis fungoides or Sézary syndrome, and have been observed in some reactive conditions.^{11,17,18} Assessment of T-cell clonality using molecular methods is therefore recommended by the International Society for Cutaneous Lymphomas, even in the presence of flow cytometric immunophenotypic aberrancies.

T-cell clonality has been traditionally assessed by Southern blot hybridization analysis, but more recently, polymerase chain reaction (PCR) methods to assess the T-cell receptor γ or β chain genes have become the most commonly used approach, as PCR is less labor intensive and requires less DNA for analysis.¹⁹ However, routine PCR assays to assess T-cell receptor gene clonality are qualitative, not quantitative. In addition, monoclonal T-cell receptor gene rearrangements have been detected in the peripheral blood of a small subset of patients with benign non-lymphoma skin disorders, autoimmune diseases, and even some healthy elderly volunteer blood donors.^{20–23} The term ‘T-cell expansion of undetermined significance,’ analogous to monoclonal gammopathy of undetermined significance, has been suggested for the presence of a small monoclonal T-cell population in an otherwise healthy individual, usually over 60 years of age.²⁴

Because of these diagnostic difficulties, the International Society for Cutaneous Lymphomas/European Organization for Research and Treatment of Cancer tried to simplify and clarify the definitions of low-level peripheral blood involvement in their staging system.⁶ In stage B0, Sézary cells comprise <5% of the nucleated cells, whereas in stage B1 Sézary cells are >5% but <1.0 K/ μ l, or \geq 1.0 K/ μ l but negative for monoclonal T-cell receptor gene rearrangement. Higher level peripheral blood involvement in stage B2 is defined as monoclonal T-cell receptor gene rearrangement in blood with either

\geq 1.0 K/ μ l Sézary cells, increased CD4+ T cells with a CD4/CD8 ratio \geq 10, or increased CD4+ T cells showing an abnormal immunophenotype (\geq 40% CD4+/CD7– or \geq 30% CD4+/CD26– of the CD4 T cells). These new definitions combine morphologic, flow cytometric, and molecular criteria to define the nature of the peripheral blood lymphocytes, as well as to quantify the neoplastic cells in mycosis fungoides or Sézary syndrome patients.

Flow cytometric analysis of T-cell receptor-V β expression is a potential alternative method to assess and quantify a T-cell clone. Earlier studies showed promising results by using a panel of antibodies covering over 65% of the T-cell receptor-V β repertoire.^{25,26} Langerak *et al*²⁶ showed in their study of 47 T-cell lymphomas that flow cytometry detected single V β domain usage in 31 (66%) patients, and was non-reactive in 16 (34%) additional patients, showing a 100% concordance with PCR analysis of the T-cell receptor β gene. Subsequently, several studies validated and expanded the use of flow cytometry to assess T-cell receptor V β in T-cell malignancies.^{13,25,27–30} Morice *et al*³⁰ studied 29 T-cell lymphoproliferative disorders by flow cytometric V β analysis, including 10 cases of cutaneous T-cell lymphoma, and showed a good correlation with molecular methods. This group expanded their study to include 11 Sézary syndrome and 6 mycosis fungoides cases, and showed that V β analysis by flow cytometry was helpful for assessing clonality and quantifying the peripheral blood tumor burden.²⁸ These studies have provided useful information, but validation by larger-scale studies with clinical follow-up is needed.

To assess the clinical utility of flow cytometric T-cell receptor-V β analysis in blood staging of mycosis fungoides and Sézary syndrome patients, we retrospectively reviewed results obtained on peripheral blood samples in 82 mycosis fungoides and 6 Sézary syndrome patients. These cases were selected because T cells exhibited immunophenotypic aberrancies or an increased CD4:CD8 ratio during routine flow cytometric immunophenotyping of 723 peripheral blood specimens from mycosis fungoides or Sézary syndrome patients. Results of flow cytometric V β analysis were compared with results from molecular studies in a large subset of cases. The relatively large number of cases in this series allowed us to correlate flow cytometric results with hematologic parameters, tumor cell counts by morphology, CD4:CD8 ratios, and other flow cytometric immunophenotyping results. The utility of flow cytometric V β analysis was also examined in monitoring mycosis fungoides/Sézary syndrome patients after therapy.

Materials and methods

Study Group

All patients were seen at The University of Texas MD Anderson Cancer Center and had a confirmed

diagnosis of mycosis fungoides or Sézary syndrome by a combination of clinical findings, peripheral blood examination, histological assessment of a skin biopsy specimen, and flow cytometric immunophenotyping of peripheral blood, with T-cell receptor gene rearrangement analysis performed by PCR in a subset of cases. Many patients had received various treatments for mycosis fungoides before being referred to MD Anderson Cancer Center. All patients had at least one peripheral blood sample submitted for flow cytometric immunophenotyping at MD Anderson Cancer Center. The cases were collected during a 4-year period beginning in May 2004 when the flow cytometric analysis of T-cell receptor V β expression was first implemented in the clinical laboratory. From a total of 723 mycosis fungoides/Sézary syndrome patients with peripheral blood analyzed by flow cytometry, V β analysis was selectively performed in patients whose T cells exhibited an atypical flow cytometric immunophenotype and/or an increased CD4:CD8 ratio (≥ 10) by flow cytometry. Eighteen specimens from patients without a T-cell malignancy were included as a control group. The clinical information was obtained by review of the medical records in accordance with an institutional review board approved protocol. Overall survival was measured from the day of diagnosis of blood stage B2, either by morphologic assessment or flow cytometric V β analysis until death from any cause, censored for patients known to be alive to the last follow-up.

