Identification of the Tumor Cells in Peripheral T-Cell Lymphomas by Combined Polymerase Chain Reaction-Based T-Cell Receptor β Spectrotyping and Immunohistological Detection with T-Cell Receptor β Chain Variable Region Segment-Specific Antibodies

Eva Geissinger,* Irina Bonzheim,*
László Krenács,[†] Sabine Roth,* Philipp Ströbel,*
German Ott,* Peter Reimer,[‡] Martin Wilhelm,[§]
Hans Konrad Müller-Hermelink,* and
Thomas Rüdiger*

From the Institute of Pathology,* University of Wuerzburg, Germany; the Institute of Biotechnology,[†] Bay Zoltan Foundation for Applied Research Laboratory of Tumor Pathology and Molecular Diagnostics, Szeged, Hungary; the Medizinische Poliklinik,[‡] University of Wuerzburg, Germany; and the Medizinische Klinik 5,[§] Klinikum Nuernberg, Germany

Most nodal peripheral T-cell lymphomas (PTCL) originate from $\alpha\beta$ -T cells, and they often contain reactive T cells that may hamper immunophenotyping. To specifically identify the neoplastic population in immunohistochemically stained slides, we assessed the heterogeneity of the T-cell receptor β chain variable region (TCRV β). This region contains 65 gene segments, of which only one is expressed after rearrangement. To investigate PTCL, we developed a polymerase chain reaction assay to define the clonally rearranged TCRV β segment. Detecting the corresponding epitope with segment-specific antibodies enabled identification of tumor cells among the T cells. The TCRV β segment of the tumor cells was defined in 13 of 13 PTCL not otherwise specified and 11 of 13 angioimmunoblastic T-cell lymphomas. Antibodies corresponding to the respective TCRV β segment of the tumor were available for seven cases from each group. After applying these antibodies in combination with antibodies against CD3, CD5, CD4, CD8, and cytotoxic molecules, double stains were evaluated by confocal laser scanning microscopy. In 9 of 14 cases, less than 50% of T cells expressed the clonally rearranged TCRV β segment. Phenotypes defined in double stains differed from those obtained by conventional immunohistochemistry in 11 of 14 cases. The combination of TCRV β polymerase chain

reaction and immunohistochemistry may facilitate more reliable detection and characterization of tumor cells in PTCL. (*J Mol Diagn* 2005, 7:455–464)

Peripheral T-cell lymphomas (PTCLs) are rare neoplasms that constitute approximately 8% of newly diagnosed lymphomas in Western countries.¹ Phenotypes published for recognized disease entities are far from being consistent, probably because PTCL may contain variable numbers of reactive T cells in addition to T-cell-derived neoplastic cells.^{2–6} However, the tumor cells can be distinguished from normal cells by their T-cell receptor (TCR), which is clonally rearranged in the tumor cells and expressed on the cell surface.

TCRs are expressed as heterodimers (α/β and γ/δ) on the surface of the respective T cells. Most PTCL are derived from T cells expressing the α/β TCRs.^{7,8} Antibodies are available against individual variable segments of the TCR β but not of the TCR α chain.⁹ We therefore focused on the TCR β chains. On the DNA level, the TCR β locus is complexly arranged, containing 65 variable (V β), 2 diversity (D β), 13 joining (J β), and 2 constant (C β) segments. Based on a 75% identity at the nucleotide level, the 65 TCRV β segments form 32 subfamilies ranging in size from one to nine members. Among them, 46 segments belonging to 25 subfamilies are functional.^{10,11} Because of this variability, each individual TCRV β segment is expressed in only a small percentage of reactive T cells.^{12,13} An antibody against a TCRV β segment that is clonally rearranged and expressed by a malignant clone can therefore specifically identify the tumor cells in a PTCL containing reactive and neoplastic T cells.

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Address reprint requests to Dr. Eva Geissinger, Institute of Pathology, University of Wuerzburg, Josef-Schneider-Strasse 2, D-97080 Wuerzburg, Germany. E-mail: geissinger@mail.uni-wuerzburg.de.

