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TCRγ-Chain Gene Rearrangement by PCR-Based GeneScan: Diagnostic Accuracy Improvement and Clonal Heterogeneity Analysis in Multiple Cutaneous T-Cell Lymphoma Samples

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Cutaneous T-cell lymphomas are a heterogeneous group of lymphomas where the tumor population emerges within a multiple subclone pattern ("clonal heterogeneity"). PCR analysis has been shown to be useful in the diagnosis of mycosis fungoides (MF) and Sézary Syndrome (SS). Focusing the attention on clonal heterogeneity, the efficacy of the multiplex/heteroduplex (HD) PCR and the GeneScan (GS) capillary electrophoresis analysis was compared in the early diagnosis of MF/SS, using a multiple sample approach. Indeed, GS demonstrated TCRγ gene rearrangement (GR) in all the 57 SS (100%) and in 123/146 (84%) of the MF samples, whereas the multiplex/HD PCR was less sensitive. An increase in clonality was observed in connection with both a worsening of the cutaneous disease (79% T1/T2; 100% T3/T4) and an increase in the histopathological score (HS <5, 76%; HS $\geqslant 5$, 94%). Clonal heterogeneity with adjunctive reproducible skin TCRγ-GRs was also observed. "Clonal instability," with different GRs, was present in a small percentage of patients. Therefore, it can be concluded that GS analysis in TCRγ-GR is able to improve diagnosis in MF/SS patients and the multiple sample approach is helpful for a correct interpretation of clonal patterns in skin lesions, especially in early-stage MF and in SS skin/blood samples.

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INTRODUCTION

Clonal TCR gene rearrangement (GR), demonstrated by PCR, is the most effective method to evaluate clonality in T-cell malignant neoplasms. When T cells develop in the thymus, they rearrange the TCR genes (Hayday *et al.*, 1985). The γ-chain gene, although typically not expressed on the cell surface, remains rearranged in the T-cell genome and provides a conventional clonality marker (Theodorou *et al.*, 1994; Wood *et al.*, 1994a, b). Various PCR methods, based on different techniques to detect clone-specific PCR products, have been developed (Beaubier *et al.*, 2000; Sprouse *et al.*, 2000; Sandberg *et al.*, 2003; Costa *et al.*, 2004). We reported a multiplex/heteroduplex (HD) PCR method for the detection of T-cell clonality (Ponti *et al.*, 2005). This method, used routinely on cutaneous T-cell lymphomas (CTCL), offers a rapid and practical tool with a high specificity (97.7%) and sensitivity (83.5%) (Ponti *et al.*, 2005). However, sensitivity reached 100% only at the tumor stage and erythrodermic mycosis fungoides (MF) (T3/T4) stage, whereas it was lower in the early stages (T1, 71.4%; T2, 76.1%).

There is increasing evidence that CTCL are a heterogeneous group of lymphoid malignant neoplasms, in which the tumor population, of monoclonal origin, emerges frequently within a multiple subclone pattern.

Rubben *et al.* (2004) studied the mutation pattern within microsatellite DNA and suggested that the tumor cell population in MF is often made up of multiple subclones and that tumor evolution in CTCL may display multilineage progression. Only a few studies have analyzed the influence that clonal heterogeneity has on the correct interpretation of clonal pattern and, consequently, on the diagnosis of CTCL samples (Dippel *et al.* 2001; Klemke *et al.*, 2002, 2006).

These data prompted us to conduct a prospective study of cutaneous T-cell clonality in a large series of 270 biopsies from patients with a final diagnosis of either CTCL (141) or chronic benign inflammatory dermatoses (ID; 55) and in blood samples from erythrodermic patients with a clinical

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Abbreviations: CTCL, cutaneous T-cell lymphoma; GR, gene rearrangement; GS, GeneScan; HD, heteroduplex; HS, histopathological score; ID, inflammatory dermatosis; MF, mycosis fungoides; MRD, minimal residual disease; SS, Sézary syndrome; TCR-Vβ+, TCR β chain phenotypical restriction; TNM, tumor node metastasis

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suspicion of Sézary syndrome (SS). The TCRγ-GR was studied by PCR and GeneScan (GS) analysis using fluorescently labelled PCR primers and capillary electrophoresis. Although GS protocol allows an accurate comparative evaluation of the size and V-family type of clonal TCRγ-GR products, only a few studies, on a limited number of CTCL samples, are available (Dippel *et al.*, 1999, 2001; Sprouse *et al.*, 2000; Assaf *et al.*, 2000; Klemke *et al.*, 2002; Lukowsky *et al.*, 2002; Vega *et al.*, 2002; Sandberg *et al.*, 2003; Costa *et al.*, 2004). The PCR results were analyzed and compared to the clinical diagnosis, the stage of the disease, the histopathological score (HS), according to Guitart *et al.* (2001), as well as to the immunophenotypical data.

Our aim was to determine if this method combined with multiple sample approach may be useful to enhance the sensitivity of CTCL diagnosis and to overcome the bias related to the influence clonal heterogeneity has on the correct interpretation of the sample clonal patterns.

RESULTS

A prospective investigation of DNA isolated from skin specimens of 196 patients submitted for the determination of T-cell clonality, in support of clinical suspicion of CTCL, was carried out. Two or more skin biopsies were available in 58 (29%) patients (Table 1). A total of 270 skin biopsies were analyzed, confirming suspicion of CTCL by histology in 178 samples (66%). Fifty-eight samples were classified as inflammatory dermatoses (psoriasis, contact dermatitis, atopic dermatitis, drug reaction) on the basis of histological data and clinical findings. The remaining 34 samples were initially considered non-diagnostic, as their clinicopathological features were not consistent with either MF or any specific type of inflammatory dermatosis (ID). Twenty-five of these samples were harvested from patients with a previous or subsequent histological diagnosis of MF/SS, and 18 of them were reclassified as ''early MF/SS'' owing to the rescue of a specific clonal stable population by the multiple sample GS analysis (Tables 1 and 2).

