Molecular immunoglobulin/T- cell receptor clonality analysis in cutaneous lymphoproliferations. Experience with the BIOMED-2 standardized polymerase chain reaction protocol

YORICK SANDBERG, FREERK HEULE, KING LAM, PIETERNELLA J. LUGTENBURG, INGRID L.M. WOLVERS-TETTERO, JACQUES J.M. VAN DONGEN, ANTON W. LANGERAK

Background and Objectives. Molecular clonality analysis of immunoglobulin (Ig) and T-cell receptor (TCR) genes is a widely used diagnostic tool for discriminating between polyclonal, oligoclonal, and monoclonal lymphoproliferative skin lesions. We studied Ig/TCR clonality in a series of 60 patients with an initial suspicion of (primary) cutaneous B- or T-cell lymphoma (CBCL/CTCL). Clonality of Ig/TCR gene rearrangements was assessed by Southern blot (SB) and polymerase chain reaction (PCR) analysis using standardized PCR primers and protocols of the BIOMED-2 Concerted Action BMH4-CT98-3936. The obtained PCR products were subjected to heteroduplex (HD) and GeneScan (GS) analysis. We compared the data of 154 samples with the histopathologic diagnosis, based on the EORTC classification of skin lymphomas.

Design and Methods. Molecular results were largely concordant with histopathology. In 12 CBCL patients PCR analysis of Ig gene rearrangements detected clonality in 83% of cases whereas SB did so in 92%. Clonal TCR gene rearrangements were detected by SB in 68% of CTCL patients, whereas *TCRG* and *TCRB* PCR analysis detected clonality in 76% and 66% of cases, respectively. PCR GS analysis of TCR rearrangements appeared to be slightly more informative than HD analysis. Clonality assessment was particularly informative for studying involvement of extracutaneous sites, such as regional lymph nodes, peripheral blood, and bone marrow.

Interpretation and Conclusions. Our study shows that the BIOMED-2 multiplex PCR analysis strategy is a reliable and useful technique in the diagnostic process of patients with an initial suspicion of (primary) CBCL/CTCL and for assessment of extracutaneous dissemination, provided that the results are interpreted in the context of clinical, histologic and immunophenotypic data.

Key words: primary cutaneous lymphoma, Ig/TCR gene rearrangements, clonality analysis, heteroduplex analysis, GeneScan analysis.

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From the Departments of Immunology (YS, ILMW-T, JJMvD, AWL) Dermatology (FH), Clinical Pathology (KL), and Hematology (PJL), Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands.

Correspondence: Dr. Anton W. Langerak, Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands. E-mail: a.langerak@erasmusmc.nl

Cutaneous lymphoproliferations represent a heterogeneous group of benign and malignant disease entities. In cutaneous lymphoproliferations it is of great prognostic importance, though still not easy, to differentiate between a clinically malignant aggressive primary cutaneous lymphoma, such as mycosis fungoides (MF), and a more benign disease entity, such as dermatitis. Molecular diagnostic tools with a high specificity and sensitivity might contribute to an early and correct diagnosis and thereby better treatment, improving the patient's prognosis.

The first step in diagnosing patients with a clinical suspicion of primary cutaneous B- or T-cell lymphoma (CBCL/CTCL) consists of histomorphology, immunophenotyping, and cytological analysis on various tissue samples such as (involved) skin, (enlarged) lymph nodes (LN), bone marrow (BM), and peripheral blood (PB). Molecular clonality studies employing Southern blot (SB) analysis and more recently also polymerase chain reaction (PCR) analysis have been introduced as an additional diagnostic step.[1-13]

Since cutaneous lymphomas are clonal diseases that are derived from a single malignantly transformed lymphoid cell, all malignant cells contain clonal (identical) rearrangements of immunoglobulin (Ig) or T-cell receptor (TCR) genes.[14,15] Clonal or polyclonal B- or T-cell populations can be discriminated based on the presence or absence of clonally rearranged Ig or TCR genes.[16] Provided that optimal probe/restriction enzyme combinations are used, SB analysis can be considered the gold-standard molecular technique for clonality studies, because the risk of false-negative and/or false-positive results is very low.[17] Detectability of (clonal) Ig and TCR rearrangements by PCR analysis is limited by the choice of oligonucleotide primers. PCR analysis can yield more false-negative results than does SB analysis in the cases when the applied PCR primer sets are inappropriate for recognizing each rearranged Ig or TCR gene segment. False-negative results can also be caused by the occurrence of somatic mutations in Ig genes of (post-)follicular B-cell lymphomas. However, the higher efficiency and sensitivity of PCR techniques compensate for these disadvantages and may be particularly important for detecting small numbers of malignant cells, for example in early stage CBCL/CTCL. Also, SB analysis requires larger amounts of high quality DNA and cannot be performed on paraffin-embedded tissues. This implies a strong need to replace SB analysis by reliable PCR techniques.

In the present study we evaluated the contribution of both SB and PCR analyses of rearranged Ig and TCR genes to the diagnostic process of patients with an initial suspicion of (primary) CBCL/CTCL. SB analysis was performed with optimized DNA probes for the immunoglobulin heavy chain (*IGH*) locus and T-cell receptor β (*TCRB*) locus.[18,19]

For PCR-based analysis of the *IGH*, *TCRB*, and T-cell receptor γ (*TCRG*), loci we used the well-defined and fully standardized set of oligonucleotide primers and PCR protocols of the BIOMED-2 Concerted Action BMH4-CT98-3936, entitled *PCR-based clonality studies for early diagnosis of lymphoproliferative disorders*.[20] To determine their homogeneous or heterogeneous character, the PCR products were analyzed by heteroduplex (HD) as well as GeneScan (GS) analysis. GS analysis has been described to be fast, accurate, sensitive, and easy to interpret.[21-23] HD analysis can be a cheap and reliable alternative in a diagnostic setting, not requiring expensive automated sequencing equipment or fluorochrome-labeled oligonucleotides.

A total of 154 different samples (including skin, LN, BM, PB, ascites, and synovium) from 60 patients with an initial suspicion of (primary) CBCL or CTCL were subjected to clonality assessment by SB and PCR. The results were compared with the clinical, histologic, cytological, and immunophenotypic data.

Our results show that the BIOMED-2 PCR-based GS analysis of *TCRB* and *TCRG* rearrangements is a very reliable and sensitive method for detecting clonal T-cells in CTCL. This technique seems to be as informative as SB analysis of the *TCRB* locus. Although SB analysis should still be considered as the most informative method in CBCL, our BIOMED-2 multiplex PCR approach of Ig clonality detection is only slightly less informative. The implications of our results for diagnosing suspected cutaneous lymphoproliferations are discussed.