Table 1. Antibodies Used for Single and Double Stains

Antibody	Clone	Company	Dilution	Conjugation	Detection
V β 2	MPB2D5	BC	1:200		Donkey anti-mouse CY3
V β 5.1	IMMU157	BC	1:400		Donkey anti-mouse CY3
V β 5.2	36213	BC	1:100		Donkey anti-mouse CY3
V β 5.3	3D11	BC	1: 50		Donkey anti-mouse CY3
V β 8.1 and V β 8.2	56C5	BC	1:200		Donkey anti-mouse CY3
V β 9	FIN9	BC	1:100		Donkey anti-mouse CY3
V β 14	CAS1.1.3	BC	1: 50		Donkey anti-mouse CY3
V β 17	E17.5F3	BC	1: 50		Donkey anti-mouse CY3
V β 18	BA62	BC	1:20		Donkey anti-mouse CY3
V β 22	IMMU546	BC	1:20		Donkey anti-mouse CY3
CD 3	Polyclonal	Dako	1: 2		Donkey anti-rabbit CY2
CD 4	MT310	Dako	1: 10	FITC	
CD 4	BL4	BC	1:100		Avidin-biotin POX
CD 5	BL1A	BC	1: 10	FITC	
CD 5	BL1A	BC	1:400		Avidin-biotin POX
CD 8	B9.11	BC	1: 10	FITC	
CD 8	5C2-2H2	Dianova	1:20		Donkey anti-mouse IgM Cy5
CD 8	C8/144B	Dako	1:1600		Avidin-biotin POX
TIA-1	2G9	BC	1:200		Donkey anti-mouse CY3
TIA-1	2G9	BC	1:200		Avidin-biotin POX
GranzymeB	GrB-7	Monosan	1:100		Donkey anti-mouse CY3
GranzymeB	GrB-7	Monosan	1:20		Avidin-biotin POX
Perforin	5B10	Novocastra	1:20		Donkey anti-mouse CY3
Perforin	5B10	Novocastra	1:10		Avidin-biotin POX
CD94	HP-3D9	Dako	1:100		Avidin-biotin POX
PanTCRa β	8A3	Serotec	1:100		Avidin-biotin POX
PanTCR $\gamma\delta$	5A6.E9	BC	1:100		Avidin-biotin POX

Detection with fluorescent-labeled antibodies (1:100) (Dianova) or Zenon mouse IgG Labeling kits (Molecular Probes, Leiden, The Netherlands). Beckman Coulter [BC], Marseille, France; Dako Cytomation, Hamburg, Germany; Serotec, Duesseldorf, Germany; Monosan, Uden, The Netherlands; Novocastra, Newcastle upon Tyne, United Kingdom.

We have designed subfamily-specific TCRV β primers covering all 46 functional and most of the nonfunctional segments that, in combination with two different sets of segment-specific TCRJ β primers, allow us to define the rearranged TCRV β subfamily of the tumor clone by polymerase chain reaction (PCR). In a second step, the respective TCRV β -specific antibody can detect the tumor cells in frozen sections and allows investigation of their phenotype in double stains.

Here, we report that the phenotypes (CD3, CD5, CD4, CD8, TIA-1, GranzymeB, and Perforin expression) of the tumor cells defined by this method for seven angioimmunoblastic T-cell lymphomas (AILTs) and seven PTCLs-not otherwise specified (PTCLs-NOS) differed significantly from those defined by conventional immunohistochemistry in 11 of 14 cases. The great variety in published immunophenotypes for these lymphomas may therefore be explained as being due to problems in identifying the tumor cells in immunohistochemically stained sections.

Materials and Methods

Patient Samples

Twenty-six frozen samples from 25 patients with PTCL (13 PTCLs-NOS and 13 AILTs) were analyzed for their TCR β rearrangement. One natural killer (NK) cell lymphoma, two T-cell-rich B-cell lymphomas, peripheral blood from four healthy donors, and two T-cell lines (Jurkat and A3.01) served as controls.

All cases had been diagnosed according to the World Health Organization classification and were characterized by conventional immunohistochemistry with antibodies against CD3, CD5, CD4, CD8, TIA-1, GranzymeB, Perforin and TCR β chain, TCR δ chain, and CD94 (Table 1), thus identifying the tumor cells morphologically.