Flow Cytometric Immunophenotyping and Analysis of T-Cell Receptor V β Repertoires

Peripheral blood specimens were collected in EDTA tubes. After incubation of whole peripheral blood with monoclonal antibodies for 10 min at 4°C, erythrocytes were lysed with NH₄CL for 10 min, followed by two washing steps using phosphate-buffered saline solution. The cells were then resuspended and fixed with 1% paraformaldehyde. Four-color flow cytometry was performed using FACSCalibur cytometers (BD Biosciences, San Jose, CA). Flow cytometric immunophenotyping included a panel of monoclonal antibodies specific for CD2, CD3, CD4, CD5, CD7, CD8, CD25, CD26, CD56, T-cell receptor α/β and T-cell receptor γ/δ (BD Biosciences) for the initial peripheral blood assessment, and CD3, CD4, CD8, and CD26 for follow-up peripheral blood samples. Flow cytometric immunophenotypic aberrancies could be divided into three groups: (1) altered expression levels of CD2, CD3, CD4, CD5; (2) loss CD7 in $\geq 40\%$ of CD4 T cells; and (3) CD4-PE positive/CD26-FITC negative cells accounting for 35% or more of CD4 + T cells. The cutoff for CD4 + CD26- cells used in the earlier samples was 30%, in keeping with the International Society for Cutaneous Lymphomas criteria; but later increased to 35% based on the normal range

established from healthy donors in our laboratory to improve specificity in detecting abnormal T cells.¹⁴

Flow cytometric analysis of T-cell receptor V β expression was assessed by using an eight-tube panel containing 24 monoclonal antibodies known to react with specific T-cell receptor-V β families (IOtest Beta Mark T-cell receptor-V β Repertoire Kit, Beckman Coulter). Each tube contained a mixture of three different antibodies conjugated to PE, FITC, or PE and FITC, thus permitting simultaneous analysis of expression of three V β families. This panel of antibodies covers $\sim 70\%$ of the T-cell receptor-V β repertoires of normal T cells.³¹ In most cases, CD3-APC and CD4-PerCP-Cy5.5 were included in each tube for gating. Flow cytometric V β analysis was performed primarily on CD3 + CD4 + T cells. In one case with CD8 + neoplastic cells, CD8 was used for gating instead of CD4. To improve detection sensitivity, if an aberrant immunophenotype was observed, V β expression was analyzed specifically on the aberrant T cells. The flow cytometric results were considered to be positive for clonality if (1) a single T-cell receptor-V β was expressed by $\geq 50\%$ of a gated T-cell subset; (2) a T-cell receptor-V β was expressed at a frequency ≥ 10 times above its normal limit, based on the manufacturer's reported ranges for total T cells or T-cell subsets; or (3) $\geq 70\%$ of gated T cells failed to react to any of the T-cell receptor-V β antibodies, presumably because of expression of a T-cell receptor-V β not recognized by the antibody panel.²⁸ Henceforth, the latter cases are referred to as 'non-reactive' in the text.

Quantitative T-Cell Subset Analysis

Absolute cell counts were obtained using dual platform analysis. The total white blood cell count was derived from a hematology analyzer (CellDyn 3500; Abbott Diagnostics). Instrument performance was checked daily by recording fluorescence intensity with calibrating beads (BD Biosciences). Reagent and antibody performance was checked by analyzing control cells (CDChex; Streck Laboratories, Omaha, NE, USA) and peripheral blood from blood bank donors at MD Anderson Cancer Center.

T-cell Receptor Gene Rearrangement Analyses by Molecular Techniques

Genomic DNA was extracted from peripheral blood samples and the T-cell receptor β gene was analyzed by multiplex PCR using four sets of fluorescently labeled, multicolor primers and capillary electrophoresis.³² The T-cell receptor β gene was assessed by multiplex PCR using a two-tube multicolor system, capillary electrophoresis, and BIOMED-2 primers.³³ A monoclonal pattern was defined as one or two peaks, with a peak height substantially higher than the baseline, with or without a low-level

or more prominent smear (polyclonal amplification) pattern in the background. An oligoclonal pattern was defined as three prominent peaks, with peak height substantially higher than the baseline. The analytic sensitivity of the T-cell receptor γ and β PCR assays was 5–10 and 10–15%, respectively. In selected cases, the T-cell receptor V β gene was analyzed by Southern blot analysis using EcoRI, HindIII, and BamHI restriction enzyme digests and a radioactively labeled constant region probe (C β). To establish the presence of monoclonal gene rearrangement, non-germline bands were required in two digests.

Statistical Analysis

Statistical comparisons were performed as follows (1) continuous numeric data were compared using the two-sided Student's *t*-test, or one-way ANOVA; (2) proportions were compared using Fisher's exact test or the χ^2 -test. For all statistical tests, statistical significance was defined by a *P*-value of 0.05 or less.

Results

Patient Characteristics

A total of 83 patients with mycosis fungoides and 6 patients with Sézary syndrome had an atypical immunophenotype or a markedly increased CD4:CD8 ratio (≥ 10) (Table 1) and were studied by flow cytometric V β analysis. This group was culled from 723 mycosis fungoides or Sézary syndrome patients who underwent T-cell immunophenotyping of peripheral blood specimens. In consideration of the cost, cases with no evidence of absolute lymphocytosis, immunophenotypic aberrancies, or an increased CD4:CD8 ratio, were not studied by flow cytometric V β analysis.

The immunophenotype of the neoplasms was CD3 + CD4 + in 88 cases. One mycosis fungoides case had a CD3 + CD8 + immunophenotype and was excluded from this analysis leaving a study group of 88 patients. Of the 88 patients selected for flow cytometric V β analysis, 31 (35%) cases showed altered expression levels of CD2, CD3, CD4, or CD5; 36 cases showed loss of CD7 expression in $>40\%$ of CD3 + CD4 + T cells; 62 (70%) cases showed loss of CD26 in $\geq 35\%$ of CD3 + CD4 + T cells. Overall, immunophenotypic aberrancies were detected in 71 of 88 (92%) cases. Seventeen cases with no definitive immunophenotypic aberrancies were studied by flow cytometric V β because these cases either showed an increased CD4:CD8 ratio ≥ 10 (8 cases), or borderline increased CD4 + CD26– cells in the range of 30–35% (9 cases) when a 30% cutoff was used in the earlier samples (please refer to Materials and methods for CD4 + CD26– cutoff).

