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Accurate detection of the tumor clone in peripheral T-cell lymphoma biopsies by flow cytometric analysis of TCR-Vβ repertoire

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Multiparametric flow cytometry has proven to be a powerful method for detection and immunophenotypic characterization of clonal subsets, particularly in lymphoproliferative disorders of the B-cell lineage. Although in theory promising, this approach has not been comparably fulfilled in mature T-cell malignancies. Specifically, the T-cell receptor-Vβ repertoire analysis in blood can provide strong evidence of clonality, particularly when a single expanded Vß family is detected. The purpose of this study was to determine the relevance of this approach when applied to biopsies, at the site of tumor involvement. To this end, 30 peripheral T-cell lymphoma and 94 control biopsies were prospectively studied. Vβ expansions were commonly detected within CD4+ or CD8+ T cells (97% of peripheral T-cell lymphoma and 54% of non-peripheral T-cell lymphoma cases); thus, not differentiating malignant from reactive processes. Interestingly, we demonstrated that using a standardized evaluation, the detection of a high Vβ expansion was closely associated with diagnosis of peripheral T-cell lymphoma, with remarkable specificity (98%) and sensitivity (90%). This approach also identified eight cases of peripheral T-cell lymphoma that were not detectable by other forms of immunophenotyping. Moreover, focusing Vβ expression analysis to T-cell subsets with aberrant immunophenotypes, we demonstrated that the T-cell clone might be heterogeneous with regard to surface CD7 or CD10 expression (4/11 cases), providing indication on 'phenotypic plasticity'. Finally, among the wide variety of Vβ families, the occurrence of a Vβ17 expansion in five cases was striking. To our knowledge, this is the first report demonstrating the power of T-cell receptor-Vβ repertoire analysis by flow cytometry in biopsies as a basis for peripheral T-cell lymphoma diagnosis and precise T-cell clone identification and characterization.
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Peripheral T-cell lymphomas are hematological malignancies that are thought to develop from transformed lymphocytes of post-thymic origin. These disorders account for about 10% of all non-Hodgkin's lymphomas in Western countries.[1,2] Their diagnosis relies on clinical, morphological and immunohistochemical evaluation. However, since none of these criteria are disease-specific, peripheral T-cell lymphoma diagnosis remains challenging, as evidenced by a large multicentric study where misclassification occurred in 10.4% of peripheral T-cell lymphoma cases.[3] Therefore, complementary

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methods such as the assessment of T-cell clonality are generally required. Molecular techniques aiming at identifying monoclonal rearrangements of the gene loci of the T-cell receptor (TCR)γ or ß chains are the gold standard,[4–6] revealing the presence of a T-cell clone in 60–100% of peripheral T-cell lymphomas[7–10] with good specificity, albeit not 100%.[10] Although very useful in routine diagnosis, molecular assays are not quantitative, and do not allow the immunological characterization of the T-cell clone, which is a requisite for improving our knowledge of the malignant T cells involved in peripheral T-cell lymphomas.

In this setting, TCR repertoire analysis by flow cytometry offers significant opportunities, since it allows assessment of T-cell clonality, as well as immunophenotyping of malignant cells and quantification of tumor burden.[11] Current immunophenotyping panels include a total of 25 monoclonal antibodies directed against epitopes of the variable region of the TCR-ß chain (Vß), covering about 70% of the T-cell repertoire according to the manufacturer. This technique has proved useful in detecting malignant T cells in peripheral blood samples from patients with diverse mature T-cell leukemias.[11–16] T-cell clones use the same Vß domain, and are thus detected by an overexpressed Vß family (direct identification) or by a restricted Vß repertoire if they are not recognized by the 25 anti-Vß antibodies of the panel (indirect identification). However, T-cell clone analysis in blood samples is not optimal for peripheral T-cell lymphomas, as (1) there may be no circulating lymphoma cells, (2) malignant circulating cells may not precisely reflect the clone at the site of tumor involvement and (3) when present, clonal subsets are not always malignant, as observed in infectious or autoimmune conditions, as well as in elderly people.[17–20] It should be noted that the applicability of the TCR-Vß repertoire analysis in various biopsy samples has already been reported by several groups including ours.[21] What remains unknown is the capacity of this approach to detect malignant T cells in peripheral organs and its power to differentiate malignant from reactive Vß expansions, which are expected to be frequent at the site of immune reactions. To answer this question, we prospectively examined a total of 30 biopsies involved by peripheral T-cell lymphomas and 94 lymphoid tissues not involved by T-cell lymphomas as control biopsies.

Immunophenotyping is part of the routine diagnosis of lymphomas as recommended by the 2008 WHO classification,[22] and is commonly performed using immunohistochemical techniques. Pan-T-cell-associated antigens such as CD2, CD3, CD7 and CD5 are important in confirming the T-cell origin of a suspect population. Demonstration of the loss of one or more of these antigens can also be used as a surrogate marker for T-cell clonality, as opposed to the complete phenotype of normal T-lymphocytes. It is worth noting that it is neither constant nor completely tumor-specific.[23–26] As their normal counterparts, T-cell clones belong to one of the CD4+, CD8+ and more rarely CD4−CD8− or CD4+CD8+ subsets. However, this may be difficult to determine, especially in the case of minor tumor involvement. As flow cytometry has proven to be efficient in peripheral T-cell lymphoma immunophenotyping, we aimed at determining the relevance of TCR-Vß repertoire analysis for the characterization of the T-cell clone.

Materials and methods

Patients and Biopsy Samples

We conducted a prospective study from November 2000 to August 2010 at Grenoble University Hospital. Patients provided informed consent, according to the protocol approved by the Institutional Review Board. Surgical biopsies were obtained from patients with suspicion of lymphoma. A single biopsy was used for morphological, flow cytometry, cytogenetic and molecular studies, according to the standard institutional protocol.[21] A total of 124 biopsies fulfilling the following criteria were included: (i) at least 5 million cells recoverable for analysis, (ii) more than 80% viable cells, (iii) expression of the TCRαβ/CD3 complex on T-cell membrane and (iv) more than 25% CD3+ cells in the whole population. The biopsy sites were lymph nodes ($n=118$), tonsil ($n=2$), orbit ($n=1$), spleen ($n=2$) or skin ($n=1$). Biopsies were collected from 124 patients aged 7–87 years (median 51). The male to female ratio was 1.61:1.

Histopathology

In all, 30 biopsies from 30 patients were diagnosed as peripheral T-cell lymphomas using histology and immunohistochemical evaluation according to the 2008 WHO classification of hematological malignancies.[22] They consisted of 13 peripheral T-cell lymphomas not otherwise specified, 14 angioimmunoblastic T-cell lymphomas, 1 Sezary syndrome, 1 adult T-cell leukemia/lymphoma and 1 T-cell prolymphocytic leukemia. The 94 remaining biopsies were controls. They were defined as lymphoid tissues not infiltrated with peripheral T-cell lymphomas, that is, with non-malignant resident T cells. They comprised 37 reactive hyperplasias, 27 Hodgkin's lymphomas, 23 B-cell lymphomas, 6 adenocarcinomas and 1 Castleman disease. In total, 81 cases had been reported previously.[21]

Immunophenotyping and TCR-Vβ Repertoire Analysis by Flow Cytometry

Biopsies were processed during the first 48 h after surgical excision as described previously.[21] Four-color immunostaining was performed using

standard techniques, and analyses were conducted with a FACScalibur and CellQuest-Pro software (BD Biosciences, San Jose, CA, USA). The panel of monoclonal antibodies routinely used to detect and classify T-lymphoid malignancies was as follows: PE-Cy5-CD3 (UCHT1), FITC-CD4 (13B8.2), PE-CD8 (B9.11) and PE-CD10 (ALB1) from Beckman Coulter (Brea, CA, USA), and PE-CD7 (M-T701), FITC-CD2 (RPA-2.10) and FITC-CD5 (UCHT2) from BD Biosciences. This panel allows appreciation of the proportion of T-cell subsets in the biopsies and detection of immunophenotypic abnormalities, that is, loss of a pan-T-cell antigen (considered aberrant when the proportion of negative T cells exceeded 3% of total lymphocytes for CD3, or 15% for CD2, CD5 or CD7), or CD10 expression (considered abnormal when positive T-cell accounted for more than 5% of total lymphocytes). These criteria were based on our experience in phenotyping control biopsies. Such characteristics were further used to focus the TCR-Vβ repertoire analysis on a particular T-cell subset.