Polymerase Chain Reaction for the Detection of TCR β Rearrangements

To detect TCR β gene rearrangements, genomic DNA was isolated from frozen tissue material according to standard procedures. Two hundred nanograms of DNA was subjected to 50 PCR reactions, each. The 25 TCRV β primers (0.5 μ mol/L each primer; V β 6a/b and V β 13a/b, respectively, were put together in the same reaction [0.25 μ mol/L each primer]; Table 2) were combined with either the J β 1 mix or the J β 2 mix (0.25 μ mol/L each primer, labeled at their 5' end with 5-carboxyfluorescein) as published previously.¹⁴ All reactions were carried out at a final volume of 25 μ l using 50 μ mol/L each of dNTP and 0.5 units of TaqDNA polymerase (Invitrogen, Life Technologies, Carlsbad, CA) in the buffer recommended by the supplier. An initial step of 95°C for 2 minutes was followed by 35 cycles of 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds and finally by 72°C for 5 minutes. Two microliters of each PCR product was separately analyzed on a sequencing gel by automatic fluorescence quantification and peak height determina-

Table 2. Sequences of the Subfamily-Specific TCRV β Primers Used for TCR β PCR (according to the TCRV β nomenclature by Arden et al)

Subfamily	Primer sequence
V β 1	5'-ACA GCG AGT GAC GCT GAG ATG-3'
V β 2	5'-TGC CGT TCC CTG GAC TTT CAG-3'
V β 3	5'-TAA CCC AGA GCT CGA GAT ATC TA-3'
V β 4	5'-GTG TCA AGT CGA TAG CCA AGT C-3'
V β 5	5'-AGC AAG TGA CTC TGA GMT GCT C-3'
V β 6a*	5'-CAG GTG CTG GAG TCT CCC AG-3'
V β 6b*	5'-GCA GAT ACT GGA GTC TCC CAG-3'
V β 7	5'-CAG ACA CCA ARA CAC CTG GTC A-3'
V β 8	5'-CRA GGT GAC AGA GAT GGG ACA A-3'
V β 9	5'-CCC AGA CTC CAA AAT ACC TGG T-3'
V β 10	5'-AGG TCA CCC AGA GAC CTA GAC-3'
V β 11	5'-TCA CTC TGG AAT GTT CTC AAA CC-3'
V β 12	5'-CCA AGA CAC AAG RTC ACA GAG A-3'
V β 13a [†]	5'-GTC ACT CAG ACC CCA AAA TTC C-3'
V β 13b [†]	5'-TCA CCC AGG CAC CAA CAT CTC-3'
V β 14	5'-TGA CCC AGA ACC CAA GAT ACC T-3'
V β 15	5'-GTT ACC CAG ACC CCA AGG AAT A-3'
V β 16	5'-TAG AAG CTG GAG TTA CTC AGT TC-3'
V β 17	5'-CTC AGT CCC CAA AGT ACC TGT T-3'
V β 18	5'-TGC AGA ACC CAA GAC ACC TGG-3'
V β 19	5'-ACA AAG ATG GAT TGT ACC CCC G-3'
V β 20	5'-AGA TCT CAG ACT ATT CAT CAA TGG-3'
V β 21	5'-TCT CCC AGA TAT AAG ATT AYA GA-3'
V β 22	5'-TCA CAC AGA TGG GAC AGG AAG T-3'
V β 23	5'-TCA AAG AAA AGA GGG AAA CAG CC-3'
V β 24	5'-AAG ATA CCA GGT TAC CCA GTT TG-3'
V β 25	5'-ACA GAA AGC AAA ATT ATA TTG TGC C-3'

M- AC; R- AG; Y- CT.

*TCRV β 6a primer amplifies rearrangements of the TCRV β segments TCRV β 6s1-3 and TCRV β 6s5-7. TCRV β 6b primer amplifies rearrangements of the TCRV β segment TCRV β 6s4.

[†]TCRV β 13a primer amplifies rearrangements of the TCRV β segments TCRV β 13s1-4 and TCRV β 13s6-9. TCRV β 13b primer amplifies rearrangements of the TCRV β segment TCRV β 13s5.

tion, using GeneScan Analysis Software 3.1 according to the recommendations of the supplier (ABI Prism 377XL collection, ABI 377 DNA Sequencer; Applied Biosystems, Foster City, CA).

Definition of Peak Height Ratio

To determine the peak height ratio (Rn; according to Luo et al¹⁵), peaks were measured using GeneScan software. We determined the Rn value as the ratio between the highest peak among all of the 50 PCR reactions (which was regarded as the "clonal peak") and the average of the second and the third highest peak.

