Clinical significance of T-cell clonality in mycosis fungoides and other cutaneous T-cell lymphomas

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Molecular biological findings in the light of the Biomed-2 approach

Chapter 5 - Molecular biological findings in the light of the Biomed-2 approach.

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5.1 Evaluation of T-cell clonality in archival skin biopsy samples of cutaneous T-cell lymphomas using the Biomed-2 PCR protocol

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Abstract.

Recently, several European centres of lymphoma diagnosis and research developed in cooperation various polymerase chain reaction (PCR) methods for clonality analysis in suspect T- and B-cell proliferations [Biomed-2 Concerted Action]. They have mainly been applied to frozen material of systemic B- and T-cell malignancies. Until now only limited data exist concerning cutaneous T-cell lymphoma (CTCL) and paraffin-embedded material. Thus, we applied the Biomed-2 T-cell receptor (TCR) γ- and TCRβ PCR as well as an in-house TCRγ PCR to a collection of 107 archival skin samples (84 CTCL, 3 systemic T-cell lymphoma and 20 controls). As a result the Biomed-2 TCRγ PCR revealed 81%, the in house TCRγ method 86%, and the Biomed-2 TCRβ 78% clonality in CTCL samples generating at least the 300 bp fragment in the Biomed-2 control PCR. We could demonstrate clonal TCRβ rearrangements in 5/17 CTCL samples which have been polyclonal in the Biomed-2 TCRγ PCR. By combining all Biomed-2 assays, one or more clonal rearrangements were detected in 87% of CTCL as well as in all 3 systemic T-cell lymphoma. By combining all TCR PCR assays applied here, clonality was demonstrated in 90% of the CTCL cases.

In conclusion, we could show that the Biomed-2 TCR PCR worked well with DNA from paraffin-embedded tissue, revealing a high clonality detection rate in CTCL and thus should be highly recommended for routine molecular analysis. In addition, the high diagnostic sensitivity and specificity of our in-house TCRγ assay verify our previously published findings on clonally expanded T-cells in CTCL.

Introduction.

The DNA sequence of a T-cell receptor (TCR) gene rearrangement provides a unique marker for each individual T-lymphocyte. Since in lymphomas all malignant cells are derived from a single transformed lymphoid cell, the presence of an expanded clonal TCR gene rearrangement indicates a neoplastic T-cell proliferation. Thus, its molecular analysis by polymerase chain reaction (PCR) is widely used in the diagnosis of various T-cell lymphomas (TCL) including cutaneous T-cell lymphoma (CTCL). In particular, TCR gene analysis is very supportive in those cases, where a differential diagnosis between reactive lesion and malignant lymphoma based on immunohistological criteria alone is challenging. This holds particularly true for the diagnosis of CTCL consisting predominantly of small tumour cells embedded in a dense inflammatory background.

In recent years, a large number of PCR assays have been designed for the detection of clonal TCR gene rearrangements. These are easier to handle and more sensitive than the previous used Southern blot methods. To date, PCR analyses of the TCRγ genes are predominantly applied in routine practice. This is based on the relatively simple TCRγ locus configuration and the large homology within the Vγ and Jγ gene segments, limiting the number of required primers. However, the limited junctional diversity also results in a high background amplification of rearrangements of reactive T-cells. Moreover, in a significant proportion of malignant proliferations the tumour clone escapes detection. To overcome these limitations, several DNA-based TCRβ PCR protocols were developed [see: 1-3]. One advantage of the TCRβ PCR is the extensive combinatorial repertoire of TCRβ rearrangements and its large hypervariable region resulting in a higher specificity. However, due to highly degenerated consensus primer or a large number of different tubes the efficacy and comparability of these TCRβ PCR protocols varies considerably. To improve and standardize PCR technologies, several known centres of lymphoma diagnosis and research from 7 European countries have elaborated and tested new protocols and primer sets for PCR-based clonality analysis in suspect T- and B-cell proliferations (Biomed-2 Concerted Action BMH4-CT98-3936) [4]. The Biomed-2 methods include two TCRγ PCR and three PCR for complete and incomplete TCRβ rearrangements. Compared to pre-existing PCR protocols or the Southern Blot methods it was shown that the Biomed-2 TCR assay is more sensitive in detecting clonal TCR rearrangements and the Southern Blot is no longer regarded as “gold standard” for TCR genotyping [3,5,6]. Recently, the value of the Biomed-2 protocol was confirmed by its application to a large series of most frequent systemic mature T-cell malignancies using fresh or frozen material [7]. So far, a representative number of formalin-fixed and paraffin embedded (archival) samples has not been investigated. However, use of archival material is essential, since paraffin-embedded tissue samples are supplied for routine diagnosis procedures in most instances. Furthermore, only limited data are currently available for clonality detection by the Biomed-2 TCR assay in lesional skin biopsies of CTCL [8, 9].