The TCR-Vβ repertoire was analyzed using 25 monoclonal antibodies directed against the variable (V) region of the TCR-ß chain, including 24 Vß families from the IOTest Beta Mark kit (Beckman Coulter) and Vß6.7 (Endogen, Woburn, MA, USA), as reported previously.[21] PE-Cy7-CD4 (SFCI2TD11) and APC-CD8 (B9.11) from Beckman Coulter were combined with Vβ monoclonal antibodies to study the TCR repertoire of the so defined T-cell subsets in a single experiment. For peripheral T-cell lymphomas, other gating monoclonal antibodies were used, according to the particular T-cell clone phenotype: PE-Cy7-CD4 and APC-CD7 (CD7.6B7; CliniScience, Montrouge, France) when a defect in CD7 expression was demonstrated ($n=8$), PE-Cy7-CD4 and APC-CD10 (H10a, BD Biosciences) when CD10 was positive on T cells ($n=1$) and PE-Cy7-CD3 (UCHT1, Beckman Coulter) when the T-cell clone expressed neither CD4 nor CD8 ($n=2$).

On the basis of our previous work assessing reference values for Vß families in CD4+ and CD8+ T-cell populations in lymph nodes,[21] T-cell expansions were defined as follows: (i) Vß percentage over the mean + 3 s.d. reference value within the corresponding T-cell subset (ie, CD4+ or CD8+), or (ii) sum of all 25 Vß percentages below the mean − 3 s.d. reference value in the corresponding T-cell subset (ie, 54% in CD4+ and 41% in CD8+ T cells). In the latter cases, expansions were not recognized directly by the monoclonal antibodies from the panel and were therefore referred to as 'out of panel' Vß expansions. For Vβ expansions assessed within the 'double-negative' CD3+CD4−CD8− T-cell subset, the same reference values as for CD4+ T cells were used. In an effort to standardize the level of Vβ expansion, we calculated a ratio (Vβ ratio) between the observed Vß percentage and the upper reference value (ie, mean + 3 s.d.) of the corresponding family.

Cytogenetic and Molecular Cytogenetic Analyses

Cytogenetic analysis was performed on the same cell suspensions as were used for flow cytometry analysis, following 17 h unstimulated culture and a colchicine block. R-banded metaphase chromosomes were prepared by standard procedures.[27]

Multiplex PCR Amplification and PCR Product Analysis

Genomic DNA was isolated from frozen tissue biopsies using standard procedures.[27] Detection of clonal *TCRγ* gene rearrangements was performed using two techniques: (1) genomic DNA was submitted to a single multiplex polymerase chain reaction (PCR) using GC-clamp primers and analyzed by denaturing gradient gel electrophoresis, as described by Theodorou *et al*.[4] (2) Samples were submitted to *TCRγ* gene rearrangement amplification followed by Genescan analysis as described previously.[28]

Results

In all, 30 patients with peripheral T-cell lymphomas were prospectively included in this study. The details of clinical, histological, immunological, cytogenetic and molecular analyses are summarized in Table 1.

Vß Expansions are Observed in Both Peripheral T-Cell Lymphomas and Control Biopsies

Vβ expansions were assessed within the CD4+ and CD8+ T-cell subsets, except for double-negative peripheral T-cell lymphoma cases for which they were evaluated within CD3+ cells. An expansion of at least one Vβ family was directly recognized by one of the 25 monoclonal antibodies from the panel in 29/30 peripheral T-cell lymphomas (97%) and 51/94 control cases (54%). Additional Vβ expansions were indirectly identified in peripheral T-cell lymphoma cases only (5/30, 17%) (Supplementary Data 1). Of note, Vß expansion within CD4+ T cells was unique in 20/27 peripheral T-cell lymphomas (74%) *vs* 18/94 control cases (19%). In contrast, 8/21 peripheral T-cell lymphomas (38%) and 23/94 control cases (24%) displayed this characteristic within CD8+ T cells. The expansion was unique in all three double-negative cases. When multiple in biopsies, Vβ expansions ranged from 2 to 6 within the CD4+ subset, and from 2 to 5 within CD8+ T cells.

Because of their high frequency in control biopsies, the presence of a Vβ expansion, whether single or multiple, was thus not sufficient to distinguish T-cell lymphomas from non T-cell lymphoma lesions.

Table 1 Multidisciplinary characteristics of patients with T-cell lymphomas and control patients demonstrating a single high Vß expansion