Sequence Analysis

To verify the results of our PCR and to obtain both the exact TCRV β segment rearranged in the tumor cell population and the sequence of the CDR3 region, we sequenced amplicates from each tumor. To circumvent subcloning and prevent sequencing errors, DNA was again amplified in separate reactions, by using the respective TCRV β primer with every segment-specific TCRJ β primer of the respective J β 1 or J β 2 mix. Using the same PCR protocol as described above, the most prom-

inent band was identified on an agarose gel and sequenced in both directions.

Immunohistochemistry

Antibodies available against TCRV β subfamilies or segments defined by PCR were purchased. To confirm their sensitivity and specificity, reactive tonsils and most of the cases were stained with all available TCRV β antibodies. Cases for which a TCRV β antibody and sufficient material were available were further investigated by double stains using antibodies against CD3, CD5, CD4, CD8, TIA-1, GranzymeB, and Perforin (Table 1). Antibodies from different species could be incubated simultaneously. If two antibodies from the same species had to be used, one of the antibodies was detected by conventional methods. To prevent cross-reactions, the second primary antibody was applied as fluorescein isothiocyanate (FITC)-labeled antibody. Blocking was achieved with mouse serum (Dianova, Hamburg, Germany; 1:20). Dilutions and sources of all primary and secondary antibodies are given in Table 1.

The staining protocols were as follows:

1. Simultaneous incubation procedure

- Antibody diluent as blocking reagent, 15 minutes
- Mouse anti-TCR β and rabbit anti CD3, 1 hour
- Donkey anti-mouse CY3 and donkey anti-rabbit CY2, 1 hour

2. Step-by-step incubation procedure

- Antibody diluent as blocking reagent, 15 minutes
- Mouse anti-TCR β , 1 hour
- Donkey anti-mouse CY3, 1 hour
- Mouse serum, 1 hour
- CD4/CD5/CD8 FITC conjugated, 1 hour

3. Step-by-step incubation procedure using a labeling kit for the second antibody

- Antibody diluent as blocking reagent, 15 minutes
- Mouse anti-GranzymeB/TIA/Perforin, 1 hour
- Donkey anti-mouse CY3, 1 hour
- Mouse serum, 1 hour
- TCR β -specific antibody labeled with Alexa Fluor 488*
- Zenon mouse IgG labeling kit, 1 hour

After each incubation, the slides were washed for 5 minutes in Tris-buffered saline three times. After staining, slides were mounted with anti-fading medium (Fluor-mount G; Southern Biotechnology Associates, Birmingham, AL) and kept in the dark at 4°C. The images were evaluated on a confocal laser scanning microscope (Leica TCS SP2; Leica, Bensheim, Germany). Immunophenotypes as defined by conventional and double stains were compared.

Results

Primer Design and Testing, Specificity, and Sensitivity of the TCR β PCR

The newly designed TCR β PCR assay was established and optimized by using DNA extracted from T cells from four healthy donors, who had given their informed con-

Figure 1. Graphical display of fluorescent TCR β PCR products. Monoclonal PTCL-NOS (**a**; case 6), oligoclonal AILT (**b**; case 19), and polyclonal control (**c**; peripheral blood from a healthy donor) analyzed with the GeneScan software. For each case, the three highest peaks are given. They define the Rn value (see text). Note the different scales on the y axes. Relative fluorescence intensities (y axis) are plotted as a function of PCR fragment size in nucleotides (x axis) for each PCR product.

sent. As expected, these samples resulted in PCR products of different but generally low intensity in a Gaussian length distribution that reflected a polyclonal rearrangement pattern. This indicates that the amplification was largely unbiased, ie, that all of the V β -J β combinations were amplified with a similar probability. In addition to these reactive controls, the TCR β chain was not clonally rearranged in the two T-cell/histiocyte-rich B-cell lymphomas and in one nasal type NK-cell lymphoma. The latter expressed the NK-cell marker CD94 but not the TCR β chain.