To evaluate the reliability and applicability of Biomed-2 methods in archival tissue of CTCL, we applied the Biomed-2 TCRγ and TCRβ assay to 107 archival paraffin-embedded tissue samples of 84 CTCL patients, 3 systemic TCL (sTCL) and 20 controls. Results of the Biomed-2 TCRγ approach [4] were compared with our in-house TCRγ assay [10, 11] that has been used in CTCL diagnosis in our department for more than 10 years, routinely.

Materials and Methods.

Patients and clinical samples. A total of 107 consecutively collected formalin-fixed and paraffin-embedded lesional skin biopsies obtained from 84 CTCL patients, 3 cases of sTCL, and 20 controls (one sample per case) were investigated. All diagnoses were based on clinical, histological and immunohistological criteria. According to the WHO-EORTC classification [12] the CTCL patients were diagnosed with the following: 56 mycosis fungoides (Mf) i.e.18 patch, 35 plaque and 3 tumor stage, 14 lymphomatoid papulosis (LyP), 6 cutaneous anaplastic large TCL (cALCL), 5 Sézary syndrome (SS) and 3 pleomorphic CTCL (pleoCTCL). The sTCL were two cases with cutaneous lesions of an angioimmunoblastic TCL and one patient with a peripheral TCL, unspecified. Samples from 20 patients without a malignant T-cell proliferation (5 cutaneous B-cell lymphoma, 13 benign inflammatory dermatoses and 2 pseudolymphoma) served as controls.The research committee of the Charité-Universitätsmedizin Berlin has approved the described studies. Informed consent for the experimental studies was obtained from the patients. The study was conducted according to the Declaration of Helsinki Principles.

T-cell lines. Five clonal human T-cells lines i.e. MyLa, SeAx, Jurkat, Molt-4, Peer and HH were investigated by the Biomed-2-PCR and used also as clonal controls, Jurkat and Peer cells were additionally applied for evaluation of analytical sensitivities.

DNA-preparation and control-PCR. From all samples genomic DNA was prepared manually by the same standard procedure using paraffin extraction with Roticlear® and proteinase K digestion as described before [11]. The quality of each DNA sample was confirmed by Biomed-2 control PCR [4] yielding amplicons of 100, 200, 300 and 400 bp in size in a multiplex assay and by subsequent electrophoresis on a 1.5% agarose gel followed by staining with GelRed^{TM} Nucleic Acid Gel Stain (Biotium, Hayward, USA). As recommended for the subsequent Biomed-2 TCR PCR only DNA samples which provided at least a faint 300 bp band were used [13]. For estimation of the detection limits in formal-fixed specimens serial dilutions of DNA from Jurkat and Peer cells in tonsillar DNA were analyzed. For this purpose, cell pellets derived from 10^8 cells of the respective cell lines were generated by centrifugation and, after removal of the supernatants, the cell pellets were fixed in neutral buffered formalin (4%) for two hours and, subsequently, embedded in paraffin. Normal tonsillar tissue was fixed for approximately 24 hours under standard conditions using 4% neutral buffered formalin. DNA was obtained from these materials and dilution series were prepared.

TCR PCR. The DNA of the skin biopsy samples were amplified by the two Biomed-2 TCRγ PCR (sets A and B) and three TCRβ PCR (sets A,B,C) according to the original Biomed-2 report [4]. The in-house TCRγ PCR method was performed for the same set of samples as described earlier [11]. The TCRγ PCR 1 and 2 were applied to all samples, whereas the TCRγ PCR 3 amplifying rearrangements of Vγ with Jγ1.1/2.1 (JP1/2) gene segments was only applied to lymphoma samples which were non-clonal in the PCR 1 and 2. All primers used in this study, were purchased from a commercial local supplier (BioTez, Berlin, Germany). The primer synthesis includes a standard purification by gelfiltration but not by HPLC. Each reaction was screened by electrophoresis on a 1.5% agarose gel followed by staining with GelRed^{TM} before fluorescence fragment analysis (FFA,see below) was applied. Each TCR PCR showing a clonal outcome in the FFA (see below) was repeated in order to confirm the individual clonal TCR fragment length and to exclude pseudoclonality. Moreover, all