Patient	*Gender*	*Age (years)*	*Histology*	*Immunophenotypic abnormalities by FC*	TCRγ *rearrangement PCR DGGE (number of bands)*	*Karyotype*	*Staging at presentation*	*Involved sites at presentation*	*B symptoms*	*Autoimmunity skin rash*	*Follow-up (months)*	*Outcome*
1	M	54	PTCL-nos	None	C	A	IV	PolyADN+SP+BM	Yes	No	10	Died of disease
2	F	38	PTCL-nos	In CD4+:CD7−	C	A	IV	PolyADN+SP+BM	Yes	No	108	Relapse
3	M	65	AITL	None	ND	ND	IV	PolyADN+SP+BM	Yes	AIHA	3	Died of disease
4	M	74	AITL	In CD4+:CD7−10+	C	N	III	PolyADN	No	No	11	Complete remission
5	M	56	ATLL	In CD4+:CD7−	ND	ND	IV	PolyADN+SP+skin+bone	Yes	No	10	Lost to follow-up
6	M	41	AITL	In CD4+:CD7−	C	A	IV	PolyADN+SP	Yes	Skin rash	6	Died of disease
7	F	74	PTCL-nos	In CD4+:CD7−10+	C	N	IV	PolyADN+SP+liver+skin+BM	Yes	Erythrodermy	—	Lost to follow-up
8	F	65	AITL	In CD4+:CD7−10+	C	N	IV	PolyADN+SP	Yes	AIHA	—	Partial remission
9	M	74	PTCL-nos	In CD4+:CD7−	ND	A	III	PolyADN	No	No	7	Lost to follow-up
10	M	63	PLL	None	C	A	IV	PolyADN+SP+skin	Yes	Nno	9	Died of disease
11	M	77	PTCL-nos	In CD4+:CD7−10+	C	ND	III	PolyADN	Yes	AIHA	1	Died of disease
12	F	57	AITL	In CD4+:CD7−10+	C	N	IV	PolyADN+SP+BM	Yes	ND	13	Relapse
13	F	56	Sezary	In CD4+:CD7−	C	A	IV	PolyADN+SP+liver+skin	Yes	Erythrodermy	46	Partial remission
14	M	76	PTCL-nos	None	C	A	IV	PolyADN	ND	ND	nd	Lost to follow-up
15	M	65	PTCL-nos	In CD3+4−8− :CD7−	C	A	III	PolyADN+SP+BM	Yes	No	37	Died of disease
16	M	57	PTCL-nos	In CD3+4−8−:CD7−	C	F	IV	PolyADN+SP	Yes	Skin rash	0	Died of disease
17	F	48	AITL	In CD4+:CD7−10+	ND	ND	IV	PolyADN+SP+liver	Yes	AIHA+skin rash	2	Ongoing evaluation
18	F	47	AITL	None	C	A	IV	PolyADN+SP+liver	Yes	Skin rash	1	Died of disease
19	M	84	AITL	In CD4+:CD7−	C	A	IV	PolyADN+BM	Yes	AIHA	3	Died of disease
20	M	71	AITL	None	ND	A	II	PolyADN	No	No	5	Ongoing evaluation
21	M	57	AITL	In CD4+:CD7−10+	C (2)	A	IV	PolyADN+SP+liver	Yes	ITP	47	Complete remission
22	F	58	AITL	None	C (2)	A	IV	PolyADN	Yes	AIHA	72	Died of AML
23	M	66	AITL	In CD4+:CD7−10+	C	A	IV	PolyADN+BM	Yes	CIC	20	Died of disease
24	F	68	PTCL-nos	In CD4+:CD7−	C	ND	IV	PolyADN+tumoral ascite	Yes	ND	1	Died of disease
25	M	69	PTCL-nos	None	C	A	II	PolyADN	Yes	No	8	Partial remission
26	F	79	PTCL-nos	In CD4+:CD7−	C	A	III	PolyADN+SP+liver+BM	Yes	No	1	Died of disease
27	M	26	PTCL-nos	In CD3+4−8−:CD7−	C	A	IV	PolyADN+BM+liver	Yes	No	160	Complete remission
28	F	79	AITL	None	C	N	IV	PolyADN+SP	Yes	ND	25	Lost to follow-up
29	F	42	AITL	None	P	A	IV	PolyADN+BM	Yes	No	33	Complete remission
30	F	57	PTCL-nos	None	P	N	IV	PolyADN+SP+liver+skin+orbi+BM	Yes	No	6	Died of disease
31	M	64	RH	None	O (3)*	A	—	Regional polyADN	No	Skin rash	58	Complete remission
32	M	68	HL	In CD8+:CD7−	O (3)*	F	I	Unique tumor of orbit	No	No	60	Complete remission

O(3)*: Clonal *TCRγ* rearrangement in gene scanning *Vf1-5y1/2*.
A: abnormal; ADN: adenopathy; AIHA: autoimmune hemolytic anemia; AITL: angioimmunoblastic lymphoma; AML: acute myeloid leukemia; ATLL: adult type T-cell leukemia/lymphoma; BM: bone marrow; C: clonal; CIC: circulating immune complex; F: failure; HL: Hodgkin's lymphoma; ITP: immune thrombocytopenic purpura; N: normal; ND: not done; O: oligoclonal; P: polyclonal; polyADN: poly-adenopathy; PLL: prolymphocytic leukemia; PTCL-nos: peripheral T-cell lymphoma, not otherwise specified; RH: reactive hyperplasia; SP: spleen.

The Extent of Vß Expansions Differentiates Peripheral T-Cell Lymphomas from Other Pathologies

As shown in representative cases of peripheral T-cell lymphomas and controls (Figure 1a), the amplitude of each Vβ family expansion was different among patients. Therefore, we subsequently investigated the diagnostic value of the size of individual Vβ expansions. As shown in a previous study, reference values established in lymphoid tissues were highly variable from one Vβ family to another.[21] Thus, to compare accurately the degree of