There were 49 men and 39 women with a median age of 62 years (range, 16–94 years) (Table 1). The

Table 1 The laboratory features of patients with mycosis fungoides/Sézary syndrome analyzed by flow cytometry for T-cell receptor V β expression

<i>Patients (N = 88)</i>	
Median age (range)	62 (16–94)
Sex (male/female)	49/39
Median WBC/ μ l (range)	11 813 (3000–67 000)
Lymphocyte %, median (range)	34% (2–89%)
Absolute count/ μ l, median (range)	5,111 (196–59 630)
CD3+CD4+ lymphocyte %, median (range)	72% (1–99%)
Absolute count/ μ l, median (range)	4,160 (12–57 792)
<i>SS cells by morphology (mean, range)</i>	
Cases with $\leq 5\%$	56 (0.6%, 0–5%)
6–19%	15 (13%, 6–18%)
$\geq 20\%$	17 (47%, 20–95%)
<i>Aberrant T-cell phenotype (altered CD2, CD3, CD4, CD5 expression levels)</i>	
Normal	57 (65% of cases)
Abnormal	31 (35%)
<i>CD4+CD7– cells</i>	
Normal ($<40\%$ of lymphocytes)	52 (59%)
Abnormal ($\geq 40\%$ of lymphocytes)	36 (41%)
<i>CD4+CD26– cells</i>	
Normal ($<35\%$ of lymphocytes)	26 (30% of cases)
Abnormal ($\geq 35\%$ of lymphocytes)	62 (70%)
<i>CD4/CD8 ratio</i>	
< 10	49 (56% of cases)
≥ 10	39 (44%)
<i>Flow cytometric Vβ analysis</i>	
Negative	22 (25% of cases)
Positive	66 (75%)
Single V β +	44 (50%)
$\geq 70\%$ Non-reactive	22 (25%)
<i>Molecular clonality analysis</i>	
Positive	28 (70% of tested cases)
Oligoclonal	4 (10%)
Negative	8 (20%)
Not performed	47

study group included 82 patients with mycosis fungoides and 6 patients who met criteria for *de novo* Sézary syndrome. The median white blood cell count was 11 813/ μ l (range, 3000–67 000/ μ l). The median lymphocyte percentage and absolute count were 34% (range, 2–89%) and 5111/ μ l (range, 196–59 630/ μ l), respectively. By flow cytometric analysis, the median CD3 + CD4 + lymphocyte percentage and absolute count were 72% (range, 15–99%) and 4160/ μ l (range, 12–57 792/ μ l), respectively. The median CD4:CD8 ratio was 6.2 (0.60–954). In our laboratory, flow cytometric V β analysis is performed weekly on peripheral blood samples obtained from healthy donors as part of the laboratory quality controls. In the same 4-year period, ~ 250 samples (median age, 55 years; range, 18–81 years) were analyzed by flow cytometric V β ; all were negative. For this study, 10 age-matched

peripheral blood samples collected from 3 female and 7 male healthy donors (median age, 60 years; range, 35–79) were tested for T-cell receptor γ and T-cell receptor β clonality by PCR methods. In addition, 18 peripheral blood samples from age-appropriate (median, 54 years; range, 17–89) patients with no evidence of T-cell malignancy were included in the control group. These patients sought medical care for a variety of illnesses including: benign dermatitis ($n=5$), Epstein–Barr virus infection ($n=2$), hypereosinophilic syndrome ($n=1$), Langerhans cell histiocytosis ($n=1$), follicular lymphoma ($n=1$), plasma cell dyscrasia ($n=1$), myelodysplastic syndrome ($n=4$), and acute myeloid leukemia ($n=3$). All control patients had no clinical or histologic evidence of T-cell lymphoma (including the patient with hypereosinophilic syndrome).

The Utility of V β Analysis by Flow Cytometry in Assessment of T-Cell Clonality

V β expression was analyzed on all the 88 cases. The gating was primarily set on the total CD3 + CD4 + cells in 61 cases. In 27 (31%) cases, aberrant levels of expression of CD3 or CD4 or both were observed, and gating was therefore set specifically on the aberrant cell population instead of the total CD3 + CD4 + cells in those cases.

Forty-four cases showed a single V β expressed by $\geq 50\%$ of gated T cells or ≥ 10 times the normal upper limits. An additional 22 cases were non-reactive, defined as $\geq 70\%$ of gated CD3 + CD4 + T cells that failed to react with any of the V β antibodies. These non-reactive cases were also considered positive for V β clonality. Overall, a total of 66 cases were positive for a clonal V β expression by flow cytometry. None of the 10 healthy donors or 18 cases in the control group showed a T-cell population with abnormal V β expression. These results indicate a specificity of 100% for flow cytometric V β analysis in the cohort analyzed.

V β family usage in the positive cases was as follows V $\beta 1$ ($n=3$), V $\beta 2$ ($n=6$), V $\beta 3$ ($n=1$), V $\beta 4.1$ ($n=1$), V $\beta 5.1$ ($n=3$), V $\beta 5.2$ ($n=1$), V $\beta 7.1$ ($n=1$), V $\beta 8$ ($n=5$), V $\beta 9$ ($n=2$), V $\beta 12$ ($n=4$), V $\beta 13.1$ ($n=4$), V $\beta 14$ ($n=3$), V $\beta 17$ ($n=6$), V $\beta 18$ ($n=1$), and V $\beta 22$ ($n=3$). Figure 1 illustrates a polyclonal V β expression pattern in a case scored as negative for V β clonality (Figure 1a), a positive case with clonal V $\beta 17$ expression (Figure 1b), and a non-reactive case (also interpreted as clonal) (Figure 1c). Figures 2a–c show that gating on cells with an aberrant immunophenotype increases the detection sensitivity of V β analysis.

Comparison of Flow Cytometric V β Analysis with Other Flow Cytometric Immunophenotypic Aberrations and CD4:CD8 Ratio

Overall, immunophenotypic aberrancies were detected in 61 of 66 (92%) V β clonal cases, and in 10 of 22 (46%) V β non-clonal cases ($P<0.001$). Specifically focusing on CD26 results, the median proportion of CD4 + CD26– cells was 24.7% of the CD4 + T cells (range, 6.5–55.5%) in the control group; 30.2% (range, 13.4–58.9%) in the V β non-clonal cases; and 70.1% (2.6–96.2%) in the V β clonal cases (Figure 3a). These differences were statistically significant ($P<0.0001$). However, using an upper limit of 35% established in our laboratory on normal peripheral blood specimens, loss of CD26 was also observed in three cases in the control group (3/18, 17%), and in six V β non-clonal cases (6/22, 27%), whereas CD26 expression was in the normal range in 12 of 66 (18%) V β clonal cases (Figure 3a; Table 2). Altered CD2, CD3, CD4, CD5 expressions were more specifically indicative of a clonal process, and identified in 29/66 (44%) V β clonal cases, and in 2/22 (9%) ($P=0.004$) V β non-clonal cases. Loss of CD7 in 40% or more of CD3 + CD4 + T cells was found in 31/66 (47%) V β clonal cases and 5/22 (23%) V β non-clonal cases ($P=0.05$).