TCR PCR of CTCL-samples showing a polyclonal result were verified by at least one replicate. For statistical evaluation of selected PCR results the McNemar test was performed. Significance was assumed at a confidence level of at least 90%. Fluorescence fragment analysis

Following PCR and a positive screening on the agarose gel, products were subjected to FFA on the ABI 310 PRISM capillary sequencing instrument using the Gene Mapper 3.7 software (Applied Biosystems, Weiterstadt, Germany). A successful PCR from DNA of polyclonal T-cells displayed approximately Gaussian profiles fitting the relevant size ranges. Peak height ratios were applied for the assessment of the FFA from all TCR PCR [14]: clonality was supposed if one peak dominated the fluorescence intensity profile providing a peak-height ratio of at least two. The peak-height ratio was calculated by dividing the clonal peak height by the mean height of left and right adjoining peaks as recorded by the Gene Mapper program. Moreover, the height of the suspected clonal peaks had to exceed the mean height of all polyclonal background peaks generated in the given set. Since a T-cell clone can exihibit up to two rearrangements of the TCRγ locus [3], patterns with more than two dominant amplification products are not compatible with a single clonal T-cell population.

Results.

The lymphoma samples, controls and T-cell lines were tested for T-cell clonality with the three PCR methods (in-house TCRγ, Biomed-2 TCRγ , Biomed-2 TCRβ). An assay was evaluated as positive if at least one of the various PCR tubes of the method in question revealed a clonal PCR product. Exemplary clonal and non-clonal (polyclonal) profiles of the Biomed-2 PCR are shown in Figure 1 and 2. If compared to profiles of products of fresh/frozen material the same types of curves were received with peaks within the expected size ranges [4]. The results are presented in detail in table 1 and summarized in table 2.

Using serial dilutions with DNA from formalin–fixed cell lines and formalin-fixed tonsils, detections limits of approx. 2.5 to 5% were demonstrable when different primer sets were applied for the detection of TCRγ and TCRβ rearrangements.

Results of the PCR assays. The in-house TCRγ assay demonstrated 72/84 clonal CTCL samples (frequency of clonality/ diagnostic sensitivity: 86%). The highest rates of T-cell clonality (100%) were seen in SS, cALCL, and pleoCTCL, the lowest rate (79%) in LyP. In Mf, 47/56 (84%) clonal specimens were detected, with a higher rate of clonality in plaque stage (89%) than in patch stage (78%). One of the three tumor stage samples remained non-clonal. All of the three sTCL and all five T-cell lines were found to be clonal, all of the controls were non-clonal.

The Biomed-2 TCRγ assay detected 68/84 clonal CTCL samples (81%). Again, all specimens derived from SS, cALCL, and pleoCTCL were found to be clonal, whereas the lowest rate of T-cell clonality was seen in LyP (71%). In Mf, 44/56 (79%) clonal specimens were detected, with a higher rate of T-cell clonality in plaque stage (83%) than patch stage (72%). One of the three tumor stage samples remained non-clonal. All three sTCL samples were found to be clonal. One of the 20 control samples exhibited T-cell clonality with a small (uncertain) peak of 147 bp in Biomed-2- TCRγ tube A indicating a V10-Jγ1.3/2.3 (Jγ1/2) recombination. This sample was obtained from a patient with a cutaneous marginal zone lymphoma. All T-cell lines investigated were found to be clonal and the

corresponding TCRγ and β amplicon sizes - which are in line with previous descriptions with one exception [15]. They are shown in table 3. In Molt 4 cells we obtained a slightly different result: set A presented a biallelic TCRγ rearrangement of 219/242 bp, whereas a value of 223/242 bp was quoted [15]. The Biomed-2 TCRβ found 62/80 clonal CTCL specimens (78%), i.e. 56/80 (70%) in the VJ-PCR (sets A and B) and 40/80 (50%) in the DJ-PCR (set C). Four CTCL samples (1 cALCL, 3 LyP) could not be analyzed, because all material was used for the TCRγ assays. The highest rates of T-cell clonality were found in SS (100%), pleoCTCL (100%), and cALCL (80%), the lowest rate (73%) in LyP. In Mf, 42/56 (75%) samples revealed clonal PCR products with lower rates in patch than plaque stages (72% vs. 77%). The TCRβ assay also failed to detect T-cell clonality in the one MF tumor stage sample which was non-clonal in both TCRγ assays. T-cell clonality was detected in 2/3 sTCL. Two of 20 control samples obtained from patients with pseudolymphoma showed small clonal peaks (clonality uncertain). Both of them were non-clonal in all other TCR PCR.