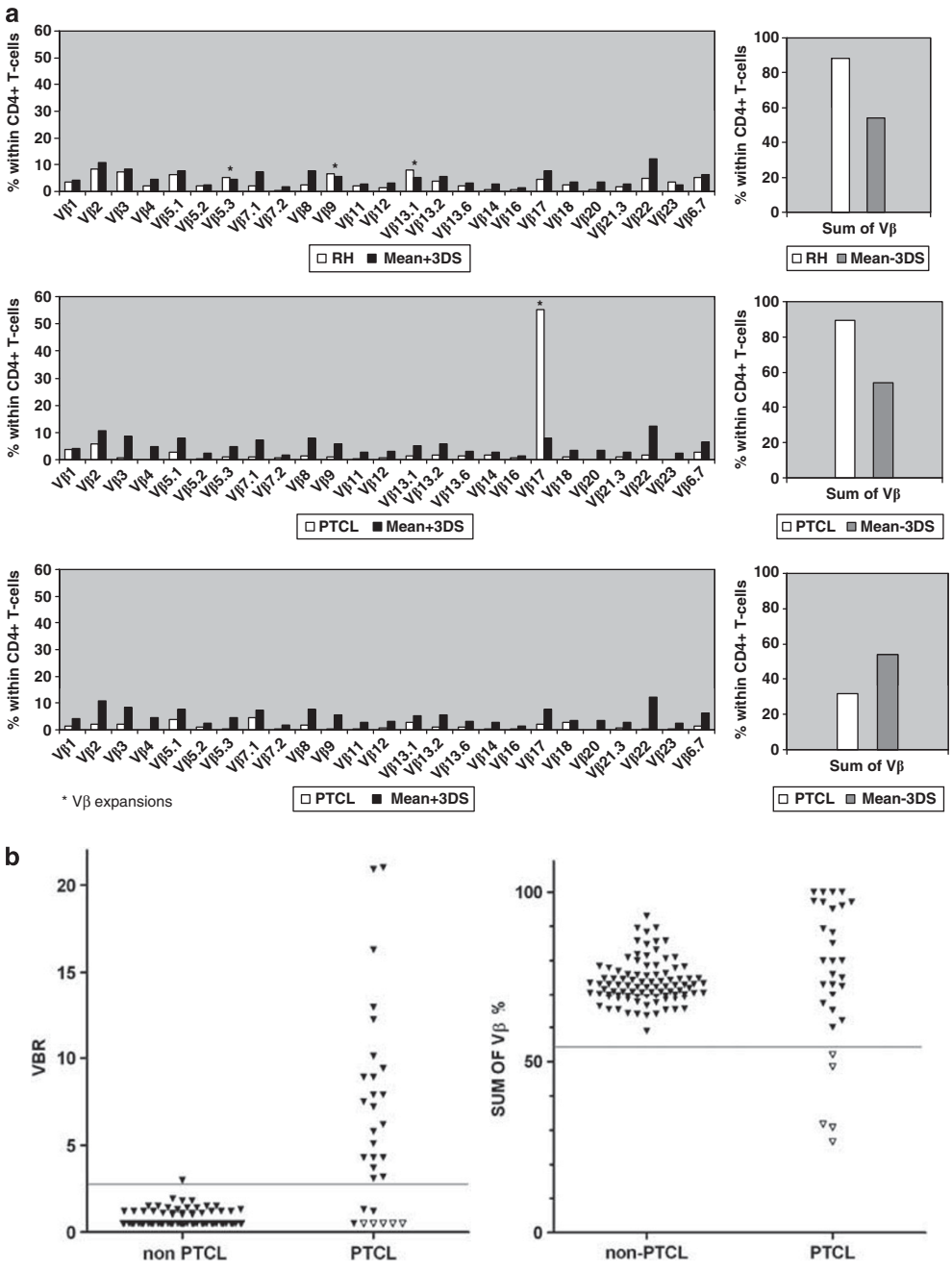

Figure 1 Sensitivity and specificity of the T-cell receptor (TCR)-Vβ repertoire analysis for the diagnosis of peripheral T-cell lymphoma (PTCL) in biopsies. (**a**) Histogram representation of CD4+ TCR-Vβ repertoire in representative cases from control (top) and PTCLs (middle and bottom). A polyclonal pattern with three Vβ low expansions (*) is shown in one case with reactive hyperplasia (RH). Monoclonal patterns with either a high Vβ expansion (middle) or an out of panel expansion (bottom) are illustrated in two cases with PTCL. In the latter case, the sum of Vβ is lower than the reference mean − 3 s.d. value. (**b**) Left: The size of CD4+ or double-negative CD4−CD8− Vβ expansions directly recognized by the monoclonal antibodies of the panel is represented by the Vβ ratio value (ratio between the Vß percentage and the upper reference value of the corresponding family). Using a Vβ ratio cutoff value of 3 (gray line), 22/30 PTCL and 1/94 control patients demonstrated a high Vβ expansion. (**b**) Right: The size of CD4+ or double-negative CD4−CD8− Vβ expansions not directly recognized by the monoclonal antibodies of the panel is defined using the sum of all Vβ percentages. A cutoff value of 54% (mean − 3 s.d. of the reference value, gray line) isolated 5/30 PTCL and 0/94 control patients with an out of panel Vβ expansion, represented with empty triangles.

expansions of individual Vβ families, we used a normalized ratio (Vβ ratio) calculated between the observed Vβ percentage and the upper reference value of the corresponding family.[21]

As shown in Table 2, Vβ ratio ranged from 1 to 21 within CD4+ T cells, and from 1.1 to 6.5 within CD8+ T cells. We graphically considered that a Vβ ratio cutoff value of three best separated patients with either high or low Vβ expansions (Figure 1b). Using this criterion, 23 cases displayed a high Vβ expansion within the CD4+ or the double-negative T-cell subsets. Of these, 22 cases (96%) were peripheral T-cell lymphomas (mean Vβ ratio: 8.7; range: 3.1–21) and one case (4%) was not (mean Vβ ratio: 3). The expansion was unique in all cases (Figure 1b). In the CD8+ subsets, all Vβ expansions presented a Vβ ratio lower than 3, except in three control cases (3/94, 3%). The expansion was unique in only one of them, a case of Hodgkin's lymphoma (patient 32, Vβ ratio: 6.5). For all cases with high Vβ expansions, the proportion of the expanded Vß family in total lymphocytes ranged from 4 to 82.8% (mean: 35.8%).

Thus, a high and unique, 'in' or 'out of panel' Vβ expansion was observed in 27/30 peripheral T-cell lymphomas (90%), exclusively within CD4+ or double-negative T cells. In control biopsies, this occurred in only 2/94 cases (2%), within CD4+ as well as CD8+ T cells.

The three peripheral T-cell lymphoma cases with no or low Vβ expansions (patients 28–30) had been classified as peripheral T-cell lymphomas not otherwise specified (patient 30) or angioimmunoblastic T-cell lymphoma (patients 28 and 29). None demonstrated an aberrant immunophenotype (Table 1). Clonal *TCRγ* rearrangements were detected in patient 28, by both denaturating gradient gel electrophoresis and Genescan analyses, whereas a faint clonal rearrangement in a polyclonal background was identified only by Genescan in patient 29. In this latter case, a pseudotetraploid (91–95 chromosomes) 'chaotic' karyotype was observed (8/21 mitoses) and DNA content assessed by flow cytometry was near tetraploid in 5% of the cells (data not shown). In contrast, patient 30 did not show any clonal aberration either by flow cytometry, cytogenetic or molecular assays (Supplementary Data 2).

Quite strikingly, both control biopsies presenting a high and unique Vβ expansion within one of the CD4+ or CD8+ T-cell subsets were found to display clonal *TCRγ* rearrangements using molecular techniques, thereby confirming the presence of a T-cell clone. Patient 31 was diagnosed with reactive hyperplasia in the context of EBV infection, confirmed by the detection of IgM EBNA antibodies and EBV PCR products. Patient 32 was diagnosed with T-lymphocyte-rich Hodgkin's lymphoma of the orbit. Loss of CD7 expression was observed on CD8+ T cells. It is worth noting that karyotypic abnormalities were detected in both cases, albeit of nonspecific nature (Supplementary Data 2).

In summary, using a Vβ ratio threshold value of 3, TCR-Vß repertoire analysis showed a sensitivity of 90% and a specificity of 98% for the diagnosis of peripheral T-cell lymphomas, and the T-cell clone could be identified as a specific Vß expansion in 73% (22/30) of cases (Figure 1).

T-cell Clones can be Heterogeneous with Respect to T-Cell Antigens or CD10 Expression

As shown in Table 1, 19/30 peripheral T-cell lymphoma cases (63%) exhibited aberrant cell surface molecule expression patterns by flow cytometry. CD7 expression was absent in 19/30 (63%) of cases and CD10 was present in 8/30 (27%). In addition, three TCRαβ-expressing cases demonstrated neither CD4 nor CD8 positivity.

Of note, Vß expansions were not only detected, directly or indirectly, in all peripheral T-cell lymphoma cases with phenotypic aberrations, but also in eight cases with normal expression of lymphocyte markers.

Interestingly, in 11 lymph node biopsies (Table 3), we could focus TCR-Vß repertoire analysis on particular T-cell subsets using specific gating antibodies: (i) CD4 and CD7 for eight cases in which the CD4+ lymphoma cells demonstrated a defect in CD7 expression; (ii) CD3 and CD7 for two CD4 and CD8 double-negative cases also presenting a CD7− phenotype; or (iii) CD4 and CD10 for one case positive for CD10. As expected, the expanded Vß family was selectively increased in the T-cell population presenting an aberrant phenotype compared with the whole CD4+ or CD3+ subsets. In four cases, the expanded Vß family was also overrepresented in T lymphocytes expressing a 'normal' phenotype, whereas in seven cases, its expression was within normal ranges (Figure 2).

A Large Set of Vß Families are Expressed in Peripheral T-Cell Lymphomas

The T-cell clone was identified by one of the 25 monoclonal antibodies from the panel in 22/30 cases of peripheral T-cell lymphomas (73%) (Table 2). A total of 14 different Vβ families were involved, most of them in one case or two cases each. Only Vß3 and Vb17 were more frequently expressed, that is, in three and five cases, respectively. In the remaining five peripheral T-cell lymphoma cases (17%), the T-cell clone could not be recognized by the monoclonal antibodies of the panel (Figure 3).