Design and Methods

Patients

From February 1990 until November 2001 154 DNA samples (skin, n=75; LN, n= 18; BM, n=12; PB, n=47; ascites, n=1; synovium, n=1) from a total of 60 patients with an initial suspicion of (primary) CBCL or CTCL were obtained. Routine biopsies were 4 mm punch biopsies, whereas occasionally *in situ* or excision biopsies were taken in case of deeply infiltrated or tumorous lesions. All patients were seen and followed at the Department of Dermatology of the Erasmus MC, University Medical Center, Rotterdam. The patients with a strong suspicion of malignant disease (n=48) were discussed in the Dutch Cutaneous Lymphoma Working Group (DCLWG), where a consensus about the definite diagnosis was reached. Twelve patients were not discussed in the DCLWG, because in an early stage of the diagnostic process a diagnosis of non-malignancy was made. The DCLWG uses the European Organization for Research and Treatment of Cancer (EORTC) classification for primary cutaneous lymphomas , which is based on a combination of clinical, histologic and immunophenotypic criteria.[24]

Twelve patients were diagnosed as having (primary or secondary) CBCL. Patients with confirmed primary CBCL (n=7) could be divided according to the EORTC classification into having the following diseases categories: primary cutaneous follicle center cell lymphoma (PCFCCL; n= 3), large B-cell lymphoma of the leg (n=3) and immunocytoma (n=1). In addition, five patients were eventually diagnosed as having primary nodal B-NHL with secondary skin involvement. These patients were classified according to the WHO classification of lymphoid neoplasms as having:[25] follicular lymphoma (n=2), diffuse large B-cell lymphoma (n=1) and lymphoplasmacytic lymphoma (n=2).

Thirty-one patients were diagnosed as having (primary or secondary) CTCL. The confirmed primary CTCL (n=28) were categorized according to the EORTC classification into: mycosis fungoides (MF; n=16), Sézary's syndrome (SS; n=3), $CD30^+$ anaplastic/pleomorphic large cell CTCL (n=5), pleomorphic small sized CTCL (n=1), and lymphomatoid papulosis (LyP; n=3). There was an extra group of three patients with a proven T-cell lymphoma which could not be placed in one of the groups of the EORTC classification. These diagnoses were T-cell non-Hodgkin's lymphoma (T-NHL) with skin involvement (n=1), $CD8^+$ CTCL (n=1), and adult T-cell leukemia lymphoma (ATLL; n=1). One additional patient was diagnosed as having a natural killer (NK)-cell lymphoma.

Patients with a confirmed diagnosis of non-CTCL, non-CBCL (n=16) lymphoproliferations were divided into the following groups: pseudo B-cell lymphoma (n=4) and benign dermatoses (n=12). The latter could be subdivided into dermatitis (n=8), histiocytosis (n=2), Jessner-Kanoff lymphocytic infiltration of the skin (n=1), and mucinosis follicularis (n=1).

Diagnosis and staging

The 48 patients with strongly suspected malignant disease underwent extensive examination to assess the diagnosis and the stage of disease. The DCLWG used the staging system according to Fuks.[26] This examination consisted of imaging techniques (chest X-ray, computed tomography (CT) of thorax and abdomen), histologic and immunophenotypic analysis of skin tissue, and cytological analysis of PB. LN excision and examination were performed in patients with palpable lymph nodes. If the PB cytomorphology was suspicious, BM aspiration and biopsy were performed as well. Immunophenotyping on cell suspensions and/or cryostat sections of

skin, LN, BM, and PB samples was performed with several distinct markers to determine the differentiation lineage of the suspicious lymphocytes (e.g. CD1, CD2, CD3, CD4, CD5, CD8, CD10, CD19, CD20, CD22 and CD30) and the presence of clonality in the case of suspicious B-lymphocytes via single Ig light chain expression (Igκ and Igλ).

DNA isolation

High molecular weight DNA was isolated from skin, LN, PB, BM, and ascites specimens that were collected during routine diagnostic procedures, using a phenol-chloroform extraction-based protocol,[14] followed by ethanol precipitation and resolution in TE buffer.

Southern blot analysis

SB analysis was performed as described elsewhere.[14] In short, 10-15 μg of high molecular weight DNA were digested with *Bgl*II, *Bam*HI/*Hind*III, *Eco*RI, or *Hind*III, electrophoresed in 0.7% (w/v) agarose gels, and transferred to nylon filters, which were hybridized with several well-defined ^{32}P-labeled probes. The IGHJ6 probe (DAKO Corporation, Carpinteria, CA, USA) was used for analysis of the *IGH* genes in combination with *Bgl*II or *Bam*HI/*Hind*III digests,[18] whereas the TCRBJ1, TCRBJ2, and TCRBC probes (DAKO Corporation) were used for analysis of the *TCRB* genes in combination with *Eco*RI and *Hind*III digests.[19] Incidentally, in case of inconclusive SB results the samples were further analyzed using specific probes for the Igκ (*IGK*) and *TCRG* genes. For the *IGK* genes this involved the IGKJ5, IGKC, and IGKDE probes (DAKO Corporation) on *Bgl*II or *Bam*HI/*Hind*III digests.[27] For the *TCRG* genes, specific probes were used for the Jγ1.1/2.1 and Jγ1.3/2.3 gene segments (in *Eco*RI digests) and for the Jγ1.2 gene segment (in *Bgl*II digests).[14] In one patient, diagnosed as having NK-cell lymphoma, assessment of the presence of (clonal) EBV genome was also performed, using the *Xho*I probe in *Bam*HI/*Hind*III digested DNA.[28]

PCR amplification

In each 50 μL PCR reaction, 200 ng DNA, 10 pmol of 5' and 3' oligonucleotide primers, 0.2 mmol/L dNTP, 5 μL 10×Gold buffer (*IGH, IGK, IGL*) or 5 μL 10 × buffer II (*TCRB*, *TCRG*, t(14;18)), and 1-2 U AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA) were used. The concentration of $MgCl_2$ ranged from 1.5 mmol/L (*IGH*, *IGK*, *TCRB* tube C, *TCRG*, t(14;18)) and 2.5 mmol/L (*IGL*) to 3 mmol/L (*TCRB* tubes A and B). All amplification reactions were performed in an automated thermocycler (model ABI 9600; Applied Biosystems) using multiplex PCR according to the BIOMED-2 protocol.[20] Cycling conditions were those described in the BIOMED-2 PCR protocol and consisted of the following steps: pre-activation (7 min, 95°C), followed by 35 cycles of denaturation at 95°C (45 sec for classical thermocyclers; 30 sec for new generation thermocyclers), annealing at 60°C (>45 sec for classical thermocyclers; >30 sec. for new generation thermocyclers), and extension at 72°C (1.5 min for classical thermocyclers; >30 sec for new generation thermocyclers), and a final extension step of at least 10 min at 72°C.