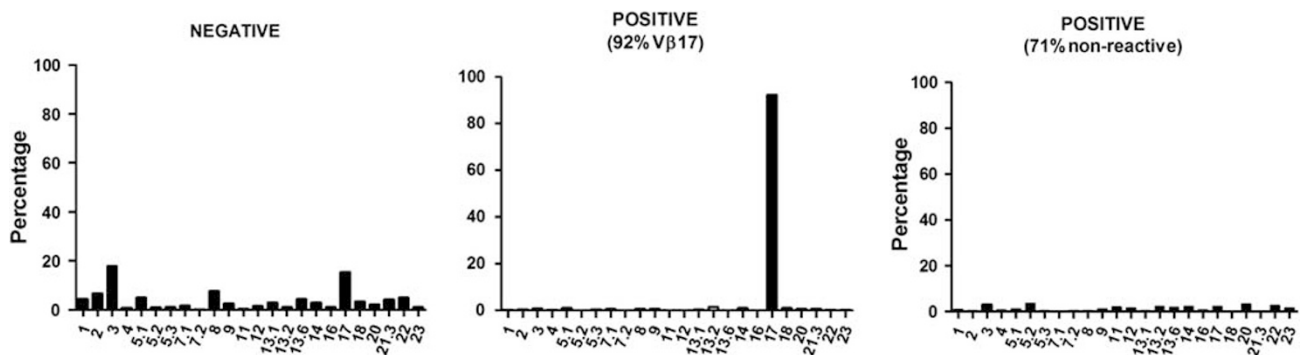


Figure 1 Flow cytometric analysis for V β repertoire. Analysis was based on CD3 + CD4 + T cells, and the percentage of each V β subtype in the total gated population is shown as a solid bar. (a) A negative case with CD4 + T cells expressing various V β subtypes. (b) A positive case with 92% CD4 + T cells expressing V $\beta 17$. (c) A positive case with $>70\%$ CD4 + T cells failed to react to any of the T-cell receptor-V β antibodies, as ‘non-reactive.’

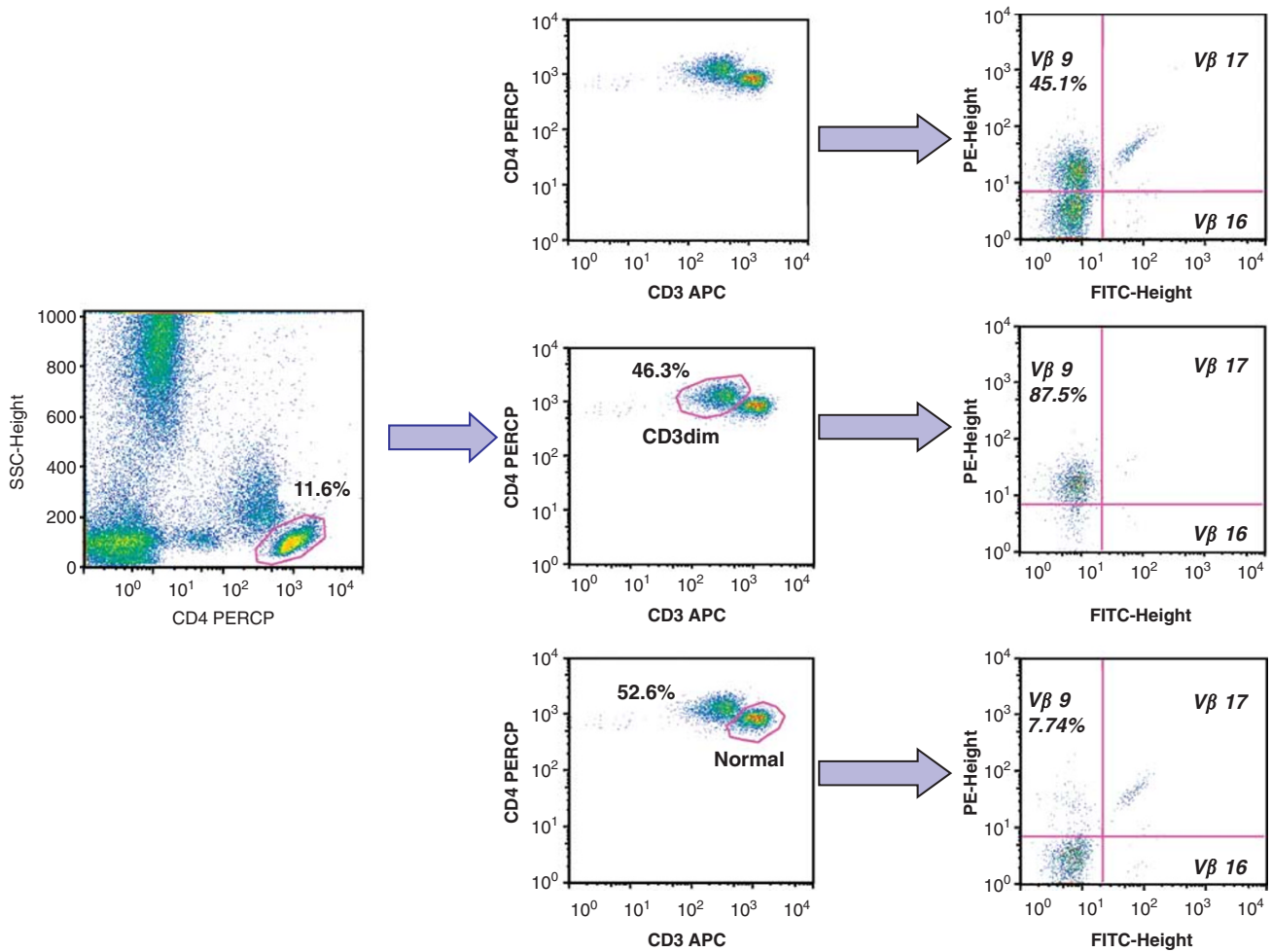


Figure 2 V β flow cytometric analysis—improving detection sensitivity by gating on T cells with an aberrant immunophenotype. Upper panel: Gating on the total CD4⁺ T cells, V β 9 expression (45.1%) was below the cutoff and scored as ‘negative.’ Mid panel: Gating on the CD3dimCD4⁺ T-cell population, V β 9 expression (87.5%) was above the cutoff and scored as ‘positive.’ Lower panel: Gating on normal CD3⁺CD4⁺ T cells, V β 9 (7.7%) was within the normal range.