Discussion

In this prospective study on a large cohort of 124 patients (30 peripheral T-cell lymphomas, 94 control cases), we demonstrated for the first time that

Table 2 Characteristics of T-cell subsets and Vβ expansions in biopsies from peripheral T-cell lymphomas and from discordant control patients

Patient	*Histology*	*CD3 (%)*	*CD4 (%)*	*CD8 (%)*	*Main Vß expanded*	*T-cell subset with the main Vß expansion*	*Size of Vß expansion within T-cell subsets (CD4+CD8+CD3+CD4−CD8−)*			*% of Vß expansion in total lymphocytes*	*Other Vß expansions in the T-CD4+ subset (VßR)*	*Other Vß expansions in the T-CD8+ subset (VßR)*
							Vß (%)	*Reference value (%)*[a]	*VBR*[b]			
1	PTCL-nos	92	81	11	Vß9	CD4	70.6	<7.93	8.9	61.4	—	Vß7.2 (1.4)
2	PTCL-nos	90	75	11	Vß13.1	CD4	31.0	<6.87	5.1	26.1	—	Vß11 (1.2) Vß16 (1.5)
3	AITL	60	30	27	Vß4	CD4	43.2	<4.27	10.1	24.6	—	—
4	AITL	93	81	14	Vß17	CD4	67.7	<9.42	7.2	54.8	—	ND
5	ATLL	78	59		Vß13.2	CD4	78.0	<6.06	12.9	46.0	—	ND
6	PTCL-nos	83	70	11	Vß7.2	CD4	58.0	<2.76	21.0	40.6	—	Vß5.2 (1.7)
7	PTCL-nos	90	73	12	Vß8	CD4	77.2	<8.23	9.4	56.4	—	ND
8	AITL	70	58	17	Vß17	CD4	40.9	<9.42	4.3	23.7	—	Vß23 (1.2)
9	PTCL-nos	75	64	7	Vß5.1	CD4	37.9	<11.9	3.1	24.3	—	Vß1 (1.2)
10	PLL	91	90	1	Vß20	CD4	88.1	<5.4	16.3	79.3	—	Vß1 (1.9) Vß20(1.8)
11	PTCL-nos	68	47	18	Vß5.1	CD4	73.5	<11.9	6.2	34.5	—	—
12	AITL	79	53	23	Vß7.1	CD4	57.3	<4.7	12.2	30.4	—	ND
13	Sezary	98	92	2	Vß12	CD4	90.0	<4.3	20.9	82.8	—	ND
14	PTCL-nos	83	74	8	Vß2	CD4	75.0	<17.55	4.3	55.5	—	Vß4 (1.3) Vß18 (1.5)
15	PTCL-nos	70	16	5	Vß3	CD3+CD4−CD8−	49.8	<11.47	4.3	24.9	ND	ND
16	PTCL-nos	59	12	19	Vß3	CD3+CD4−CD8−	37.2	<11.47	3.2	21.9	ND	ND
17	AITL	72	47	21	Vß17	CD4	71.0	<9.42	7.5	10.0	—	—
18	AITL	67	50	29	Vß13.6	CD4	37.0	<4,67	7.9	19.0	—	Vß2 (1.2)
19	AITL	59	47	6	Vß3	CD4	30.0	<11.47	7.9	27.0	—	Vß2 (1.2)
20	AITL	34	17	13	Vß13.1	CD4	25.3	<6.87	3.7	4.0	—	Vß4 (1)
21	AITL	55	44	14	Vß17	CD4	55.0	<9.42	5.8	24.2	—	Vß12 (1.8) Vß16 (1.3) Vß6.7 (1.2)
22	AITL	85	75	3	Vß17	CD4	83.7	<9.42	8.9	63.0	—	Vß8 (1) Vß12 (2.3)
23	AITL	65	39	20	OPVß	CD4	ΣVb CD4 = 31.8	>54.18	—	—	—	Vß5.1 (1.1) Vß18(1.2)
24	PTCL-nos	70	61	11	OPVß	CD4	ΣVb CD4 = 48.5	>54.18	—	—	—	ND
25	PTCL-nos	89	82	7	OPVß	CD4	ΣVbCD4 = 26.6	>54.18	—	—	—	Vß7.2(1.1)
26	PTCL-nos	65	49	20	OPVß	CD4	ΣVb CD4 = 30.8	>54.18	—	—	—	ND
27	PTCL-nos	90	23	33	OPVß	CD3+CD4−CD8−	ΣVb CD3 = 52	>54.18	—	—	ND	ND
28	AITL	91	68	18	None	—	—	—	—	—	Vß7.1 (1.2) Vß6.7 (1.2)	Vß7.1 (1.6) Vß6.7 (2.6)
29	AITL	85	76	6	None	—	—	—	—	—	—	—
30	PTCL-nos	89	34	56	None	—	—	—	—	—	Vß7.1 (1) Vß9 (1.3)	Vß7.1 (1.2) Vß9 (2.5)
31	RH	40	31	14	Vß17	CD4	28.0	<9.42	3.0	8.7	—	Vß2 (1.3) Vß4 (1.3) Vß5.3 (2.2) Vß13.2 (3.6) Vß20 (1.5)
32	HL	95	64	32	Vß13.1	CD8	48.9	<7.57	6.5	15.7	—	—

[a]Reference value: mean + 3 s.d. for each Vβ family;

[b]ND: not done; OPVβ: out of panel Vβ expansion; ΣVb: sum of all Vβ; VBR: Vß ratio.

Table 3 Immunophenotypical heterogeneity of the T-cell clone in peripheral T-cell lymphomas (11 cases)

Patient	*Vβ expanded*	*Vβ reference values (%)*	*Gate: % Vβ expansion*
2	Vβ13.1 in CD4	<6.87	CD4+: 35 CD4+CD7+: 29 CD4+CD7−: 47
4	Vβ17 in CD4	<9.42	CD4+: 68 CD4+CD7+: 61 CD4+CD7−: 88
6	Vβ7.2 in CD4	<2.76	CD4+: 58 CD4+CD7+: 5.8 CD4+CD7−: 89
9	Vβ5.1 in CD4	<11.9	CD4+: 38 CD4+CD7+: 6.3 CD4+CD7−: 81
12	Vβ7.1 in CD4	<4.7	CD4+: 57 CD4+CD10−: 32 CD4+CD10+: 97
16	Vβ3 in CD3+CD4−CD8−	<11.47	CD3+: 37 CD3+CD7+: 3.8 CD3+CD7−: 85
17	Vβ17 in CD4	<9.42	CD4+: 15 CD4+CD7+: 5 CD4+CD7−: 66
19	Vβ3 in CD4	<11.47	CD4+: 30 CD4+CD7+: 8 CD4+CD7−: 90
18	OP Vβ in CD4	>54.18	CD4+: 49 CD4+CD7+: 65 CD4+CD7−: 26
20	OP Vβ in CD4	>54.18	CD4+: 31 CD4+CD7+: 86 CD4+CD7−: 13
27	OP Vβ in CD3+CD4−CD8−	>54.18	CD3+: 52 CD3+CD7+: 66 CD3+CD7−: 19

OP: out of panel.

TCR-Vβ repertoire analysis by flow cytometry represents a sensitive and specific tool to detect, identify and quantify malignant T-cell clones in lymphoid tissue biopsies.