Despite the fact that the TCRβ assay is highly complex, the TCRβ VJ PCR (sets A and B) failed completely to generate specific products in only 4/80 CTCL samples. In the β DJ assay (set C) from 8/80 samples no products were formed. Amplification in all three sets failed in only three cases, thereby indicating the high applicability of this assay to DNA from paraffin embedded tissues. Interestingly, the TCRβ DJ PCR did not indicate T-cell clonality in any of the investigated CTCL cases exclusively as in each of the samples with a clonal TCRβ DJ rearrangement at least one further Biomed-2 PCR revealed a clonal product.

Comparison of the TCRγ assays. Compared to the Biomed-2 TCRγ assay, the in-house TCRγ assay showed a slightly higher diagnostic sensitivity (86% versus 81%), however, the difference is not yet significant (confidence level: 89%). As demonstrated by the control samples both methods revealed a high specificity.

In a subset of 11 CTCL samples, i.e. 8 Mf (4 patch, 3 plaque, 1 tumor stage) and 3 LyP, both, the Biomed-2 TCRγ PCR as well as the in-house TCRγ PCR, unanimously did not detect clonal rearrangements. Both methods revealed clonality in all three sTCL specimen.

Comparison of the Biomed-2 TCRγ and TCRβ assays. We could demonstrate that the diagnostic sensitivities of both PCR methods were comparable to each other (81% versus 78%). Interestingly, 5 CTCL (2 Mf patch, 1 Mf plaque, 2 LyP) were non-clonal in the Biomed-2 TCRγ assay but exhibited clonal TCRβ rearrangements. On the opposite, 7 CTCL (2 Mf patch, 3 Mf plaque, 1 LyP, 1 cALCL) were non-clonal or non-amplified in the Biomed-2 TCRβ assay but could be shown to be clonal in the Biomed-2 TCRγ assay. Due to the partially different outcome, the combination of both assays enhanced the diagnostic sensitivity significantly towards 87% (confidence level: 96.3%).

Combining all PCR assays. By combining all TCR PCR assays applied in our study, clonality was demonstrated in 76 of the CTCL cases (90%) whereas 8 specimens (6 Mf - 3 patch, 2 plaque, 1 tumor stage and 2 LyP) remained non-clonal.

Sample	Diagnosis	In-house			Biomed-2 γ		Biomed-2 β		
		γ1	γ 2	γ3	B2 γ A	B2 γ B	B2β A	B2 β B	B2 β C
20050294	MF-pt	c	-		c	-	-	-	-
20050305	MF-pt	c	c		c	p	c	p	c
20050340	MF-pt	c	c		c	c	c	-	p
20050498	MF-pt	p	c		p	c	-	c	c
20050527	MF-pt	p	p	c	c	p	p	c	p
20050617	MF-pt	c	-		c	p	p	p	c
20050627	MF-pt	p	p	p	p	p	p	p	p
20060163	MF-pt	p	-	-	p	p	c	-	c
20060184	MF-pt	p	c		p	p	p	c	p
20060237	MF-pt	c	p		c	p	c	p	p
20060240	MF-pt	p	c		c	c	p	c	p
20060272	MF-pt	c	c		c	c	c	c	c
20060374	MF-pt	c	p		c	p	-	-	-
20070063	MF-pt	c	c		c	p	c	p	c
20070408	MF-pt	p	p	p	p	p	p	p	p
20070416	MF-pt	p	p	p	p	p	p	p	p
20070466	MF-pt	c	p		c	-	-	c	-
20070475	MF-pt	p	c		c	c	c	p	c
20040408	MF-pl	c	p		c	p	c	p	c
20040537	MF-pl	p	p	p	c	p	c	p	c
20040687	MF-pl	c	-		c	-	-	-	c
20050068	MF-pl	c	c		c	p	p	c	c
20050287	MF-pl	-	c		c	c	-	c	c
20050315	MF-pl	p	c		p	c	p	p	c
20050327	MF-pl	p	-	c	c	p	c	p	c
20050413	MF-pl	c	c		c	p	p	c	c
20050432	MF-pl	c	p		c	-	c	p	c
20050445	MF-pl	c	p		p	c	c	p	p
20050492	MF-pl	c	p		p	p	p	p	p
20050546	MF-pl	c	p		c	p	c	p	c
20050571	MF-pl	c	p		c	p	c	c	c
20050598	MF-pl	p	p	c	c	p	p	p	p
20050608	MF-pl	c	p		c	p	p	c	p
20050659	MF-pl	c	c		c	c	c	p	c
20060072	MF-pl	p	p	p	p	p	p	c	c
20060169	MF-pl	c	c		c	p	c	p	c
20060176	MF-pl	c	-		c	-	c	-	p
20060210	MF-pl	c	p		c	p	p	c	p
20060216	MF-pl	c	p		c	p	p	c	p
20060224	MF-pl	c	p		c	p	p	c	p
20060225	MF-pl	p	c		p	p	p	p	p
20060232	MF-pl	c	c		c	c	p	c	p
20060278	MF-pl	p	p	-	p	p	p	p	p
20060312	MF-pl	p	c		c	p	p	c	c
20060315	MF-pl	c	p		c	p	c	p	c
20070098	MF-pl	c	p		c	p	c	c	c
20070103	MF-pl	c	p		c	p	p	p	p
20070206	MF-pl	p	p	p	p	p	p	p	p
20070260	MF-pl	c	c		-	c	c	c	p
20070328	MF-pl	c	p		c	p	-	p	p
20070362	MF-pl	c	p		c	p	c	c	p
20070480	MF-pl	c	p		p	p	p	p	-
20070502	MF-pl	c	c		c	p	c	p	p
20060047	MF-tm	c	-		c	p	p	p	c
20060370	MF-tm	p	p	p	p	p	p	p	p
20070330	MF-tm	c	p		c	p	p	p	c
20060350	SS	c	c		c	p	-	c	p