Identification of the Rearranged TCRV β Subfamily by PCR and Determination of the TCRV β Segment and the CDR3 Region by Sequencing of the PCR Products

In 13 of 13 PTCLs-NOS (100%) and 11 of 13 AILTs (85%), the rearranged TCRV β subfamily of the tumor cells could be identified. The two remaining AILT cases showed an oligoclonal rearrangement with several dominant bands. The Rn value was 9.8 on average (median, 7.5; range, 2.1 to 34.3) for the PTCLs-NOS and 4.5 on average (median, 3.85; range, 1.3 to 9.6) for the AILTs. In reactive controls and the T-cell/histiocyte-rich B-cell lymphomas, it was 1.6 on average (median, 1.65; range, 1.6 to 1.8; SD, 0.09). Examples of monoclonal, oligoclonal, and polyclonal results are given in Figure 1. All in all, segments of the TCRV β 5 ($n = 9$), TCRV β 9 ($n = 3$), and TCRV β 8 ($n = 3$) subfamilies were detected most frequently. Among the TCRJ β segments, there was a general preference for TCRJ β 2 family members ($n = 15$) over TCRJ β 1 family

members ($n = 8$), not counting the two oligoclonal cases and the relapse of one PTCL-NOS (case 1).

All sequences could be identified as derived from the TCR β chain by comparison with databank sequences (<http://www.ncbi.nlm.nih.gov> and <http://imgt.cines.fr>). Individual TCRV β and TCRJ β segments were identified within the subfamilies detected by PCR. Furthermore, the CDR3 region of the rearranged TCR β chain was analyzed using the junction analysis of the IMGT/V-QUEST (<http://imgt.cines.fr>). Amino acid sequences of the CDR3 region were not conserved in our cases. We did not detect any somatic hypermutations in the TCR β genes.

A relapse biopsy on one patient with PTCL-NOS (case 1), who had relapsed after 4 years, revealed the identical nucleotide sequence of the rearranged TCR β chain (V β 9s1-J1-5) as the primary tumor. All nucleotide and deduced amino acid sequences of the 12 primary PTCLs-NOS and the 11 monoclonal AILTs were submitted to GenBank (<http://www.ncbi.nlm.nih.gov>) (accession numbers of the PTCLs-NOS: AY972471, AY972473, AY972474, AY972476, AY974074, AY974075, AY974077, AY974079, AY974080, AY974081, AY974084, and AY974087; accession numbers of the AILTs: AY972472, AY973206, AY973207, AY974073, AY972475, AY974076, AY974078, AY974082, AY974083, AY974085, and AY974086.)

Immunostaining with Subfamily-/Segment-Specific Antibodies

Specific antibodies against the TCRV β protein corresponding to the clonally rearranged TCR β sequence

Figure 2. Definition of the tumor cell phenotype in PTCL-NOS (case 3). **a:** H&E; **b:** CD5 stains only small inconspicuous T cells, the large atypical cells are negative. CD4 (**c**) and CD8 (**d**) single stains detect about an equal number of T cells, but the perivascular CD8⁺ cells (**d**) appear to be large and atypical; TCRVβ14 (red) immunoreactivity does not co-localize with either CD4 (green; **e**) or CD8 (green; **f**) in a triple stain (shown as double stains with both CD4 and CD8 in green, for better visualization).

were available for 14 of the 24 clonally rearranged cases with sufficient material for immunohistochemical analysis (7 of 13 PTCLs-NOS and 7 of 11 AILTs). On frozen sections from these tumors, the antibody directed against the respective TCRVβ segment detected variable numbers of atypical cells, whereas antibodies directed against other segments identified only scattered cells, similar to the pattern in reactive tonsils. This was evident in four PTCLs-NOS and one AILT that contained sheets of large atypical tumor cells at least in some areas. In these cases, 70 to 90% of the CD3⁺ T cells were detected by

the respective TCRVβ-specific antibody in the tumor cell-rich areas.

In three PTCLs-NOS and six AILTs, the antibody directed against the clonally rearranged TCRVβ segment detected less than one-half of the CD3⁺ and CD5⁺ T cells. Morphologically, these tumor cells could not be reliably distinguished from reactive T cells in frozen sections and the more elaborate method was necessary to define the tumor cells (Figures 2 and 3).

In one AILT (case 25), the highest peak was detected in the Vβ13-Jβ2 PCR reaction at an Rn value of 1.4.