For amplification of *IGH* rearrangements six FR (framework)1-V_H family primers, seven FR2-V_H family primers, seven FR3-V_H family primers, and one FAM-labeled J_H consensus primer were used in three multiplex combinations (*IGH* multiplex tubes A, B, and C). NALM-6 DNA was employed as a positive control. In case of inconclusive *IGH* gene results, we further analyzed the samples using multiplex PCR reactions for the *IGK* and *IGL* genes. Amplification of the *IGK* locus was performed with six Vκ family primers and two FAM-labeled Jκ primers (*IGK* multiplex tube A), or the same six Vκ family primers, one intron RSS primer, and one FAM-labeled Kde primer (*IGK* multiplex tube B). For amplification of the *IGL* locus two Vλ primers and one FAM-labeled Jλ primer were used in a single multiplex tube (*IGL* tube A). For amplification of *TCRG* genes we used Vγl and Vγ10 primers (*TCRG* multiplex tube A) or Vγ9 and Vγ11 primers (*TCRG* multiplex tube B) in combination with 2 FAM-labeled Jγ primers. Positive controls consisted of MOLT 3 and ALL 1 (tube A) and Jurkat, and patient's DNA samples (tube B). For amplification of *TCRB* rearrangements 23 Vβ family primers were used with 9 FAM-labeled Jβ primers (*TCRB* multiplex tube A), the same 23 Vβ family primers with the remaining 4 FAM-labeled Jβ primers (*TCRB* multiplex tube B), and 2 Dβ primers with all 13 FAM-labeled Jβ primers (*TCRB* multiplex tube C).[20] Positive controls consisted of ALL 1 (tube A), CML-T1 (tube B), and Jurkat (tube C).

Following Ig/TCR amplifications, 10 μL of PCR product were loaded on 1% agarose gels to check whether any product had been formed. Subsequently the rearranged Ig/TCR PCR products were further analyzed by heteroduplex analysis (*see below*) and GeneScan analysis (*see below*) to assess whether the obtained PCR products were derived from monoclonal or polyclonal cell populations.

PCR amplification of the t(14;18) translocation was performed according to BIOMED-2 guidelines in three multiplex reactions covering the MBR, 3'MBR, and mcr regions.[20] This analysis was performed in all CBCL and pseudo B-cell lymphoma patients. PCR analysis for the detection of the two main types of *TAL1* gene deletions (types 1 and 2) was essentially performed as described previously.[29] All CTCL patients with a clonal T-cell population were analyzed using this PCR.

Heteroduplex (HD) analysis

The PCR products for HD analysis were denatured at 94°C for 5 min and subsequently cooled at 4°C for 60 min to induce duplex formation.[30] After duplex formation, 20 μL of the hetero- and/or homoduplexes were immediately loaded on 6% non-denaturing polyacrylamide gels in 0.5 × Tris-Boric acid-EDTA (TBE) buffer, run at room temperature, and visualized by ethidium bromide staining. A 100 base pair (bp) size marker was used to determine the correct size of the PCR products.

Fluorescent fragment analysis (GeneScan (GS) analysis)

Two microliters of 10-fold diluted PCR products were mixed with 2.0 μL of formamide, 0.5 μL of 6-carboxytetramethylrhodamine-labeled internal standard (Genescan 500-TAMRA, Applied Biosystems), and 0.5 μL of loading buffer (blue dextran). After denaturation at 95°C for 2 min and cooling, 3 μL of the mixture were size-separated on a high-resolution polyacrylamide gel and analyzed using an automated ABI PRISM 377 fluorescent sequencer (Applied Biosystems). The size and profile of the PCR products were determined using GeneScan 672 computer software (Applied Biosystems).[31,32]

Results

We investigated 154 DNA samples from 60 patients in whom a clinical diagnosis of (primary) cutaneous lymphoma was initially considered. PCR analysis of Ig/TCR gene rearrangements was performed on all samples, whereas SB analysis could be performed on 144 of these samples (i.e. all skin samples and the vast majority of non-skin samples) (Tables 1-3). PCR and SB analyses were, in principle, performed once. In practice this means that Ig clonality was evaluated with the IGHJ6 probe and in three different FR *IGH* multiplex PCR tubes, whereas TCR clonality was analyzed with several *TCRB* probes and in *TCRB* as well as *TCRG* multiplex PCR tubes. In case of doubtful or discrepant results, PCR assays were repeated and SB was checked using *IGK* and/or *TCRG* probes. Although skin biopsies were available from all patients, in three patients the amount of DNA isolated was not sufficient for reliable molecular analysis; in these patients DNA from suspicious LN samples was analyzed.

Ig/TCR gene rearrangement analysis in CBCL

Three patients diagnosed with *primary cutaneous follicle center cell lymphoma* (*PCFCCL*) and one with *immunocytoma* (Table 1) all demonstrated clonal IGH rearrangements by PCR HD/GS and SB analysis. In two of the three patients with *large B-cell lymphoma of the legs*, PCR HD and PCR GS analysis showed clonal B-cell populations in skin samples (Figure 1) whereas clonally rearranged *IGH* genes were detected in all three skin samples by SB analysis. Clonal *IGH* gene rearrangements were detected in four out of five patients with a primary nodal B-NHL and skin involvement by PCR-based HD/GS and SB analysis. Remarkably two of the CBCL patients showed large clonal PCR products after *IGH* PCR analysis. These products were outside the expected size range of V_H-D_H-J_H couplings, and concerned amplifications of V_H-D_H-J_H rearrangements in which the consensus JH primer appeared to anneal better to the more downstream J segment. Additional *IGK* PCR analysis confirmed clonality in both cases.

Molecular analysis of the *IGH* locus was also performed on the skin samples of four patients with pseudo B-cell lymphoma (Table 1). Clonal B-cell populations were detected in two cases by SB analysis and in none by PCR analysis. In one patient, who did not show any clonal *IGH* gene rearrangements, clonal *IGK* but polyclonal *IGL* gene rearrangements were detected (*data not shown*).

Two out of twelve analyzed CBCL patients demonstrated a t(14;18) translocation in skin and PB samples. These two patients were diagnosed as having primary nodal follicular lymphoma with secondary skin involvement, whereas none of the

Table 1. Frequency of clonal IGH rearrangements in the skin of CBCL and 4 pseudo B-cell lymphoma patients, classified according to histopathologic and clinical criteria, as assessed by PCR heteroduplex/GeneScan analysis and Southern blot analysis.