We chose to investigate biopsies rather than blood samples since peripheral T-cell lymphoma cases exist with no leukemic involvement. Furthermore, analysis in biopsies is more adapted to unambiguous identification of the malignant T-cell clone since, as mentioned previously, clonal circulating T cells are frequently observed in non-malignant conditions, leading to potential confusion between reactive and tumor cells.[17]

On the basis of the promising results obtained from blood samples,[11–16] we used flow cytometry to assess the expression of 25 Vβ chain members of the TCR. Indeed, T-cell clones use a single Vβ domain and can thus be identified by the abnormal expansion of one Vβ family or by a restricted TCR-Vβ repertoire (see Materials and methods). Using our previously reported criteria for defining Vβ expansions,[21] clonal subsets were thus found in 97% of peripheral T-cell lymphomas and 54% of control cases, and thus suggesting that this parameter is not suitable for distinguishing malignant and reactive processes,[29–31] despite significant statistical differences. In contrast, we demonstrated that assessment of the magnitude of Vβ expansion by the calculation of a Vβ ratio is highly discriminative.[21] Using a threshold value of 3, the presence of a high Vβ expansion was found to be closely associated with a

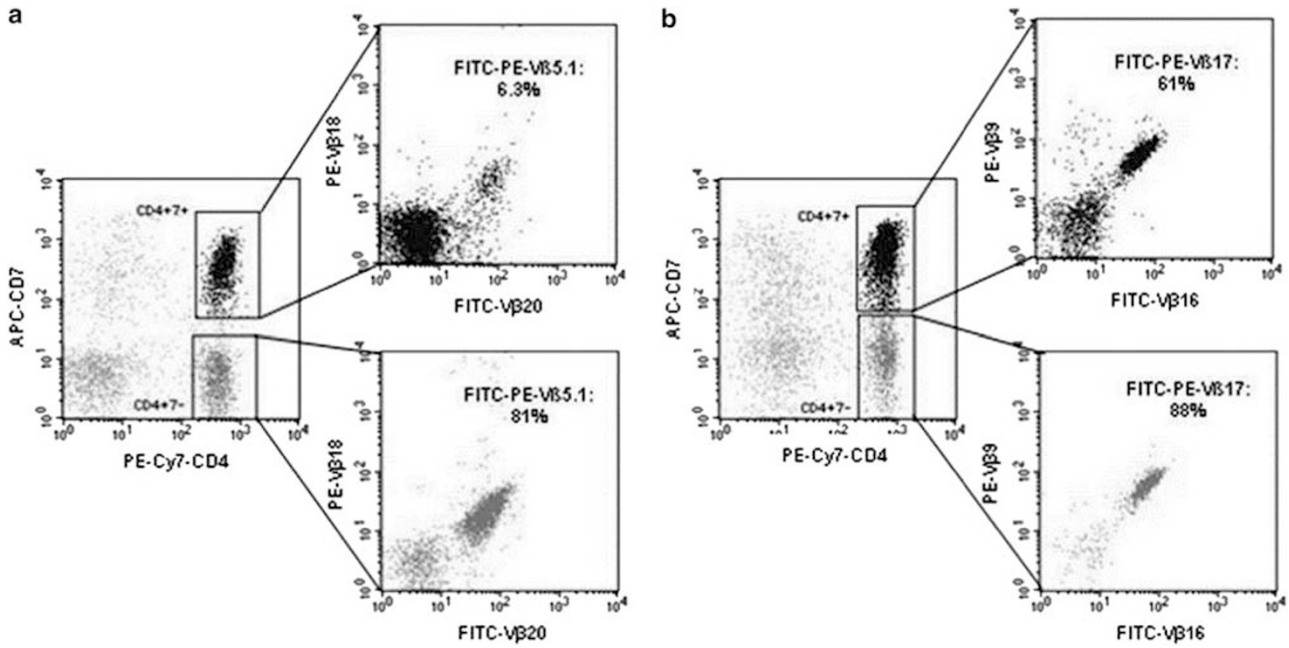

Figure 2 Simultaneous analysis of aberrant T-cell immunophenotype and Vß expansion. Lymphocytes from patient 9 (**a**) and 4 (**b**) lymph nodes were stained with the anti-Vß combination of the IOTest Beta Mark kit containing the previously identified expanded family (FITC-PE-Vß5.1 for patient 9 and FITC-PE-Vß17 for patient 4), in combination with PECy7-CD4 and APC-CD7 antibodies. CD4 + CD7 + and CD4 + CD7− T cells were gated separately (**a** and **b**, left), and the frequency of the expanded Vβ family was reported within each subset (**a** and **b**, right). In patient 9, the expanded Vβ family was mainly CD7−, whereas for patient 4, it consisted of both CD7− and CD7 + cells.

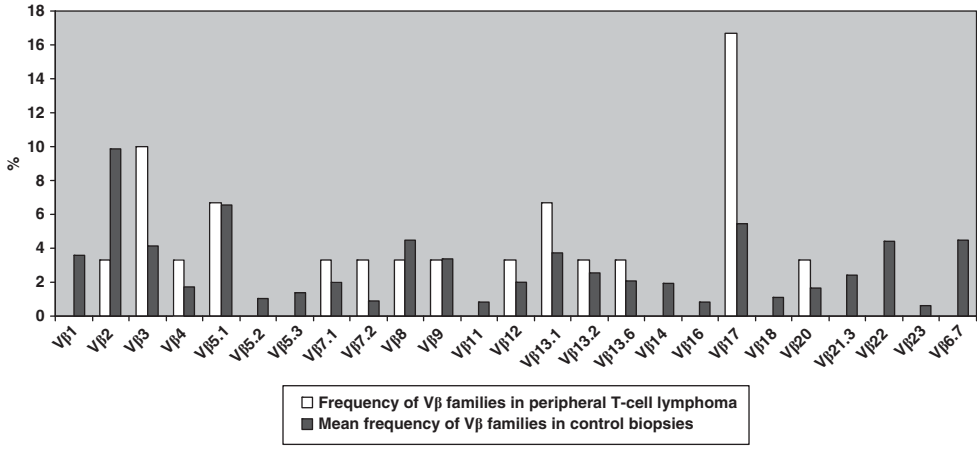

Figure 3 T-cell receptor (TCR)-Vβ families involved in peripheral T-cell lymphomas. Vβ17 and Vβ3 are the most frequently expanded families in this series of 30 peripheral T-cell lymphomas.

diagnosis of peripheral T-cell lymphoma, with a remarkable specificity (98%) and sensitivity (90%).

By this method, false-positive results were observed in only 2 out of 94 control biopsies that demonstrated a high and unique Vβ expansion within CD4+ (patient 31) or CD8+ (patient 32) T-cell populations. These two false-positive cases most likely reflect true clonal expansions as monoclonal *TCRγ* gene rearrangements and chromosomal aberrations were also detected. Of note, such characteristics do not necessarily imply the presence of a malignant disorder, as chromosomal abnormalities have also been demonstrated in a wide variety of benign tumors as well as in reactive processes. (Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer.) This raises the central question of the relationship between clonality and malignancy. Detection of non-malignant clonal T-cell populations has been widely reported in the literature in various settings, particularly within CD8+ T cells.[17–20] The high specificity of the present approach with respect to peripheral T-cell lymphoma diagnosis might result from its application to biopsy samples rather than to peripheral blood, and from its ability to precisely measure the size of the clone,[11] which appears as an advantage over semiquantitative molecular methods. In the B-cell lineage, quantification of the clone is also a relevant parameter to distinguish some B-cell-derived disorders as myeloma or chronic lymphoid leukemia from non-malignant expansions, respectively, monoclonal gammopathy of undetermined significance and monoclonal B lymphocytosis.[22]

False-negative cases with no Vβ expansion despite the histological diagnosis of peripheral T-cell lymphoma were rare in our series (3/30). Two cases (patients 28 and 29) undoubtedly reflected technical limitations in the flow cytometry analysis as T-cell clones were detected by other techniques. In the third case (patient 30), clonality was not demonstrated in several biopsies whatever the technique used (*TCR* gene rearrangement, flow cytometry and karyotype). Whether this indicated the presence of a very minor clone or represented a false positive of histology could not be definitively judged by this analysis alone. This is in line with molecular reports of peripheral T-cell lymphoma cases without the identification of a T-cell clone, despite the use of sensitive methods.[6] Because of the criteria employed with regard to the magnitude of Vβ expansions, TCR-Vβ repertoire analysis by flow cytometry might only be able to detect large malignant populations, depending on the expanded Vβ family (three times the upper reference value of the Vβ family).[21] Nevertheless, our results demonstrated that this limitation only marginally impacts on peripheral T-cell lymphoma diagnosis, as only 3/30 peripheral T-cell lymphoma cases were not detected in our series. The sensitivity of TCR-Vβ repertoire analysis appears similar to what can be expected from molecular assays according to published studies,[6,32] although this comparison implies data derived from different cohorts of patients.