Figure 3. Definition of the tumor cell phenotype in AILT (case 21). **a:** H&E; **b:** CD3 stains both small inconspicuous and large atypical T cells. CD4 (**c**) shows a weak expression in clear cells, whereas CD8 (**d**) shows a strong expression in few cells including activated T cells. TCRV β 8.1 (red) immunoreactivity co-localizes in varying intensities with CD4 (green, **e**), but not with CD8 (green, **f**).

However, sequencing revealed only artifactual products. Interestingly, sequencing the PCR product of the second highest peak resulted in the identification of an in-frame rearranged TCR β fragment (V β 9s1-J β 1-1). When using the TCRV β 9 antibody, immunohistochemical analysis detected an abundance of CD3⁺ cells that showed a homogenous phenotype in the double stains.

Multiple Immunofluorescent Stains—Detection of Tumor Cell Phenotypes

Tumor cell immunophenotype was defined by double stains using antibodies against CD3, CD5, CD4, CD8,

TIA-1, GranzymeB, and Perforin in combination with the TCRV β subfamily-/segment-specific antibody to identify the tumor cells. Based on these double stains, five of seven PTCLs-NOS expressed CD3 and CD5, respectively. Four tumors were CD4⁺, and none was CD8⁺; three tumors were double negative for both CD4 and CD8. Five AILTs were positive for CD3 and CD5, and all expressed CD4 at varying levels. A cytotoxic phenotype could be confirmed in only one AILT, expressing TIA-1 and GranzymeB but not Perforin. None of the PTCLs-NOS expressed any of the cytotoxic proteins.

In most cases, the immunophenotype determined on double stains differed from the one determined on single

Table 3. Results of TCR β PCR and Immunohistochemical Single and Double Stains in PTCL-NOS and AILT

ID	Dx	Single stains						Double stains					
		CD3	CD5	TIA	grB	Perf	Lineage	CD3	CD5	TIA	grB	Perf	Lineage
1	NOS	+	+	—	—	—	CD4	+	+	—	—	—	CD4
3	NOS	+	—	+	+	—	CD8	—	—	—	—	—	DN
4	NOS	+	+	—	—	—	DN	+	+	—	—	—	CD4
6	NOS	+	+	—	—	—	DN	—	—	—	—	—	DN
7	NOS	+	—	—	—	—	CD4	+	+	—	—	—	CD4
9	NOS	+	—	—	—	—	DN	+	+	—	—	—	DN
12	NOS	—	—	—	—	—	CD4	+	+	—	—	—	CD4
16	AILT	+	+	—	—	—	CD4	+	—	—	—	—	CD4
18	AILT	+	+	—	—	—	CD4	—	+	—	—	—	CD4
20	AILT	—	+	—	+	—	CD4	—	—	—	—	—	CD4
21	AILT	+	—	—	+	—	CD4	+	+	—	—	—	CD4
22	AILT	+	+	+	+	—	CD4	+	+	+	+	+	CD4
25	AILT	+	+	—	—	—	CD4	+	+	—	—	—	CD4
26	AILT	+	+	—	+	—	CD4	+	+	—	—	—	CD4

GrB, GranzymeB; Perf, Perforin.

stains when identifying the tumor cells morphologically (Table 3). The expression of CD3 and CD5 was differently evaluated in four (three PTCLs-NOS and one AILTs) and seven (four PTCLs-NOS and three AILTs) cases, respectively. The differing results for CD4 and CD8 led to a disparate lineage assignment in two of seven PTCLs-NOS: One tumor that had been assessed as double negative (DN) in the single stains could be defined as weakly CD4⁺, probably because of the higher sensitivity of the fluorescent stains. On the other hand, one tumor shown to be DN in the double stains had been considered CD8⁺ when evaluating the single stains. Furthermore, the expression of cytotoxic proteins could not be confirmed in all but one AILT (one PTCL-NOS and three AILTs).