20070010	SS	c	p		c	c	p	c	c
20070161	SS	c	c		c	-	-	c	-
20070207	SS	ol	ol	c	c	p	-	c	-
20070477	SS	c	c		c	c	c	p	c
20030777	LyP	c	p		c	p	c	p	c
20040070	LyP	p	p	-	p	p	p	p	p
20040245	LyP	c	p		p	c	nd	nd	nd
20040413	LyP	ol	p	c	p	p	p	c	-
20040610	LyP	c	c		c	c	nd	nd	nd
20040678	LyP	p	p	p	p	p	p	p	p
20050325	LyP	p	p	p	p	p	p	c	p
20050483	LyP	c	p		c	p	nd	nd	nd
20060068	LyP	p	c		c	p	c	p	c
20060069	LyP	c	c		c	p	p	p	p
20060106	LyP	p	c		c	c	c	p	c
20060179	LyP	c	p		c	p	c	p	c
20060185	LyP	c	p		c	p	c	p	c
20070180	LyP	c	p		c	p	c	p	c
20050187	cALCL	c	c		c	c	nd	nd	nd
20050482	cALCL	p	c		c	-	c	p	c
20050624	cALCL	-	c		-	c	-	-	-
20060102	cALCL	c	p		c	p	p	p	c
20060113	cALCL	c	c		c	p	p	c	c
20060180	cALCL	p	c		c	c	c	-	c
20050341	pleoCTCL	c	p		c	p	c	c	c
20070044	pleoCTCL	p	p	c	c	p	c	p	c
20070080	pleoCTCL	c	p		c	p	p	c	p
20050381	AILT	c	p		c	p	-	p	p
20050441	AILT	c	c		c	c	p	p	c
20050596	PTCL	c	c		c	c	p	c	c

Table 1. Analysis of the CTCL and sTCL samples: results MF, mycosis fungoides; pt, patch stage; pl, plaque stage; tm, tumor stage; SS, Sézary syndrome; LyP, Lymphomatoid papulosis; cALCL, cutaneous anaplastic large cell lymphoma; pleoCTCL, pleomorphic CTCL; AILT, angioimunoblastic TCL; PTCL, peripheral TCL, unspecified; c, clonal; p, polyclonal; ol, oligoclonal (classified as polyclonal); -, no PCR amplification; nd, not done. Highlighted in grey: at least one of the PCR of an assay (in-house TCRγ, Biomed-2 TCRγ or Biomed-2 TCRβ) reveals a clonal PCR product.