	Number (%) of patients with clonal IGH rearrangements IGH			
Diagnosis (no. of patients)	HD	GS	SB	t(14;18)
Primary cutaneous follicle center cell lymphoma (n=3)[a]	3/3 (100)	3/3 (100)	3/3 (100)	0/3 (0)
Immunocytoma (n=1)	1/1 (100)	1/1 (100)	1/1 (100)	0/1 (0)
Large B-cell lymphoma of the legs (n=3)[a]	2/3 (67)	2/3 (67)	3/3 (100)	0/3 (0)
Nodal B-NHL with skin involvement (n=5)	4/5 (80)	4/5 (80)	4/5 (80)	2/5 (40)
All CBCL (n=12)	10/12 (83)	10/12 (83)	11/12 (92)	2/12 (17)
Pseudo B-cell lymphoma (n=4)[b]	0/4 (0)	0/4 (0)	2/4 (50)	1/4 (25)

HD: heteroduplex analysis; GS: GeneScan analysis; SB: Southern blot analysis; IGH: immunoglobulin heavy chain gene; ND, not done.
[a]In one patient with a primary cutaneous follicle center cell lymphoma and one diagnosed with large B-cell lymphoma of the legs large clonal IGH PCR products (see text for explanation) were detected. After additional PCR analysis of the IGK locus, clonal IGK gene rearrangements were detected in both patients.
[b]Clonal IGK gene rearrangements were detected in one patient.

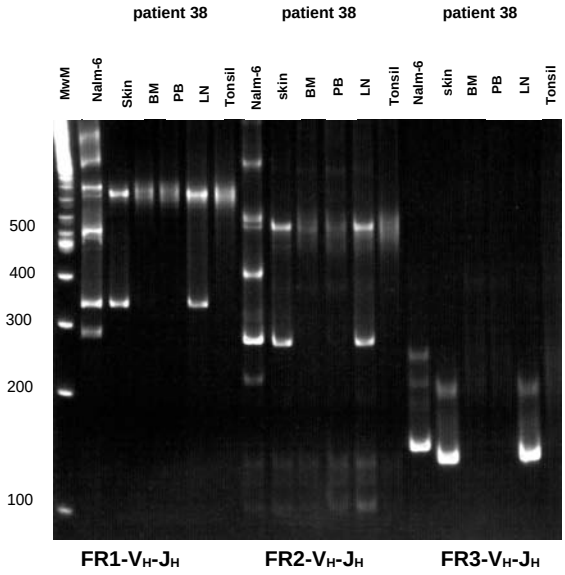

Figure 1. Heteroduplex analysis of IGH PCR products from a CBCL patient. After amplification of the IGH gene rearrangements with FR1-VH, FR2-VH, FR3-VH and a JH consensus primer, PCR products were denatured for 5 min at 94°C and renatured for 1 h at 4°C. Electrophoresis was performed in 6% non-denaturing polyacrylamide gels, using a 100 bp size marker. Homoduplexes can be identified in control cell line DNA (Nalm-6) and in skin and LN DNA from patient 38, diagnosed with a large B-cell lymphoma of the leg. Heteroduplexes, as identified in polyclonal tonsillar DNA and in BM and PB DNA from patient 38, suggest the absence of the B-cell clone in BM and PB.

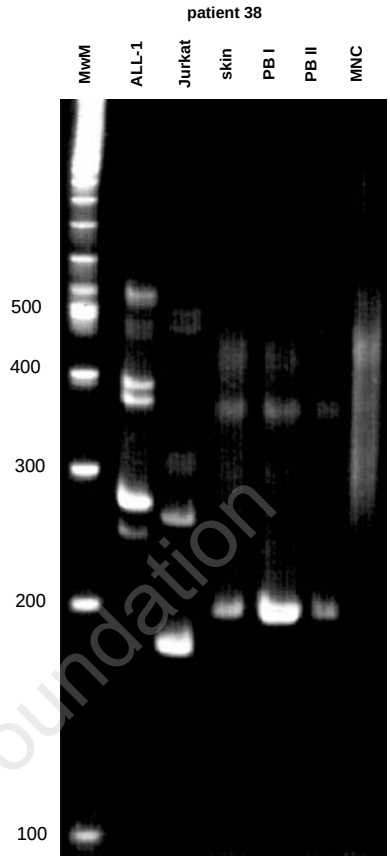

Figure 2. Heteroduplex analysis of *TCRG* PCR products from a CTCL patient. After amplification of the *TCRG* gene rearrangements with Vγ1, Vγ10 and Jγ1.3/2.3, Jγ1.1/2.1 primers, PCR products were denatured for 5 min and renatured for 1 h at 4°C. Electrophoresis was performed in 6% non-denaturing polyacrylamide gels, using a 100 bp size marker. Homoduplexes can be identified in control cell line DNA (ALL-1 and Jurkat) and in skin and PB DNA of patient 13, diagnosed with Sézary syndrome. The PB II sample was taken 4 years after treatment, indicating a relapse. Heteroduplexes can be identified in polyclonal MNC DNA.

patients with primary cutaneous CBCL were positive. Remarkably, one out of four patients diagnosed with a pseudo B-cell lymphoma showed a t(14;18) translocation in the skin sample as well. Although no clonal Ig gene rearrangements could be detected by SB and PCR analysis, this finding is suggestive of an early stage primary nodal follicular lymphoma, but requires further investigation and intensive follow-up.

Clonal TCR rearrangements were not detected in any of the skin and/or LN samples of the CBCL or pseudo B-cell lymphoma patients.

Ig/TCR gene rearrangement analysis in CTCL

Our study population included 16 patients with *mycosis fungoides* (*MF)* stage I to IV. Molecular analysis was performed of skin and/or LN samples from all these patients. Nine patients presented in an early stage of the disease with patch/plaque cutaneous lesions (stage I-II). Clonally rearranged *TCRB/TCRG* genes were detected in four out of eight cases (50%) by PCR HD analysis and five out of eight (63%) by PCR GS analysis. Clonal *TCRB* gene rearrangements were found in the skin samples of six out of nine stage I-II patients (67%) by SB analysis. Seven patients were diagnosed with MF stage III-IV. In the skin of four out of six of these patients (67%) clonal T-cell populations were detected by PCR-based *TCRB/TCRG* gene rearrangement analysis and SB analysis of the *TCRB* locus. In the seventh patient, from whom only LN and PB samples could be studied molecularly, a T-cell clone was found in the LN sample by both PCR and SB analysis.