Discussion

The major aim of this study was to establish a method for the specific detection of neoplastic T cells in a mixed infiltrate of T lymphocytes. In previous studies, we found it difficult to reliably distinguish neoplastic from reactive T cells in PTCL,^{5,6} because cytological atypia may be subtle, and clear cells cannot be readily identified in immunostained slides. Because reactive T cells may be abundant in PTCL,^{2–4} they may result in an incorrect definition of the tumor cell phenotype. This might be one reason for the great variation in published phenotypes for AILTs^{16–32} and PTCLs-NOS.^{26,27,33–38}

To define tumor cell lineage (CD4 or CD8 expression), PTCL were double stained for Ki-67 and CD4 or CD8, respectively.^{39–41} and the more proliferating subset was assumed to be neoplastic. Yet proliferation does not define neoplasia, and some authors have found it difficult to co-localize their signals in frozen sections.⁴² Alternatively, CD4⁺ and CD8⁺ subpopulations were compared quantitatively, and the predominant one was regarded as neoplastic.^{26,43} However, the numbers of both subsets in the infiltrate were often similar.⁴⁴ Tumor lineage thus remained undetermined in 36% of 174 PTCLs,³⁶ and in flow cytometry, 6 of 31

PTCLs did not show any subtype predominance.⁴⁵ Consequently, double-negative tumors are difficult to detect using this approach, and they might, for this reason, not receive the attention they warrant.

We therefore developed a method to specifically identify tumor cells in PTCLs. It is based on a combination of a TCR β PCR with the subsequent identification of tumor cells by immunohistochemistry, using antibodies against the TCRV β segments of the tumors. We designed primers that would bind to all functional and most of the nonfunctional TCRV β segments (Table 2) on genomic DNA to detect virtually every possible TCR β gene rearrangement and used them in combination with previously published mixtures of TCRJ β segment-specific primers.¹⁴ Alternatively, TCR β gene rearrangements could also be studied from mRNA by RT-PCR, but the transcription levels of individual TCRV β segments vary depending on their promoters⁴⁶ and/or cellular activation.^{47–49} This may lead to erroneous results when analyzing clonality in whole-tissue specimens.

To achieve a largely unbiased amplification, our TCRV β primers were designed to be almost completely homologous to their target sequences. In addition, all primer pairs required the same annealing temperature and resulted in products of similar size (260–340 bp), a size that also enables the analysis of formalin-fixed paraffin-embedded tissue (data not detailed). The primer-binding sites of the TCRV β primers were chosen fairly upstream in the TCRV β segments to permit determination of the respective segment in a consecutive sequence analysis. Using subfamily-specific instead of consensus primers, we could directly identify the TCRV β subfamily rearranged in the tumor cells.

On the basis of the peak height ratio (Rn) determined by Luo et al,¹⁵ we tried to define a reproducible objective threshold that differentiates between monoclonal and polyclonal populations in the PCR analysis. The higher background infiltrate in AILT was also reflected by the average Rn value, which was less than half of the average Rn value in PTCL-NOS, thus representing a lower number of tumor cells. As in all controls that did not

contain a clonal T-cell population, the Rn value was less than 1.8 (average, 1.65; SD, 0.09). We therefore chose a value greater than 2.0 as evidence of clonality, because, statistically, less than 1% of polyclonal cases can be expected to reach this value. In all cases with an Rn value greater than 2.0, sequencing and immunohistochemistry confirmed the PCR results, but cases with an Rn value lower than 2.0 have to be assessed more carefully, as illustrated by case 25. In this AILT, the PCR product representing the highest peak yielded only unreadable sequences. In all probability, these PCR products represented unrelated rearrangements involving the same TCRV β 13 subfamily. This is one of the largest subfamilies, containing five to seven functional members.⁵⁰ We were able to sequence the PCR product that had the second highest peak (V β 9) and an antibody against TCRV β 9s1 detected cells with a coherent phenotype in double stains.

The other two AILTs were regarded as oligoclonal, but it could not be ruled out with certainty that these cases might also contain a clonal T-cell population. Antibodies against some of the dominant peaks were not available, and double stains with the available TCRV β antibodies did not result in a coherent phenotype.

In clonal cases, after determining the TCRV β segment rearranged in the tumor cells, we applied antibodies directed against the respective TCRV β epitopes to identify neoplastic cells in immunohistochemical analyses. Such subfamily- and/or segment-specific antibodies do not yet cover the whole spectrum of segments,⁹ therefore, only 14 of the 24 PTCL with identifiable TCRV β segments could be further investigated.

Immunostains independently confirmed the TCRV β PCR results: Antibodies directed against the rearranged TCRV β segment as defined by PCR detected more cells than other TCRV β antibodies. Most notably, the vast majority of these cells stained uniformly in the double stains, especially with CD4 and CD8, which suggests their clonal origin. In contrast, double stains with antibodies against TCRV β segments regarded as polyclonal detected cells with different phenotypes. Thus, immunohistochemistry may serve as an independent control of molecular analyses.