Another approach for T-cell clonality relies on the detection of an aberrant immunophenotype, that is, absence of expression of a T-cell marker—CD2, CD3, CD5, CD7—or positivity for CD10 (75% of peripheral T-cell lymphomas in our institution (data not shown), in accordance with the literature).[23] In the present series of 30 peripheral T-cell lymphomas, CD3− clones have been excluded because of the associated absence of TCR. They account for 35% of all peripheral T-cell lymphomas using flow cytometry analysis. Interestingly, we showed that high Vβ expansions were always associated with phenotypic abnormalities in biopsies, arguing that this criterion is very specific for the diagnosis of peripheral T-cell lymphomas. Furthermore, high Vβ expansions were also observed in eight biopsies without detectable membrane marker expression loss or CD10 expression, indicating that the TCR-Vβ analysis increased the sensitivity of immunophenotypic diagnosis of peripheral T-cell lymphomas with expression of the

CD3/TCRαβ complex at the membrane. Hence, it is postulated that TCR-Vβ repertoire analysis by flow cytometry could be regarded as a promising approach in the peripheral T-cell lymphoma diagnosis strategy.

Importantly, the demonstration that malignant T-cell clones could be unambiguously identified by one of the 25 anti-Vβ monoclonal antibodies in 73% of peripheral T-cell lymphomas provides a powerful marker for more precise characterization of the tumour clone without confusion with reactive T cells. Such an approach proved to be very informative to phenotype T-cell clones using immunohistochemistry after Vβ identification by spectratyping.[32] Interestingly, our strategy appears easier to perform and allows the simultaneous analysis of a larger set of markers. In our series, we thus determined that the T-cell clone belonged to the CD4+ subset or was double negative, but never CD8+. We also established that immunophenotypic aberrations may only partially involve the clonal population, as observed in 4 out of 11 cases showing a CD4+CD7− or CD4−CD10+ phenotype. Such a result is not in favor of the hypothesis that peripheral T-cell lymphomas would arise from the expansion of a CD7− normal counterpart that underwent oncogenic transformation. It may rather reflect 'phenotypic plasticity'. Progressive downregulation of membrane protein expression has been described in Sezary's syndrome,[33,34] and may play a role in the progression of T-cell lymphoma.[35] Intraclonal heterogeneity is indeed a well-known phenomenon illustrated on karyotypic studies demonstrating variable additional genetic abnormalities within a single clone. (Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer.) These results outline that TCR-Vβ repertoire analysis is a more precise approach to identify and characterize a T-cell clone than abnormal expression of T-cell markers. Such an observation is of great importance when aiming at differentiating malignant T cells from normal resident subsets.

Moreover, characterization of a malignant clone by its Vβ specificity is also a promising approach for disease monitoring. Indeed, a single tube including the appropriate Vβ can be used to assess tumor burden and treatment response in diverse sites such as blood, bone marrow or needle aspirates.[36] Aberrant expression of T-cell markers by malignant T cells further allows for improved gating for Vβ clonality analysis, leading to increase detection sensitivity and specificity.[12,37]

In this series of 30 peripheral T-cell lymphomas, a wide variety of Vβ families was involved (14 out of 25 specific and 5 out of panel families). They were observed at low frequencies, except Vβ17 that appeared to be expanded in 5/30 cases (Figure 3). Of note, all five Vβ17 peripheral T-cell lymphomas were classified as angioimmunoblastic T-cell lymphoma. Moreover, the EBV-infected case (patient 31) also demonstrated a high Vβ17 expansion. Considering the known association between EBV and angioimmunoblastic T-cell lymphoma,[37] its role as a common antigen or superantigen in the pathogenesis of this peripheral T-cell lymphoma subtype could be hypothesized. In addition, preferential use of some Vβ families has been reported in leukemic phase of cutaneous T-cell lymphoma[16] and T-CD4+ large granular lymphocytic proliferations in which the clone specificity for hCMV peptides has been demonstrated.[11,39] By contrast, Lima *et al*[11] reported that CD8+ T-cell large granular lymphocytic proliferations in blood samples showed a pattern of Vβ distribution that mimics the frequency at which individual Vβ families are represented in normal CD8+ T cells, suggesting that CD8+ T-cell large granular lymphocytic leukemia cells were clonally transformed in a random manner. Overall, there is some evidence to encourage further evaluation of the TCR-Vβ profile in a larger number of peripheral T-cell lymphoma cases.

In summary, TCR-Vβ repertoire analysis in biopsy samples proved to be a highly specific and sensitive technique for peripheral T-cell lymphoma diagnosis when combined with conventional approaches. Once the Vβ family involved has been identified, it may also prove a promising tool for the assessment of bone marrow involvement, and for follow-up evaluation in blood or needle aspirates. Most interestingly, the precise identification of the T-cell clone allows accurate evaluation of its size and immunophenotype.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

References

1 Jaffe ES. Pathobiology of peripheral T-cell lymphomas. Hematol Am Soc Hematol Educ Program 2006;1: 317–322.
2 de Leval L, Gaulard P. Pathobiology and molecular profiling of peripheral T-cell lymphomas. Hematol Am Soc Hematol Educ Program 2008;1:272–279.