Molecular analysis of the skin and/or LN samples of all three patients classified as having Sézary's syndrome (SS) showed clear clonal *TCRG* and *TCRB* gene rearrangements by PCR and SB analysis (Figures 2 and 3).

Table 2. Frequency of clonal *TCRB* and *TCRG* rearrangements in the skin or lymph node (LN) of CTCL, ATLL and NK-cell lymphoma patients, classified according to histopathologic and clinical criteria as assessed by PCR heteroduplex/GeneScan analysis and *TCRB* Southern blot analysis.

	Number (%) of patients with clonal TCR gene rearrangements in skin and/or LN									
	Skin					*Skin and/or LN*				
	TCRG		*TCRB*			*TCRG*		*TCRB*		
Diagnosis (no.of patients)	*HD*	*GS*	*HD*	*GS*	*SB*	*HD*	*GS*	*HD*	*GS*	*SB*
Mycosis fungoides (n=16)										
early; stage I-II (n=9)	4/8 (50)	5/8 (63)	4/8 (50)	5/8 (63)	6/9 (67)	4/8 (50)	5/8 (63)	4/8 (50)	5/8 (63)	6/9 (67)
late; stage III-IV (n=7)	4/6 (67)	4/6 (67)	4/6 (67)	4/6 (67)	4/6 (67)	5/7 (71)	5/7 (71)	5/7 (71)	5/7 (71)	5/7 (71)
Sézary's syndrome (n=3)	2/2 (100)	2/2 (100)	2/2 (100)	2/2 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)
Large cell CD30+ CTCL,										
Anaplastic and pleomorphic (n=5)	3/4 (75)	4/4 (100)	3/4 (75)	3/4 (75)	3/4 (75)	3/4 (75)	4/4 (100)	3/4 (75)	3/4 (75)	3/5 (60)
Lymphomatoid papulosis (n=3)	1/3 (33)	1/3 (33)	1/3 (33)	1/3 (33)	0/3 (0)	1/3 (33)	1/3 (33)	1/3 (33)	1/3 (33)	0/3 (0)
Pleomorphic small sized CTCL (n=1)	1/1 (100)	1/1 (100)	0/1 (0)	0/1 (0)	1/1 (100)	1/1 (100)	1/1 (100)	0/1 (0)	0/1 (0)	1/1 (100)
CD8+ CTCL (n=1)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)
T-NHL (with skin involvement) (n=1)	1/1 (100)	1/1 (100)	0/1 (0)	0/1 (0)	1/1 (100)	1/1 (100)	1/1 (100)	0/1 (0)	0/1 (0)	1/1 (100)
ATLL (with skin involvement) (n=1)	ND	ND	ND	ND	1/1 (100)	0/1 (0)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)
All CTCL (n=31)	17/26 (65)	19/26 (73)	15/26 (58)	16/26 (62)	20/29 (69)	19/29 (66)	22/29 (76)	18/29 (62)	19/29 (66)	21/31 (68)
NK-cell lymphoma (n=1)[a]	ND	ND	ND	ND	ND	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)

HD: heteroduplex analysis; GS: GeneScan analysis; SB: Southern blot analysis; TCRB: T-cell receptor β gene; TCRG, T-cell receptor γgene; ND: not done;
[a]Clonal (episomal) EBV genome was observed.

Skin samples of four patients with anaplastic/pleomorphic CD30+ CTCL were analyzed molecularly. Three samples showed clonal *TCRG* gene rearrangements by PCR HD analysis and all four by GS analysis. Molecular analysis of the *TCRB* locus demonstrated clonal T-cell populations in three out of four skin samples (Figure 4). SB analysis of a LN sample of a fifth patient did not show clonal *TCRB* gene rearrangements. In one of the three patients diagnosed with lymphomatoid papulosis (*LyP*), a clonal T-cell population could be detected by TCR PCR analysis of the skin sample.

The remaining four cases in our study were patients with T-cell lymphoma/ leukemia with skin involvement: pleomorphic small sized CTCL (n=1), CD8+ CTCL (n=1), primary nodal T-NHL (n=1) and adult T-cell leukemia lymphoma (ATLL; n=1). PCR GS analysis of the *TCRG* locus showed clonal rearrangements in all four patients (100%). Clonal *TCRG* gene rearrangements in the LN sample of the ATLL patient were not detected by HD analysis. Clonally rearranged *TCRB* genes were only detected in skin and/or LN DNA of the CD8+ CTCL and ATLL patients by PCR analysis but in all four by SB analysis.

We also investigated the presence of deletions affecting the *TAL1* gene, but were not able to detect *SIL/TAL1* fusion genes in any of the analyzed CTCL patients. Moreover clonal Ig gene rearrangements were not found in skin and/or LN samples of any of the CTCL patients either.

Finally one case of NK-cell lymphoma was included in this study. A LN sample from this patient was analyzed (Table 2). No clonal *IGH* and *TCR* gene rearrangements could be detected by PCR or SB analysis. However, we did detect clonal EBV genome in the involved LN by SB analysis.

Ig/TCR gene rearrangement analysis in benign dermatoses

Our study included eight patients with dermatitis. Polyclonal TCR and Ig gene rearrangements were detected in cutaneous lesions in seven out of eight (88%) patients confirming the presence of reactive T and B cells. Remarkably one patient showed clonal *TCRB/TCRG* gene rearrangements in skin and PB by PCR HD/GS analysis, but not by SB analysis. However, on clinical, histomorphologic, cytomorphologic and immunphenotypic grounds there was no suspicion of a CTCL in this patient.

Furthermore, two patients diagnosed with *histiocytosis*, one with *M. Jessner* and one with *mucinosis follicularis* only showed polyclonal T and B cells in their skin samples.

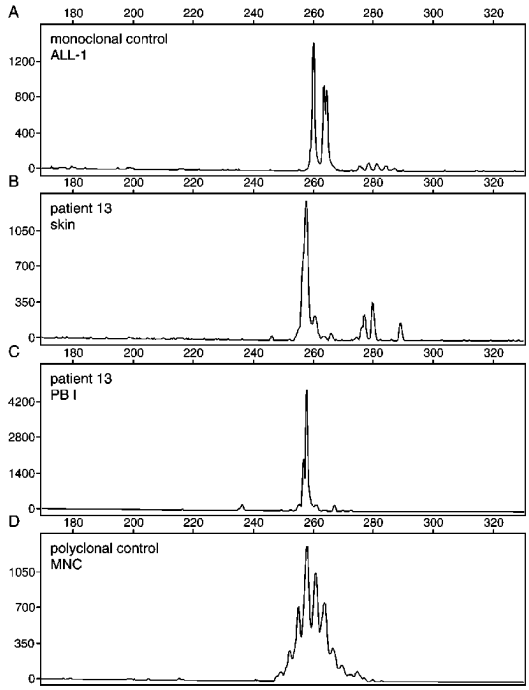

Figure 3. GeneScan analysis of PCR products of *TCRB* gene rearrangements using multiplex combination A. Monoclonal *TCRB* gene rearrangements can be identified in ALL-1 cell line DNA and skin and PB DNA from patient 13, diagnosed with Sézary's syndrome. Polyclonal *TCRB* gene rearrangements can be identified in MNC DNA.