Our method, which identifies tumor cells by their clonally rearranged TCRV β segments in double stains, is much more specific than the above-mentioned methods of detecting tumor cells in PTCL. Because each individual TCRV β segment is expressed in a few percentage of reactive T cells,^{12,13} these cells also reacted with the antibody against the TCRV β segment of the tumor clone. They exhibited deviating phenotypes in double stains, suggesting that they were not clonally related to the tumor. In our experience, these minor populations never impeded analysis.

In accordance with our previous results,⁵ all AILTs expressed CD4. Four of the seven PTCLs-NOS (57%) analyzed in double stains were CD4⁺, and the remaining three were CD4⁻CD8⁻. This represents a remarkably low percentage of CD4⁺ tumors, whereas the double-negative group was much more common than previously published.^{26,27,33-38} Tumor cell lineage as defined by double

stains differed from that defined by single stains in two of seven PTCLs-NOS. In the conventional stains, one of the three DN lymphomas had been regarded as CD8⁺. Probably, it may be difficult to recognize a CD4⁻CD8⁻ population in serial single stains when trying to decide between CD4⁺ and CD8⁺.

In the present study, 13 of 56 single stains (23%) for CD3, CD4, CD5, and CD8 in 7 PTCLs-NOS and 7 AILTs could not be confirmed by double stains. In addition, a cytotoxic phenotype had been defined in one PTCL-NOS and four AILTs when evaluating single stains, but the expression of cytotoxic proteins could only be confirmed in one AILT by double stains. Taking these data together, a concordant phenotype was observed in only one PTCL-NOS and two AILTs (21% of cases) during both approaches. This indicates that immunophenotypes as published on the basis of a morphological identification of tumor cells alone may not be reliable for the majority of PTCL. This may be one reason why immunophenotyping has such a low impact on the classification of PTCL. A reliable and consistent determination of the tumor cell phenotype in PTCL may in the future increase the importance of immunophenotypes for the diagnosis and classification of PTCL and allow us to distinguish entities on the basis of their characteristic marker expressions.

Although our major aim was to base an immunophenotypic analysis on our newly developed TCR β PCR, the latter also provided some insight on itself: Among the TCRV β segments, those of the TCRV β 5 ($n = 9$), TCRV β 9 ($n = 3$), and TCRV β 8 ($n = 3$) subfamilies were detected most frequently. The few studies addressing the TCRV β repertoire analysis in PTCL found either no evidence for an overrepresentation of certain subfamilies^{14,51,52} or a bias in the TCRV β subfamily frequency of the tumors different from ours.⁵³⁻⁵⁸ Taking all of these data together, it is unlikely that there is a general TCRV β subfamily/segment preference in PTCL, and it seems that TCRV β subfamily usage in a large series merely follows the random distribution of TCRV β subfamilies in normal individuals.^{12,59-63} The preference of the TCRJ β 2 cluster (14 tumors) to the TCRJ β 1 cluster (9 tumors) in our study fits in with the predominance of TCRJ β 2 rearrangements in precursor T-cell malignancies⁶⁴ and reflects differences in TCRJ β usage in reactive T-cell populations.^{12,59}

The diversity of the CDR3 region increases when adding or deleting nucleotides at the TCRV β -D β and TCRD β -J β junctions during rearrangement, and this affects antigen recognition. Although antigen recognition by T cells also depends on the MHC molecule presenting the antigen,⁶⁵ T cells that detect identical peptides share conserved CDR3 amino acid sequence motifs.^{66,67} The CDR3 amino acid sequences were diverse in our PTCL, therefore a common antigen specificity of the tumors is unlikely. However, this cannot be formally ruled out, because different epitopes of the same antigen may be recognized by different TCRs.

In conclusion, the TCR β PCR combined with immunohistochemical detection of the tumor cells using the respective TCRV β antibody as described in this work is a reliable and highly efficient way to identify the rearranged TCRV β segment of the tumor cells, to characterize the

tumor cells in more detail, and to arrive at new insights into the pathogenesis of these lymphomas. This could lead to a change in the classification of PTCL with regard to an increased accuracy of the individual prognosis and the choice of the best therapy.

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