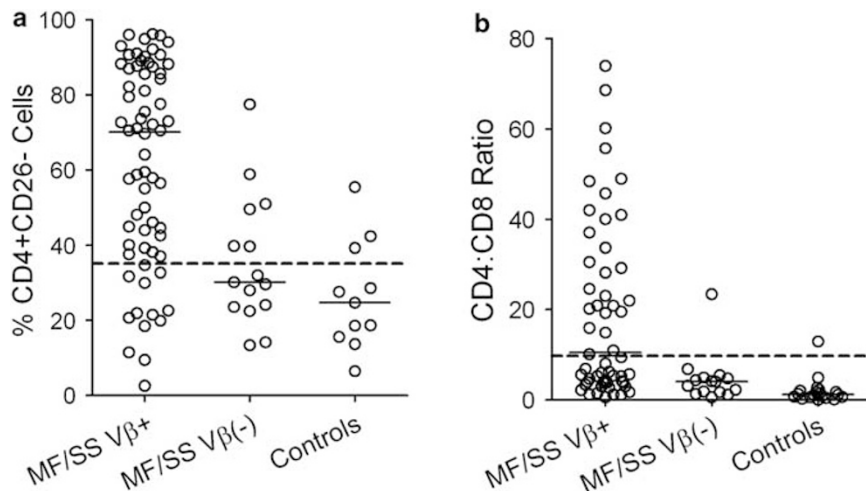


Figure 3 CD26 expression and CD4:CD8 ratio. (a) The proportions of CD4⁺CD26⁻ T cells in V β positive cases (median, 70.1%; range, 2.6–96.2%); V β negative cases (median, 30.2%; range, 13.4–58.9%) and controls (median, 24.7%, range, 6.5–55.5%). (b) The CD4:CD8 ratio in V β positive cases (median, 10.5; range, 0.6–495.0); in V β negative cases (median, 4.1; range, 0.6–23) and in controls (median, 1.2; range, 0.07–12.9).

Table 2 Correlation of abnormal cells detected by morphology, standard flow cytometric immunophenotyping, flow cytometric V β analysis, and T-cell receptor gene rearrangement in blood staging

<i>SS by morphology</i>	Control (N = 18)	$\leq 5\%$ (N = 56)	$> 5\%$ but $\leq 1000/\mu\text{l}$	$\geq 1000/\mu\text{l}$ (N = 27)
% Sézary cells (mean, range)	(0.2%, 0–3%;	(0.6%, 0–5%;	(N = 5) (10%, 6–14%;	(35%, 10–95%;
Absolute count (mean, range)/ μl	15, 0–110)	40, 0–532)	740, 492–880)	7436, 1115–41 195)
<i>Flow cytometric immunophenotype</i>				
Aberrant CD2, CD3, CD4, CD5	1	17	1	13
Increased CD4+CD7– ($\geq 40\%$)	3	17	1	18
Increased CD4+CD26– ($\geq 35\%$)	3	32	5	25
Normal	13	14	0	0
CD4:CD8 ratio ($\geq 10:1$)	2 (11% of cases)	16 (28%)	1 (53%)	22 (81%)
<i>Flow cytometric Vβ analysis</i>				
Negative	18	21	1	0
Positive (single V β /non-reactive)	0	35 (26/9)	4 (3/1)	27 (15/12)
<i>TCR gene rearrangement</i>				
Positive	3	16	1	11
Oligoclonal	3	4	0	0
Negative	6	7	0	1
Not performed	6	29	4	15
^a Final blood staging (n = 88)	NA		B0: 24 B1: 25 B2: 39	

^aTumor cells calculated by flow cytometric V β analysis.

The CD4:CD8 ratio, one of the parameters incorporated in the B-staging recommended by International Society for Cutaneous Lymphomas, showed a median of 10.5 in V β clonal cases (range, 0.6–495.0), 4.1 (range, 0.6–23) in V β non-clonal cases, and 1.2 (range, 0.07–12.9) in control cases, respectively (Figure 3b). Although these differences were statistically significant ($P=0.041$), the sensitivity of detecting tumor cells using a CD4:CD8 ratio of ≥ 10 was low, as only 29 of 66 V β clonal cases (44%) showed an elevated ratio above this threshold.

Correlation of V β Flow Cytometric Clonality Analyses with PCR Assays for T-Cell Receptor Clonality

Forty-one cases were assessed for T-cell receptor clonality by PCR: T-cell receptor γ in all 41 and T-cell receptor β in 16. In eight cases, Southern blot analysis was performed to assess the T-cell receptor β gene. Twenty-eight cases had monoclonal T-cell receptor gene rearrangements: 7 cases by T-cell receptor γ and T-cell receptor β ; 20 cases by T-cell receptor γ , and 1 case by T-cell receptor β . Twelve cases were negative: 7 cases tested for T-cell receptor γ and T-cell receptor β , and 5 cases by T-cell receptor γ . Of the 28 cases with monoclonal T-cell receptor gene rearrangements, 24 (86%) also showed abnormal V β expression by flow cytometry. Of the four cases polyclonal for V β by flow cytometry but positive by molecular studies, three cases showed an atypical V β expression pattern, with high numbers of T cells non-reactive for all tested V β chains, representing 55, 57, and 63% of gated regions, respectively. However, these cases

were judged as negative for clonality by flow cytometry because all were below the 70% threshold. The other case showed a CD4:CD8 ratio of 16. All 4 of these discordant cases had 0% Sézary cells by morphology, and only one case showed an abnormal immunophenotype.