Entity	**total** n	**clonal** n (%)									
		In-house γ		Biomed-2 γ		Biomed-2 β		Biomed-2		All assays	
Mf	56	47	(84)	44	(79)	42	(75)	47	(84)	50	(89)
pt	*18*	*14*	*(78)*	*13*	*(72)*	*13*	*(72)*	*15*	*(83)*	*15*	*(83)*
pl	*35*	*31*	*(89)*	*29*	*(83)*	*27*	*(77)*	*30*	*(86)*	*33*	*(94)*
tm	*3*	*2*		*2*		*2*		*2*		*2*	
SS	5	5		5		5		5		5	
LyP	14	11	(79)	10	(71)	8/11[*]	(73)	12	(86)	12	(86)
cALCL	6	6		6		4/5[§]		6		6	
pleoCTCL	3	3		3		3		3		3	
CTCL total	84	72	(86)	68	(81)	62/80	(78)	73	(87)	76	(90)
sTCL	3	3		3		2		3		3	
controls	20	0		1[#]		2[#]		3[#]		3[#]	

Table 2. Analysis of the CTCL, sTCL and control samples: summary. Mf, mycosis fungoides; pt, patch stage; pl, plaque stage; tm, tumor stage; SS, Sézary syndrome; LyP, Lymphomatoid papulosis; cALCL, cutaneous anaplastic large cell lymphoma; pleoCTCL, pleomorphic CTCL; sTCL, systemic TCL; *, three samples were not investigated; [§], one sample was not investigated; #, small clonal peaks - clonality uncertain.

	clonal fragment length [bp]				
cell line	TCRγ set A	TCRγ set B	TCRβ set A	TCRβ set B	TCRβ set C
MyLa	196	182	266	-	307
SeAx	216	-	-	-	295
HH	213/233	-	255	254	-
Jurkat	212	116	266	-	309
Peer	212	167	260	269	-
Molt-4	219/242	-	-	265 / 273	-

Table 3. Biomed-2 TCR PCR in clonal human T-cell lines: results. -, no PCR product.

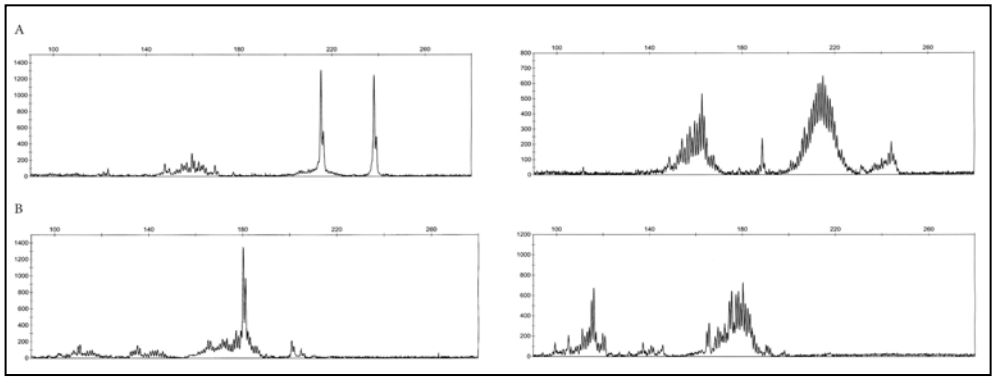

Figure 1. Fragment profiles of Biomed-2 TCRγ PCR products (examples). (A), tube A; (B), tube B; left, monoclonal; right, polyclonal.