3 Vose J, Armitage J, Weisenburger D. International peripheral T-cell and natural killer/T-cell lymphoma study: pathology findings and clinical outcomes. J Clin Oncol 2008;26:4124–4130.
4 Theodorou I, Delfau-Larue MH, Bigorgne C, *et al.* Cutaneous T-cell infiltrates: analysis of T-cell receptor gamma gene rearrangement by polymerase chain reaction and denaturing gradient gel electrophoresis. Blood 1995;86:305–310.
5 van Dongen JJ, Langerak AW, Bruggemann M, *et al.* Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003;17: 2257–2317.
6 Bruggemann M, White H, Gaulard P, *et al.* Powerful strategy for polymerase chain reaction-based clonality assessment in T-cell malignancies. Report of the BIOMED-2 Concerted Action BHM4 CT98-3936. Leukemia 2007;21:215–221.
7 Theodorou I, Bigorgne C, Delfau MH, *et al.* VJ rearrangements of the TCR gamma locus in peripheral T-cell lymphomas: analysis by polymerase chain reaction and denaturing gradient gel electrophoresis. J Pathol 1996;178:303–310.
8 Droese J, Langerak AW, Groenen PJ, *et al.* Validation of BIOMED-2 multiplex PCR tubes for detection of TCRB gene rearrangements in T-cell malignancies. Leukemia 2004;18:1531–1538.
9 Attygalle AD, Chuang SS, Diss TC, *et al.* Distinguishing angioimmunoblastic T-cell lymphoma from peripheral T-cell lymphoma, unspecified, using morphology, immunophenotype and molecular genetics. Histopathology 2007;50:498–508.
10 Cairns SM, Taylor JM, Gould PR, *et al.* Comparative evaluation of PCR-based methods for the assessment of T cell clonality in the diagnosis of T cell lymphoma. Pathology 2002;34:320–325.
11 Lima M, Almeida J, Santos AH, *et al.* Immunophenotypic analysis of the TCR-Vbeta repertoire in 98 persistent expansions of CD3(+)/TCR-alphabeta(+) large granular lymphocytes: utility in assessing clonality and insights into the pathogenesis of the disease. Am J Pathol 2001;159:1861–1868.
12 Feng B, Jorgensen JL, Jones D, *et al.* Flow cytometric detection of peripheral blood involvement by mycosis fungoides and Sezary syndrome using T-cell receptor Vbeta chain antibodies and its application in blood staging. Mod Pathol 2010;23:284–295.
13 Beck RC, Stahl S, O'Keefe CL, *et al.* Detection of mature T-cell leukemias by flow cytometry using anti-T-cell receptor V beta antibodies. Am J Clin Pathol 2003;120:785–794.
14 Morice WG, Kimlinger T, Katzmann JA, *et al.* Flow cytometric assessment of TCR-Vbeta expression in the evaluation of peripheral blood involvement by T-cell lymphoproliferative disorders: a comparison with conventional T-cell immunophenotyping and molecular genetic techniques. Am J Clin Pathol 2004;121: 373–383.
15 Langerak AW, van Den Beemd R, Wolvers-Tettero IL, *et al.* Molecular and flow cytometric analysis of the Vbeta repertoire for clonality assessment in mature TCRalphabeta T-cell proliferations. Blood 2001;98:165–173.
16 Vonderheid EC, Boselli CM, Conroy M, *et al.* Evidence for restricted Vbeta usage in the leukemic phase of cutaneous T cell lymphoma. J Invest Dermatol 2005; 124:651–661.
17 Delfau-Larue MH, Laroche L, Wechsler J, *et al.* Diagnostic value of dominant T-cell clones in peripheral blood in 363 patients presenting consecutively with a clinical suspicion of cutaneous lymphoma. Blood 2000;96:2987–2992.
18 Posnett DN, Sinha R, Kabak S, *et al.* Clonal populations of T cells in normal elderly humans: the T cell equivalent to 'benign monoclonal gammapathy'. J Exp Med 1994;179:609–618.
19 Lima M, Teixeira Mdos A, Queiros ML, *et al.* Immunophenotype and TCR-Vbeta repertoire of peripheral blood T-cells in acute infectious mononucleosis. Blood Cells Mol Dis 2003;30:1–12.
20 Lin MT, Chang HM, Huang CJ, *et al.* Massive expansion of EBV+ monoclonal T cells with CD5 down regulation in EBV-associated haemophagocytic lymphohistiocytosis. J Clin Pathol 2007;60:101–103.
21 Salameire D, Le Bris Y, Fabre B, *et al.* Efficient characterization of the TCR repertoire in lymph nodes by flow cytometry. Cytometry A 2009;75:743–751.
22 Swerdlow SH, Campo E, Harris NL, *et al.* WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, 4th edn, vol 2. IARC: Lyon, 2008.
23 Jamal S, Picker LJ, Aquino DB, *et al.* Immunophenotypic analysis of peripheral T-cell neoplasms. A multiparameter flow cytometric approach. Am J Clin Pathol 2001;116:512–526.
24 Gorczyca W, Weisberger J, Liu Z, *et al.* An approach to diagnosis of T-cell lymphoproliferative disorders by flow cytometry. Cytometry 2002;50:177–190.
25 Weisberger J, Cornfield D, Gorczyca W, *et al.* Down-regulation of pan-T-cell antigens, particularly CD7, in acute infectious mononucleosis. Am J Clin Pathol 2003;120:49–55.
26 Lee SS, Rudiger T, Odenwald T, *et al.* Angioimmunoblastic T cell lymphoma is derived from mature T-helper cells with varying expression and loss of detectable CD4. Int J Cancer 2003;103:12–20.
27 Lefebvre C, Fabre B, Vettier C, *et al.* Composite splenic marginal zone lymphoma and mantle cell lymphoma arising from 2 independent B-cell clones. Hum Pathol 2007;38:660–667.
28 Delabesse E, Burtin ML, Millien C, *et al.* Rapid, multifluorescent TCRG Vgamma and Jgamma typing: application to T cell acute lymphoblastic leukemia and to the detection of minor clonal populations. Leukemia 2000;14:1143–1152.
29 Langerak AW, Molina TJ, Lavender FL, *et al.* Polymerase chain reaction-based clonality testing in tissue samples with reactive lymphoproliferations: usefulness and pitfalls. A report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2007;21:222–229.
30 Derniame S, Vignaud JM, Faure GC, *et al.* Comparative T-cell oligoclonality in lung, tumor and lymph nodes in human non-small cell lung cancer. Oncol Rep 2005;13:509–515.
31 Ramzaoui S, Jouen-Beades F, Michot F, *et al.* Comparison of activation marker and TCR V beta gene product expression by CD4+ and CD8+ T cells in peripheral blood and lymph nodes from HIV-infected patients. Clin Exp Immunol 1995;99:182–188.
32 Tan BT, Warnke RA, Arber DA. The frequency of B- and T-cell gene rearrangements and Epstein–Barr virus in T-cell lymphomas: a comparison between angioimmunoblastic T-cell lymphoma and peripheral

T-cell lymphoma, unspecified with and without associated B-cell proliferations. J Mol Diagn 2006;8:466–475; quiz 527.
33 Geissinger E, Bonzheim I, Krenacs L, *et al.* Identification of the tumor cells in peripheral T-cell lymphomas by combined polymerase chain reaction-based T-cell receptor beta spectrotyping and immunohistological detection with T-cell receptor beta chain variable region segment-specific antibodies. J Mol Diagn 2005;7:455–464.
34 Dummer R, Nestle FO, Niederer E, *et al.* Genotypic, phenotypic and functional analysis of CD4+CD7+ and CD4+CD7− T lymphocyte subsets in Sezary syndrome. Arch Dermatol Res 1999;291:307–311.
35 Washington LT, Huh YO, Powers LC, *et al.* A stable aberrant immunophenotype characterizes nearly all cases of cutaneous T-cell lymphoma in blood and can be used to monitor response to therapy. BMC Clin Pathol 2002;2:5.
36 Koh HS, Lee C, Lee KS, *et al.* Twist2 regulates CD7 expression and galectin-1-induced apoptosis in mature T-cells. Mol Cells 2009;28:553–558.
37 Tembhare P, Yuan CM, Morris JC, *et al.* Flow cytometric immunophenotypic assessment of T-cell clonality by vbeta repertoire analysis in fine-needle aspirates and cerebrospinal fluid. Am J Clin Pathol 2010;137:220–226.
38 Dunleavy K, Wilson WH, Jaffe ES. Angioimmunoblastic T cell lymphoma: pathobiological insights and clinical implications. Curr Opin Hematol 2007;14:348–353.
39 Rodriguez-Caballero A, Garcia-Montero AC, Barcena P, *et al.* Expanded cells in monoclonal TCR-alphabeta+/CD4+/NKa+/CD8−/+dim T-LGL lymphocytosis recognize hCMV antigens. Blood 2008;112:4609–4616.

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