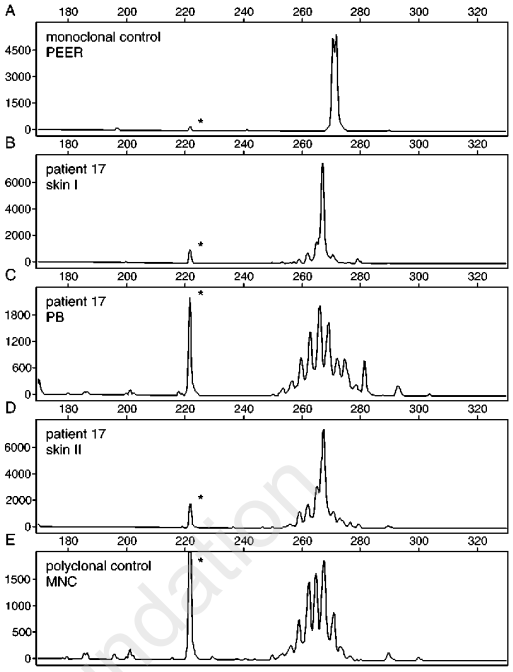

Figure 4. GeneScan analysis of PCR products of *TCRB* gene rearrangements using multiplex combination B. Monoclonal *TCRB* gene rearrangements can be identified in PEER cell line DNA and skin DNA from patient 17, diagnosed with CD30⁺ CTCL. Skin samples were taken from two different sites. Polyclonal *TCRB* gene rearrangements can be identified in PB DNA from patient 17 and MNC DNA. Asterisks indicate an aspecific peak.

Detection of identical clonal Ig/TCR rearrangements in extracutaneous tissues

The presence of identical clonal Ig/TCR gene rearrangements in LN, PB, BM, ascites and/or synovium specimens was investigated in patients with a clonal B-/T-cell population in the skin (Table 4).

Table 4 shows that gene rearrangement analysis of the draining LN is of additional diagnostic value, because an identical clonal B- or T-cell population could be detected in 9/12 (75%) CBCL/CTCL cases. Identical clonal TCR gene rearrangements were detected in 4/7 (57%) BM samples of CTCL patients and clonally rearranged *IGH* genes, as identified in the skin, were detected in one out of two BM samples of CBCL patients. In these patients with clonal rearrangements in the BM samples, suspicious atypical cells were identified by cytomorphologic analysis of the BM biopsy specimen.

Identical clonal TCR gene rearrangements were found in the PB of two out of three patients with late stage MF and in all patients with SS. In these patients, data from molecular clonality analysis and cytomorphologic analysis were concordant. Clonally rearranged *IGH* genes were detected in the PB of four out of five B-NHL patients with secondary skin involvement. However, cytomorphologic analysis of the PB of these patients did not show any atypical lymphocytes.

Remarkably, clonally rearranged *IGH* genes were repeatedly found in PB samples of a patient with MF stage I, suggesting that this patient was not only suffering from MF (in the skin), but probably also from an indolent chronic B-cell leukemia. Dual genotypes in the same patient have recently been described to be a quite frequent event.[33] On the other hand, clear clonal *TCRG/TCRB* gene rearrangements were detected in the PB sample by PCR HD/GS analysis in a patient diagnosed with a nodal B-NHL as having secondary skin involvement.

Although unexpected, clonal *TCRG* and *TCRB* gene rearrangements were found in PB and BM by PCR analysis in one patient diagnosed with dermatitis (Table 3). Cytomorphologic analysis did not reveal any atypical lymphocytes in these samples.

One MF stage IV patient showed ascites according to CT scanning. Gene rearrangement analysis of the ascites demonstrated clear clonal *TCRG* and *TCRB* gene rearrangements, identical to those found

Table 3. Frequency of clonal IGH/TCR rearrangements in the skin of patients with benign dermatoses, according to histopathologic and clinical criteria, as assessed by PCR heteroduplex/Genescan analysis and Southern blot analysis.

	Number (%) of patients with clonal IGH/TCR rearrangements		
	Skin		
	IGH/TCR		
Diagnosis (no. of patients)	HD	GS	SB
Dermatitis[a] (n=8)	1[a]/8 (13)	1[a]/8 (13)	0/7 (0)
Histiocytosis[b] (n=2)	0/2 (0)	0/2 (0)	0/2 (0)
M. Jessner (n=1)	0/1 (0)	0/1 (0)	0/1 (0)
Mucinosis follicularis (n=1)	0/1 (0)	(0/1)	0/1 (0)
Benign lymphoproliferations (n=12)	1/12 (8)	1/12 (8)	0/12 (0)

HD: heteroduplex analysis; GS: GeneScan analysis; SB: Southern blot analysis; IGH: immunoglobulin heavy chain gene; TCR: T-cell receptor gene.
[a]The clonal TCRG and TCRB gene rearrangements were observed by PCR analysis only. [b]In one case of histiocytosis clonal TCRG and TCRB gene rearrangements were observed in BM by PCR analysis and in PB by PCR and SB analysis.

Table 4. Detection of identical clonal Ig/TCR rearrangements in extracutaneous tissues in CBCL/CTCL patients, as assessed by PCR and/or Southern blot analysis.