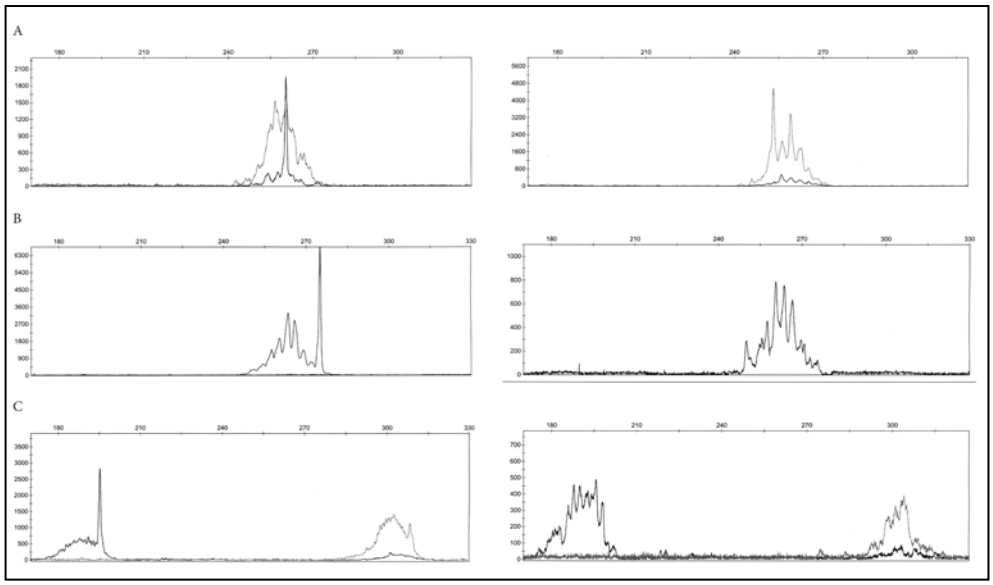

Figure 2. Fragment profiles of Biomed-2 TCRβ PCR products (examples). (A), tube A; (B), tube B; (C), tube C; left, monoclonal; right, polyclonal.

Discussion.

Up to date, PCR analysis of rearranged TCR genes in combination with high resolution electrophoresis is used commonly and represents an important technique in the diagnosis of CTL including CTCL. Since 1989 a large number of different PCR protocols for the detection of clonally rearranged TCR genes were applied in lymphoma diagnosis and research. In addition, many different electrophoretic techniques like heteroduplex polyacrylamid gel electrophoresis or FFA were used for subsequent PCR product analysis. Therefore, it was difficult to compare results from different reports or laboratories. In view of this fact a comprehensive panel of standardized PCR methods has been developed in cooperation within the European Biomed-2 project [4]. Owing to the high reliability of the Biomed-2 primer protocol in fresh or frozen material of systemic lymphomas and owing to the lack of data on its applicability in paraffin-embedded tissue and in CTCL we applied the Biomed-2 TCR assay to a representative number of archival skin samples of patients with various CTCL. The results were compared with our in-house PCR assay, which we have applied for more than 10 years. A similar evaluation of the Biomed-2 IgH-PCR in archival samples of patients with cutaneous B-cell lymphoma has been performed already [16].

In our study investigating paraffin-embedded CTCL tissues, Biomed-2 TCRγ PCR and in-house TCRγ PCR revealed comparable diagnostic sensitivities of 81%, and 86%, respectively. With the Biomed-2 TCRβ assay, 78% of CTCL cases were found to be clonal. These results show similarity with a small series of ten archival Mf samples (including five cases of Mf tumor stage) demonstrating clonality in 80% of the cases using the Biomed-2 protocol [9]. Either comparable or slightly lower detection rates have also been stated in numerous former reports using various other TCRγ assays in paraffin-embedded tissues [see: 17, 9]. Assaf et al. revealed T-cell clonality even in 100% of 24 archival CTCL samples applying a semi-nested consensus TCRβ PCR/ FFA [1]. However, in contrast to our study, only advanced stages of disease were investigated. Moreover, nested and semi-nested assays are particularly prone to generate pseudoclonal results and require repeated analyses of each clonal DNA sample.

To our knowledge the only published study applying the Biomed-2 protocols in CTCL detected clonal rearrangements in 73% by the Biomed-2 TCRγ and in 62% by the Biomed-2 TCRβ assay with subsequent FFA. Only fresh/frozen samples were used [8]. Recently, Ponti et al. demonstrated T-cell clonality in 84% of Mf cases (with approximately 70-76% in the early stages) and in 100% of SS cases applying a different TCRγ PCR protocol and FFA to 203 frozen skin samples. A slightly lower percentage of clonal Mf cases was detected when using heteroduplex polyacrylamide gel electrophoresis for PCR product separation [17]. Fairly similar to our results, Morgan et al found 85% of clonally expanded TCRγ rearrangements and 75% of clonally expanded TCRβ rearrangements when applying the Biomed-2 protocols to a group of 20 CTCL (17 Mf, 3 SS). In that study DNA was prepared from both, fresh and paraffin embedded skin sections, but the portions of both materials were not quoted [18]. Our data from paraffin embedded CTCL tissues are also similar to those from fresh or frozen samples. It should be emphasized that all samples generated the 300 bp fragment in the Biomed-2 control tube. On this condition, the lower integrity of DNA as expected after extraction from paraffin obviously did not reduce the diagnostic sensitivity of the PCR-based clonality analysis. Thus, even the more complex Biomed-2 TCRβ assay revealed comparable frequencies of T-cell clonality. However, a higher complexity of the PCR assay may result in amplification of