Notably, all four cases with an oligoclonal T-cell receptor gene rearrangement pattern were positive for V β clonality by flow cytometry, preferentially expressing the single V β chains V β 2, V β 5.1, V β 13.1, or V β 17 in $\geq 50\%$ of gated cells in one case each. Two of these cases showed dim CD3 expression, assisting in specific gating. Similarly, three of eight cases negative for T-cell receptor gene rearrangements by PCR were positive for V β clonality by flow cytometry: one with 94% of the gated cells expressing V β 2; and the other two cases with 77 and 96% non-reactive cells. One of the flow cytometric V β non-reactive cases also showed 70% Sézary cells by morphology. In the subgroup of 10 healthy donor samples, a small T-cell receptor γ clone in an oligoclonal background was detected in two samples (20%). In the control group without T-cell malignancy, monoclonal T-cell receptor gene rearrangement was positive in 3 out of 12 cases (25%), including one case of AML (no circulating blasts), one case of MDS and one patient with spongiotic dermatitis. The specificity of PCR testing in this cohort was therefore 77% (5/22).

The above results show that the concordance between flow cytometric V β analysis and T-cell receptor molecular testing in this study was 86%; the two methods are complimentary to each other in detecting T-cell clonality; but our results suggest that flow cytometric V β analysis is more specific.

Comparison of Staging Results by Morphology vs Flow Cytometric V β Analysis, and Correlation with Patient Outcomes

Identification and manual differential counting of neoplastic (Sézary) cells in peripheral blood smears were performed by senior medical technologists, with abnormal results confirmed by hematopathologists. The staging and classification of mycosis fungoides and Sézary syndrome proposed by International Society for Cutaneous Lymphomas and European Organization for Research and Treatment of Cancer traditionally divides patients into three groups based on the percentage of the circulating Sézary cells: 0–5% (negative), 6–19%, and $\geq 20\%$ of the total lymphocytes. In this study, significant numbers of Sézary cells were observed by morphologic examination in 34 (38%) patients. The percentage of tumor cells was between 0 and 5% (ie negative) in 56 (64%); 6–19% in 15 (17%), and $>20\%$ in 17 (19%) patients, respectively (Table 1). In these three groups, flow cytometric analysis of V β expression confirmed clonality in a total of 66/88 (75%) patients, but in many patients identified much higher numbers of tumor cells, with 22, 11, and 55 cases in the groups with $\leq 5\%$, 6–19%, and $\geq 20\%$ aberrant cells by flow cytometry, respectively. This frequency of detection is significantly higher than that achieved by morphological assessment ($P < 0.0001$) (Figure 4a). In the 18 control patients, the median number of Sézary cells observed by morphology was 0.3% (range, 0–3%).

In the revised International Society for Cutaneous Lymphomas criteria, peripheral blood staging is not only determined by the percentage of neoplastic cells, but also by the absolute tumor cell count. In 27 cases with $\geq 1000/\mu\text{l}$ Sézary cells by morphologic assessment, clonal V β expression was confirmed in all 27 (100%) cases. In the remaining cases, of the 22 cases with CD3+CD4+ cells $\geq 1000/\mu\text{l}$ but Sézary cells $< 1000/\mu\text{l}$ by morphologic examination, 12

cases showed $\geq 1000/\mu\text{l}$ monotypic V β cells, and were considered as B2 (Figure 4b). Overall, peripheral blood stage as assessed by morphologic criteria was 56 (64%) B0, 5 (6%) B1, and 27 (30%) B2. By flow cytometric V β analysis in conjunction with flow cytometric immunophenotyping, the peripheral blood stage assigned was 24 (27%) B0, 25 (28%) B1, and 39 (44%) B2. These differences were statistically significant ($P < 0.0001$).

In our series, T-cell receptor gene rearrangement results were available in 41 cases, therefore, a complete comparison of B-staging by flow cytometric V β analysis vs a combined staging by T-cell receptor gene rearrangement, flow cytometric immunophenotyping, CD4:CD8 ratio, and absolute lymphocyte count was not possible. Nevertheless, of the 12 cases upstaged to B2 by flow cytometric V β , 6 cases had molecular studies performed, 5 were positive, and 1 showed an oligoclonal pattern. For the cases with absolute flow cytometric V β clonal cells $< 1000/\mu\text{l}$, 5 cases were positive for T-cell receptor gene rearrangement, but none of these would be upstaged to B2 because of low tumor burden.

The clinical outcomes of patients with a blood stage of B2 defined by flow cytometric V β analysis but no morphologic evidence ($n = 12$) was compared to patients with a B2 stage defined by traditional morphologic assessment ($n = 27$). These 39 patients received various treatment modalities including topical therapy, radiation, non-chemotherapy systemic treatment, combined chemotherapy, and bone marrow transplant ($n = 5$). The median follow-up for these 39 patients was 30 months (range, 5–59 months). Patients with blood stage B2 upstaged only on the basis of flow cytometric V β analysis ($n = 12$) had an overall survival of 27 months (range, 9–59 months), comparable to patients with blood stage B2 defined by traditional morphologic assessment ($n = 27$) (median overall survival, 41 months; range, 5–54) (Kaplan–Meier log-rank, $P = 0.701$) (Figure 5).

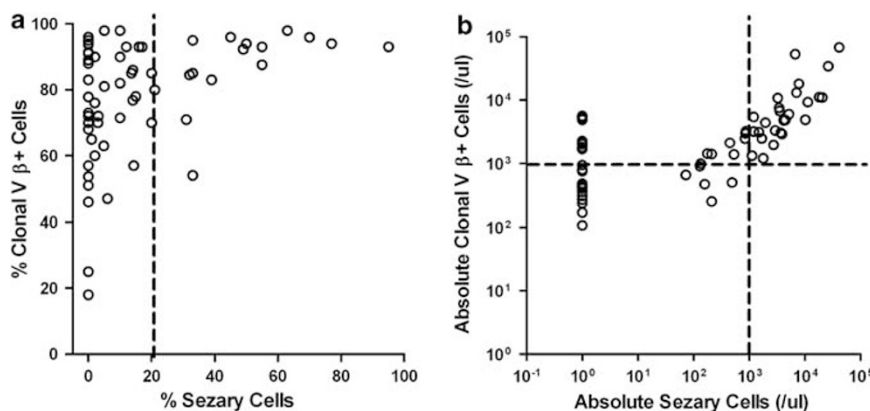


Figure 4 Correlation between tumor cells identified by morphology and by flow cytometry V β analysis. The proportion of tumor cells (a) and the absolute tumor cell count (b) identified by morphology was significantly lower than that identified by flow cytometric V β analysis ($P < 0.0001$ and $P < 0.001$).