Diagnosis	*LN*	*BM*	*PB*	*Other*
CBCL				
Primary cutaneous follicle center cell lymphoma	n.a.	n.a.	0/3	n.a.
Large B-cell lymphoma of the legs	1/1	0/1	0/2	n.a.
Immunocytoma	n.a.	n.a.	n.a.	n.a.
B-NHL with skin involvement[a]	n.a.	1/1	4/5	n.a.
Pseudo B-cell lymphoma	n.a.	n.a.	1/2	n.a.
CTCL				
Mycosis fungoides				
early; stage I-II[b]	0/1	0/2	0/6	n.a.
late; stage III-IV	2/3	1/1	2/3	2/2[c]
Sézary's syndrome[d]	3/3	2/2	3/3	n.a.
Anaplastic pleomorphic CD30+ CTCL	n.a.	0/1	0/3	n.a.
Lymphomatoid papulosis	0/1	n.a.	0/3	n.a.
Pleomorphic small sized CTCL	1/1	1/1	n.a.	n.a.
CD8+ CTCL	n.a.	n.a.	0/1	n.a.
T-NHL (with skin involvement)	1/1	n.a.	n.a.	n.a.
ATLL (with skin involvement)	1/1	n.a.	1/1	n.a.

n.a.; not applicable; aClonal TCRG/TCRB gene rearrangements were found by PCR analysis in the PB of one patient as well; bClonal IGH gene rearrangements were repeatedly detected in PB samples of one MF stage I patient; cRearrangement analysis was performed on ascites and a synovium sample; dA fourth SS patient showed identical T-cell clones in PB and BM samples, but no skin/LN samples were available.

in skin, LN, BM and PB (Figure 5). From another MF stage IV patient, suffering from arthritis deformans of the hands,[34] a synovium biopsy was taken. Gene rearrangement analysis demonstrated clonal *TCRB* gene rearrangements that were identical to the rearrangements observed in the skin lesion of this patient.

Discussion

We studied a group of 60 patients with an initial suspicion of (primary) cutaneous lymphoma to evaluate the contribution of molecular analyses in the diagnostic process of this special group of non-Hodgkin's lymphomas (NHL). A series of 144 (tissue) DNA samples was analyzed by SB analysis. The same samples and ten additional ones were analyzed by two PCR-based strategies (PCR HD and PCR GS analyses). In these strategies, we used well-defined sets of primers and PCR protocols, optimized and standardized in a recently finished European BIOMED-2 Concerted Action.[20] The results of all three techniques were compared and related to the clinico-pathologic diagnosis.

In skin samples of patients with histomorphologically proven CBCL clear clonal *IGH* rearrangements were observed in 92% of patients as assessed by SB analysis, but in 67% by PCR analysis. However, PCR analysis of the FR1 *IGH* locus demonstrated clonal products of a larger size (*see explanation in Results*

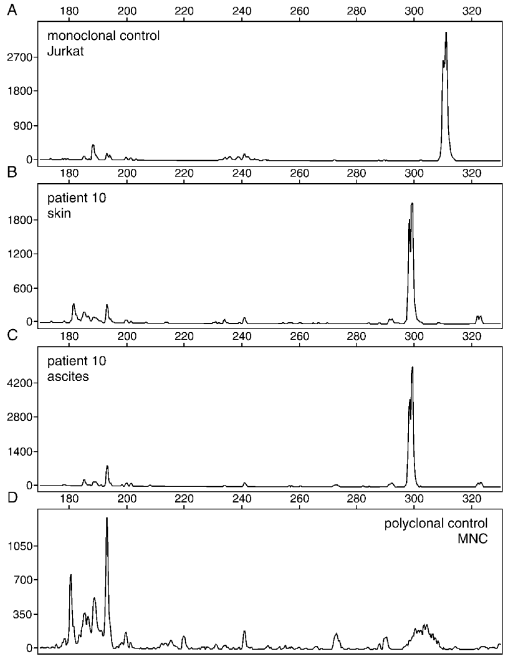

Figure 5. GeneScan analysis of PCR products of *TCRB* gene rearrangements using multiplex combination C. Monoclonal *TCRB* gene rearrangements can be identified in Jurkat cell line DNA and skin and ascites DNA from patient 10, diagnosed with mycosis fungoides, stage IV. Polyclonal *TCRB* gene rearrangements can be identified in MNC DNA.

section) in two additional patients, which was confirmed by *IGK* clonality in both patients. This raised the overall PCR-based clonality detection to 83% among CBCL patients. Thus, despite the additional *IGK* analyses, a 100% score was not reached by PCR analysis. This can partly be explained by somatic hypermutation in the V_H-D_H-J_H gene rearrangements of primary CBCL,[35] which may hamper the annealing of V_H-FR primers and, to a lesser extent, of J_H primers.[21,36] Polyclonal *IGH* PCR results in clinically suspicious skin samples should, therefore, be interpreted with caution and should at any time be checked by additional PCR assays using *IGK* and *IGL* primers, or by performing SB analysis. Two patients diagnosed with primary nodal follicular lymphoma with secondary skin involvement, but none of our primary CBCL patients, demonstrated a t(14;18) translocation in skin and PB. This confirms earlier findings that the t(14;18) translocation does not occur in primary CBCL.[37]

Interestingly, two out of four patients with pseudo B-cell lymphoma in our study had clonal *IGH* rearrangements in the skin lesions detectable by SB analysis, but not by PCR analysis. In one of these, clonal *IGK* gene rearrangements were detected by PCR analysis. Although pseudo B-cell lymphomas should be considered as benign lymphoid infiltrates, clonal B-cell populations have been found in a significant number of cases.[38,39] This may support the concept that pseudo B-cell lymphomas and primary B-cell lymphomas are part of a continuous and progressive spectrum of CBCL.[38] In addition, a t(14;18) translocation was detected in skin samples of a patient with pseudo B-cell lymphoma without clonal Ig gene rearrangements. Regular follow-up of these three patients with clonality is therefore recommended.

Of the 31 patients with histomorphologically proven CTCL (ATLL included, NK-cell lymphoma excluded) in our study, clonal *TCRG* gene rearrangements were detected in 66% (PCR HD analysis) and 76% (PCR GS analysis) in skin and/or LN biopsies. Gene rearrangement analysis of the *TCRB* locus showed dominant T-cell clones in 68% by SB analysis, in 62% by PCR HD analysis, and in 66% by PCR GS analysis in skin and/or LN samples. Overall GS analysis had a slightly higher detection rate than HD analysis. Combination of *TCRG* and *TCRB* gene rearrangement analyses resulted in a slightly higher PCR HD clonality detection rate (69%), but the detection rate reached by PCR GS analysis did not change. Previous studies reported the involvement of translocation t(2;5) in a small number of primary CTCL.[9,40] In our study no involvement of t(2;5) could be detected (*data not shown*).