pseudoclonal PCR products resulting in a lower diagnostic specificity. If this holds true for the Biomed-2 assays can only be evaluated with more control samples. Complexity of the PCR primers may also explain the differences between in-house TCRγ assay and Biomed-2 TCRγ assay: By splitting the investigation of the possible TCRγ rearrangements in three PCR, the in-house assay revealed slightly more clonal rearrangements in CTCL (see table 2), however the difference is not significant. In fact, the Biomed-2 TCRγ assay failed to detect T-cell clonality in 5 CTCL where clonality was shown by the in-house PCR. Conversely, the Biomed-2 TCRγ PCR exclusively showed clonality in one case. The in-house approach and the Biomed-2 test revealed almost identical analytical sensitivities of 2.5-10% in formalin-fixed material, depending on the T-cell line. We have received equivalent detection thresholds with dilutions of DNA from freshly collected clonal T-cells applying the TCRγ Biomed-2 protocol [19] or in-house PCR [14]. Thus, the different results of both TCRγ assays are rather caused by different primer binding positions than by different analytical sensitivities.

Insufficient priming of the TCR gene segments due to germ line configuration, incomplete, deleterious or trans-rearrangements may explain the non-clonality of 8 CTCL cases in all PCR assays. In general, clonal rearrangements are not detectable using TCRγ PCR in at least 10% of lesional skin samples from CTCL, independently of the PCR and electrophoresis method applied [20]. Priming at different genes explains the differences between the TCRγ and TCRβ analyses. Here, the consistency is lower when compared to the differences of the TCRγ assays. Combining the Biomed-2 TCRγ and TCRβ methods provided a significantly increased diagnostic sensitivity. Therefore, the Biomed-2 TCRβ assay (with exception of the TCRβ DJ PCR which did not exclusively indicate a clonal rearrangement) should supplement the TCRγ tests in routine CTCL analysis. This is in line with a recent analysis of 188 fresh frozen samples of systemic T-cell malignancies, where only 4.3% of the clonal rearrangements were detected by the TCRβ assay [7]. This procedure in clonality testing has already been recommended by others [3] and, as shown here, applies also to paraffin-embedded samples. In this investigation, combination of all TCR tests used enhances the frequency of detected T-cell clonality in CTCL to 90%. In the PCR based clonality analysis of DNA from archival samples, a substantial part of clonal cases may be missed. Christensen et al found in 4/18 sTCL cases (22%) clonal TCRγ rearrangements only with frozen but not with paraffin-embedded tissue, however, they did not employ the Biomed-2 methods or any TCRβ assay [21].

In comparison with a former report [15] we repeatedly received a slightly smaller amplicon for one rearranged allele of Molt 4 cells in the Biomed-2 TCRγ test (219 versus 223 bp). The difference seems to be too large for a normal variation of FFA sizing and may be caused by genetic aberrations occuring during long-time maintenance of the cell line.

Using the Biomed-2 assays we received an “uncertain-clonal” outcome of three control samples. Accordingly, a combination of these assays could reduce the diagnostic specificity, however, more controls have to be investigated for a reliable statement. This matter is of special concern since a clonal result of the assay is used as meaningful supplementary information to confirm a malignant lymphoproliferation. Nevertheless, due to the fact that the presence of a clonal TCR rearrangement does not always indicate malignancy, it has to be emphasized that accurate integration of clinical, histomorphological, immunohistochemical and molecular biological data is still mandatory. In conclusion, our data underline the reliability and applicability of the standardized Biomed-2 primers

and protocols and show its applicability in the diagnosis of paraffin-embedded skin biopsies of CTCL. The Biomed-2 TCRγ PCR and in-house TCRγ PCR revealed similar diagnostic sensitivities and specificities. However, the Biomed-2 TCRγ protocol is nowadays highly recommended for routine analysis of CTCL, in particular to achieve a standardization of TCR PCR techniques. This permits a much better data comparability and exchange of experience in TCL/CTCL diagnosis. Moreover, the in-house TCRγ PCR is more laborious, requiring a third tube set. As shown here the Biomed-2 TCRβ method may be helpful, particularly in cases suspected of having CTCL without detectable clonal TCRγ rearrangement. The Biomed-2 methods are also qualified to detect reliably oligoclonal expansions as well as clonal heterogeneities in CTCL. However, the confirmed diagnostic sensitivity and specificity of our in-house TCRγ assay, being routinely used in CTCL diagnosis in our lab for more than 10 years, verifies our previously published findings on clonally expanded T-cells in CTCL [10, 22].

Conflict of Interest.

The authors state no conflict of interest.

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