V β Flow Cytometric Clonality Analyses for Monitoring Treatment Responses

The quantitative nature of flow cytometric V β expression analysis allowed for effective quantitation of peripheral blood tumor burden, and thus was useful for monitoring disease progression and response to therapeutic modalities. Among the 44 patients with a tumor-associated V β chain identifiable with this antibody panel, 30 patients were followed by flow cytometric for up to 4 years, using a single relevant anti-V β tube. As shown in Figure 6, responses to therapy and disease progression could be measured approximately by the CD3 + CD4 + cell

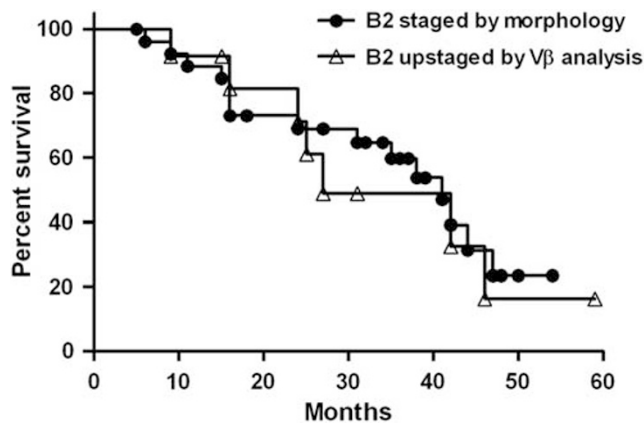


Figure 5 Comparison of overall survival of 12 patients with a blood stage B2 upstaged by flow cytometric V β analysis with 27 patients with B2 staged by traditional morphologic assessment (Kaplan–Meier log-rank, $P=0.701$).

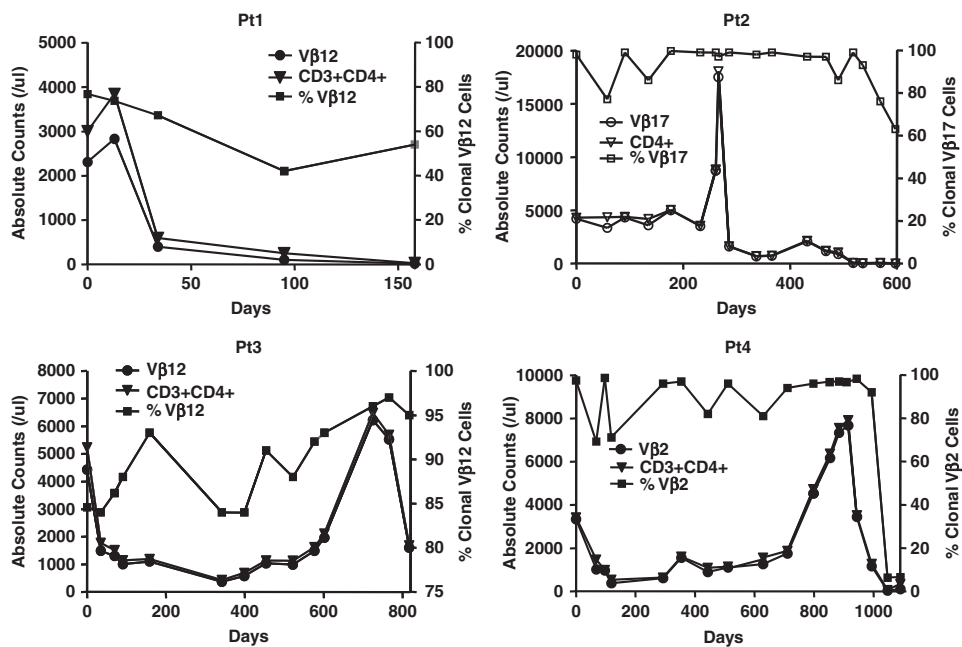


Figure 6 One-tube V β flow cytometry in monitoring tumor burden and treatment responses. Left 'y' axis: absolute cell count/ μ l; right 'y' axis: percentage of V β positive cells; 'x' axis: time after initial peripheral blood diagnosis. The clinical courses of four patients are illustrated here. V β flow cytometry showed persistent diseases even when the total CD3 + T cells fell in the normal range in patients 1, 2, and 3; and the tumor cells were eradicated only after total body radiation beam and bone marrow transplant treatment (patient 4).

count, and more precisely by the CD3 + CD4 + CD26– absolute count if the tumor cells were CD26–. However, the percentages of V β positive cells were consistently high even when the absolute numbers of CD3 + CD4 + cells declined to the normal range, and/or the total T cells showed a normal CD4:CD8 ratio. The flow cytometric V β analysis showed polyclonality only after very aggressive therapies, such as whole body beam radiation and bone marrow transplant.

Discussion

In this study, we retrospectively reviewed the utility of flow cytometric V β analysis performed on peripheral blood in 82 patients with mycosis fungoides or 6 with Sézary syndrome, and correlated the results with other flow cytometric aberrations, manual Sézary cell counts, and T-cell receptor clonality assessed by molecular methods. The results show that V β flow cytometric analysis is useful in establishing T-cell clonality, assessing tumor burden, and monitoring disease responses to therapy.

Aberrant expression of T-cell markers such as CD2, CD3, CD4, CD5, CD7, or CD26 was identified in 92% cases positive for malignant T-cell clones, with loss of CD26 being the most common (70%). However, increased CD26– cells, or CD7– cells, and even altered expression levels of other more specific T-cell markers, such as CD3 and CD4, were also observed in non-neoplastic samples. This indicates that some flow cytometric surface aberrations are not necessarily indicative of T-cell

malignancies. These findings agree with earlier observations that loss or dim expression of CD7 and CD26 can be found in patients with benign inflammatory dermatoses^{17,34,35} and other reactive conditions,¹⁴ and aberrant dim CD3 can be observed non-neoplastic T cells.²⁹ Similarly, T-cell receptor gene rearrangements have been detected in samples of patients without clinical or histologic evidence of lymphoma or evidence of T-cell clonality shown by other methods. In this study group, V β flow cytometric clonality assessment correlated with PCR testing for T-cell receptor gene rearrangements in 86% of cases, but flow cytometric V β analysis had 100% specificity vs 77% for PCR. The specificity of PCR testing in this setting would likely be greatly improved if both skin and peripheral blood samples were tested, and showed the same clone.³⁶