Molecular analysis of TCR genes appears to be a very sensitive technique for detecting clonal T-cell populations. Previous studies reported higher TCR clonality detection rates by PCR analysis.[8,11,41] However, our series included many patients who were diagnosed with early stage MF (29%), a condition at least partly known for its oligoclonal nature.[42] The PCR clonality detection rate in early stage MF I-II patients was relatively low in our series (Table 2). PCR GS analysis reached the highest rate of detecting clonality (63%), this rate being comparable to that found by others.[43] Because of the oligoclonal nature, polyclonal/oligoclonal profiles have been detected in early MF skin lesions.[44] Data in the literature report infiltration of numerous reactive non-malignant lymphocytes in early MF lesions.[45,46] The ratio of clonal tumor cells to reactive polyclonal cells might, therefore, be lower than the detection threshold of the PCR analysis.[47] In as many as 38% of early forms of MF no clonal T-cell population could be detected after PCR analysis of the *TCRG* locus.[47] Therefore, the finding of polyclonality/oligoclonality in patients with a cutaneous lymphoproliferation does not completely exclude a CTCL. Our study also included three patients with LyP. Only some patients diagnosed with LyP have a monoclonal T-cell proliferation.[48] When early stage MF and LyP were not considered, *TCRG* clonality was detected in 78% (PCR HD) and 89% (PCR GS) whereas *TCRB* clonality was detected in 72% (PCR HD and GS) and 79% (SB).

In patients with an initial suspicion of cutaneous T- or B-cell lymphoma, the finding of a molecularly confirmed clonal/polyclonal B- or T-cell population in skin and/or other tissues can be of additional diagnostic value. PCR and SB analyses can give relevant information, especially in patients who cannot be diagnosed by conventional methods, such as histomorphology, immunophenotyping, and cytological analysis. Detection of a clonal B- or T-cell population can be an early sign of malignancy which should therefore result in careful follow-up of the involved patients. The finding of a molecularly confirmed polyclonal/oligoclonal lymphocyte proliferation, especially in patients without true signs of malignancy, makes a benign process more likely.

One out of twelve patients (8%) with benign dermatoses showed clonal T-cell populations by PCR analysis of the *TCRG* and *TCRB* genes in the skin sample, but not by SB analysis of *TCRB* genes. Also, the same clonal TCR rearrangements were demonstrated after independently repeated *TCRG* and *TCRB* PCR analysis, thereby excluding the possibility of *pseudoclonality* due to a low frequency of polyclonal T-lymphocytes.[49] Although *clonal dermatitis* is known to progress to overt CTCL in 25% of cases,[50] monoclonality does not necessarily imply malignancy and molecular results should therefore always be correlated with the clinical, histopathologic and phenotypic data of the individual

patient.[51] Clinical and histologic follow-up of the skin lesions of this and similar patients is thus strongly recommended. Unfortunately, our patient died of heart failure preventing further molecular and clinical follow-up in order to evaluate possible progression of the clonal skin lesions to overt CTCL.

Our BIOMED-2 multiplex PCR approach is a rapid, cheap, and simple procedure, which is therefore very suitable in a diagnostic setting. Because of the large number of concordant results between the histopathologic diagnosis and the PCR analyses, we can conclude that both HD and GS analyses of Ig/TCR rearrangements are very helpful in diagnosing patients with cutaneous lymphoproliferative disorders.

In general GS analysis might be slightly favored over HD analysis because of the former's speed, accuracy, sensitivity, and easy interpretation.[21-23] However, the latter can be a cheaper and reliable alternative in a diagnostic setting, because expensive automated sequencing equipment and fluorochrome-labeled oligonucleotides are not necessary.

Extracutaneous presence of identical clonal T-cell populations is not exceptional in primary CTCL.[13,52-54] In our patients with early stage MF no clonal TCR rearrangements were identified in samples from extracutaneous sites. However, identical clones were detected in PB, LN and BM samples of 67% of patients with late stage MF. Furthermore, the same clonal B- or T-cell populations as those identified in the skin were detected in regional LN samples of nine out of twelve CBCL/CTCL cases. In two MF stage IV cases, gene rearrangement studies demonstrated the presence of the same clonal T-cell populations in skin and in uncommon extracutaneous locations, such as ascites and synovium. Dissemination of clonal lymphocytes in extracutaneous tissues can be easily detected by Ig/TCR analysis and is very helpful for staging.

In conclusion, our results show that the rate of clonality detection by the novel standardized BIOMED-2 based PCR analysis of rearranged *IGH* genes in CBCL is 83%, which is only slightly lower than the SB detection rate (92%), and can be further increased by *IGK* PCR analysis. However, because of the relatively low number of CBCL patients the small difference in sensitivity between SB and PCR analysis did not reach statistical significance. Furthermore, our study shows that BIOMED-2 PCR-based GS analysis of rearranged *TCRG* genes enables clonality detection in a rather high percentage of CTCL (76%). Because of this high detection rate and its speed, this technique is recommended for clonality analysis of tissue samples from patients initially suspected of having primary CTCL.

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Pre-publication Report & Outcomes of Peer Review

Contributions

YS is the actual laboratory investigator on the project, who did all BIOMED-2 PCR assays, interpreted data, and extensively contributed to first draft and final version. FH and PJL are the dermatologist and hematologist who saw the patients, helped to design the study, and largely contributed to the writing process. KL is the pathologist who evaluated the skin and lymph node biopsies and also critically contributed to the writing process. ILMW-T is a very experienced senior technician who started up the PCR asays in the laboratory and did most of the Southern blot assays and critically reviewed the draft and final version of the paper. JJMvD is head of Molecular Immunology and was involved in design, interpretation and critical reading. AWL is the actual project leader under whose supervision the whole study was conducted; he was actively involved in all phases from design, analysis, interpretation, and drafting and revising the article. All authors approve the final version of the manuscript as it is submitted to Haematologica.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Gianluca Gaidano, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Professor Gaidano and the Editors. Manuscript received November 26, 2002; accepted May 6, 2003.

In the following paragraphs, Professor Gaidano summarizes the peer-review process and its outcomes.

What is already known on this topic

Molecular analysis of rearrangements of IgV or TCR genes is widely used in assessing the clonality status of a given B-cell or T-cell lymphoproliferation, for which immunohistochemistry could not resolve the clonal status. Clonality of IgV and TCR genes may be assessed by a variety of molecular techniques, including Southern blot and PCR-based strategies.

What this study adds

This study provides a comparative analysis of different molecular strategies for defining IgV and TCR clonality in cutaneous lymphoproliferatons. PCR-based methods appear to display the highest yeild of rearrangement detection and may represent the approach of first choice. The primers adopted in the BIOMED-2 study may provide researchers and clinical pathologists with a well standardized assay for interlaboratory comparison.

Caveats

Molecular diagnosis should complement, and not substitute for, morphological and immunohistochemical diagnosis. Also, when using formalin-fixed tissues, the results of the molecular assays may be dependent upon the conservation quality of the tissue.