A major limitation of V β flow cytometric analysis is that the available anti-T-cell receptor β antibody panel only covers ~70% of all the T-cell receptor β families expressed. Therefore, a subset of T-cell neoplasms will not be detected by the current antibody panel, representing 22 (33%) cases in this study. In these cases, however, clonality can be inferred from the non-reactive pattern, unlike polyclonal T-cell populations that express various V β families. In addition, the flow cytometric V β expression analysis is comparative and requires a preponderance of clonal cells for their discrimination from normal T cells. As many peripheral blood specimens have both neoplastic CD3+CD4+ T cells and substantial numbers of normal lymphocytes (B cells, CD3+CD8+ T cells, NK-cells and normal CD3+CD4+ T cells), gating on lymphocytes based on forward vs side scatter (FSC/SSC), or CD45/SSC, or even total CD3+ T cells, is inadequate for V β analysis for many cases. Aberrant expression of T-cell markers by T-cell lymphomas allows for improved gating for V β clonality analysis, especially in cases when the proportion of malignant T cells in the CD4 positive subset is low. In this study, we gated primarily on CD3+CD4+ cells, further defined by FSC/SSC, to improve gating accuracy. In addition, altered expression of CD3 and CD4 or both were detected in 27 (31%) cases, and the detection sensitivity by V β flow cytometric analysis was improved by selectively gating on the aberrant cell populations. Particularly, this specific gating strategy helped to detect the presence of a T-cell clone in two of four cases in which PCR analysis of T-cell receptor gene(s) showed an oligoclonal pattern. One drawback of the 70% cutoff for non-reactive cases is that this cutoff may be too conservative. Three of four cases negative by flow cytometric V β analysis but positive by T-cell receptor gene rearrangement showed 55–63% non-reactive T cells. With the development of multi-color flow cytometry in recent years, incorporating more T-cell markers, such as CD26 and CD7, into V β flow cytometric analysis may improve the detection sensitivity.

Although earlier studies have shown that 'significant' peripheral blood involvement has independent prognostic significance in mycosis fungoides patients regardless of T or N staging, how to accurately define and quantify tumor cells in the peripheral blood remains debatable. We have shown in our study that V β flow cytometry is quantitative, and the quantitative nature of V β expression can be further improved by incorporating the other immunophenotyping data such as CD4:CD8 ratio, absolute CD3+CD4+CD26- cells, or CD3+CD4+ cells into the flow cytometric analysis, validating the proposal by earlier pilot studies.^{25,37} We have also shown that Sézary cell counting by morphologic analysis is extremely insensitive and would miss a high proportion of cases with a high tumor burden compared with flow cytometric analysis (Figure 4). Using flow cytometric V β expression analysis, at least 12 patients were upstaged from B1 to B2, a change in peripheral blood stage with prognostic implications. In addition, 20 patients were upgraded from B0 to B1 because a monoclonal T-cell population was confirmed. Although variable treatment modalities used in these blood stage B2 patients might have complicated the outcome analysis, we were able to show that patients with a blood stage B2 upstaged by flow cytometric V β analysis without a morphologic confirmation ($n=12$) had a similar aggressive clinical course to patients with a blood stage of B2 assessed by morphology ($n=27$). These survival data provide a rationale to support using flow cytometric V β analysis in mycosis fungoides/Sézary syndrome blood staging.

As only a proportion of the cases (41 cases) had molecular studies performed, we had limited cases available to compare B-staging by flow cytometric V β with B-staging by a combination of T-cell receptor gene rearrangement, flow cytometric immunophenotyping, CD4:CD8 ratio, and absolute lymphocytosis recommended by the 2007 European Organization for Research and Treatment of Cancer/International Society for Cutaneous Lymphomas criteria. We showed that flow cytometric V β analysis, which could assess the T-cell clonality and tumor quantity simultaneously, might be a better assay than the current recommended approach in B-staging in which the quantity and clonality of lymphocytes are assessed by different methodology. On the basis of our findings, we suggest that flow cytometric V β analysis, can serve at least as one of the major clonality tests for B-staging of mycosis fungoides/Sézary syndrome patients.

When a single V β family is detected, as is the case of two thirds of the flow cytometric positive cases in our study, a single V β tube can be used to assess clonality, tumor burden, and therefore treatment response. Morice *et al*²⁸ used V β flow cytometric analysis in the follow-up of 6 Sézary syndrome patients for up to 188 days. In our study, 30 out of 44 patients with single V β detected by V β flow cytometry were followed for peripheral blood tumor

burden up to 4 years. The absolute counts calculated by flow cytometric V β generally correlated well with the total CD3+CD4+ T cells. However, flow cytometric V β analysis showed that even when the total number of CD3+CD4+ cells dropped significantly to the normal range, the proportion of the neoplastic cells remained high, indicating residual disease. As a small subset of normal CD4+ T cells is CD26-, quantitation of V β + cells would provide a more accurate assessment of tumor burden than absolute CD4+CD26- cells in posttreatment samples. One concern in following patients with mycosis fungoides or Sézary syndrome is clonal evolution, which may result in loss of surface V β expression in the neoplastic T cells, thus escaping from detection. In our experience of monitoring 30 patients for 1–4 years during clinical follow-up, loss expression of V β was never encountered.

In summary, in this study that consisted of a large number of mycosis fungoides or Sézary syndrome cases with long-term clinical follow-up, we have shown that V β expression analysis by flow cytometry is a specific and sensitive assay to detect T-cell clonality. Furthermore, the sensitivity can be further improved by gating on specific subpopulations of T cells with immunophenotypic aberrancies. With recent developments of multiple color flow cytometric techniques, the sensitivity is likely improved further by incorporating multiple additional CD markers in the standard V β flow cytometric tubes. In addition, flow cytometric V β can assess T-cell clonality and quantity of the neoplastic cells simultaneously and is more sensitive than morphologic assessment in identifying patients with a true blood stage B2. Furthermore, this assay is rapid, convenient, cost-effective, quantitative and valuable in assessing tumor burden in monitoring treatment response.

Conflict of interest

The authors declare no conflict of interest

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