Multiplex/HD analysis

All the samples ($n=270$) were assessed for T-cell clonality using both multiplex/HD PCR and GS analysis (Table 2). A dominant clone was found by Multiplex/HD PCR analysis in 98/146 (67%) and in 49/57 (86%) of total samples from MF and SS patients, respectively. As reported previously (Ponti *et al.*, 2005), there was a higher percentage of clonal cases in the samples from MF subjects, not only with the worsening of the disease (60% T1/T2 *vs* 89% T3/T4, $P<0.001$), but also with the increase of HS (HS<5, 53%; HS$\geqslant 5$, 85%; $P<0.0001$). Noteworthy is the high clonality demonstrated in 23/29 (79%) T1/T2 samples with an HS$\geqslant 5$.

A T-cell clone was observed in 3/58 samples with a histological diagnosis of inflammatory dermatosis (ID) and in 14/33 lesions with a non-diagnostic histological pattern. Interestingly, 9/14 samples were obtained from patients with a previous or subsequent diagnosis of CTCL and successively reclassified in the group of ''early MF/SS'' cases.

GS analysis

The dilution series analysis of two clonal V-β-TCR (TCR-Vβ+) samples with biallelic GRs (biallelic VγI-Jp GR; biallelic VγI-Jγ/VγII-Jγ GR) showed that the sensitivity differs if the test is used for the detection of minimal residual disease (MRD) of a known monoclonal population, rather than for the identification of a new unknown population. Indeed, a sensitivity of 1 and 5% dilution was observed in the two series of TCR-Vβ+ sample dilution, used to detect an unknown clonal population. On the contrary, the MRD approach demonstrated the persistence of a known clonal rearrangement at a dilution of 0.1% in the first sample (biallelic VγI-Jp GR) and of 0.5–1% in the second (biallelic VγI-Jγ/VγII-Jγ GR).

A higher percentage of clonal cases was detected by GS than by multiplex/HD PCR (Table 2) in all the 57 biopsies from SS patients (100%) and in 123/146 (84%) MF samples. As already demonstrated with multiplex/HD analysis, the percentage of clonal cases in MF group increased, not only with the worsening of the cutaneous disease (79% T1/T2 *vs* 100% T3/T4), but also with the increase in the HS (HS<5, 76%; HS$\geqslant 5$, 94%). Indeed, the T1/T2 samples with an HS$\geqslant 5$ showed a high clonal pattern (25/29, 86%). Clonality could not be detected in any of the ID samples, whereas a T-cell clone was observed in 20/33 non-diagnostic histological samples; interestingly, 18 of these had been obtained from patients who showed a final diagnosis of MF/SS during followup.

Table 1. Immunopathological diagnosis on skin samples (n=270)

		Immunopathological diagnosis on skin samples					
		Non-diagnostic		ID	MF	SS	Samples
Patient's disease	Patients	Unclassifiable	Reclassified (early CTCL)				
ID	55	9		58			67
MF	108	7	11		128		146
SS	33		7			50	57
Total	196	16	18	58	128	50	270

ID, inflammatory dermatoses; MF, mycosis fungoides; SS, Sézary syndrome; non-diagnostic: samples with clinical and histopathological features not consistent with either MF or any specific type of inflammatory disease; reclassified early CTCL: samples with clinical and histopathological features not consistent with MF/SS or ID but with rescue of a stable clonal population by multiplex sample GS analysis.

Table 2. T-cell clonality: multiplex/HD PCR and GS on 270 samples taken from 196 patients

	No. of samples	Clonal HD, no. (%)	Clonal GS, no. (%)
Samples from SS patients	57	49 (86)	57 (100)
Diagnostic SS samples	50	46 (92)	50 (100)
Samples from MF patients	146	98 (67)	123 (84)
Diagnostic MF samples	128	92 (72)	112 (87)
Sample score ⩾5 from MF patients	66	56 (85)	62 (94)
Sample score <5 from MF patients	80	42 (53)	61 (76)
Samples from clinical T1/T2 patients	109	65 (60)	86 (79)
Samples from clinical T3/T4 patients	37	33 (89)	37 (100)
Samples with histological score >5 from T1/T2 patients	29	23 (79)	25 (86)
Inflammatory dermatosis (ID)	58	3 (5)	0
Reclassified early CTCL samples	18	9 (50)	18 (100)
Unclassifiable	16	5 (31)	2 (12)

As each Vγ family primer was labelled with a different fluorescent dye, it was possible to identify the Vγ family used in each clonal TCRγ-GR: VγI was the most common Vγ family (75%) observed in MF and SS, followed by VγIII/IV (24%) and VγII (7%). Two rearranged alleles were observed in 57 (40%) of the CTCL patients.

GS on multiple skin biopsies

A total of 132 multiple skin biopsies were analyzed to further investigate clonal heterogeneity. Among them, 109 were taken from 47 CTCL patients (27 MF, 20 SS), whereas the remaining 23 were performed in 11 non-CTCL patients who were compared as to their clonal TCRγ-GR pattern. A patient was considered to have a ''stable clonal pattern'' when a stable and common TCRγ-GR was present in multiple skin samples. An ''unstable clonal pattern'' was defined by the rescue of different prominent GRs in sequential samples from the same patient. A patient was considered to have a ''clonal heterogeneity'' when a common TCRγ-GR was concomitant to additional reproducible GRs in multiple samples. If the exact size of a prominent TCRγ-GR peak was not confirmed on two or more repeated GS-PCR runs of the same sample, the case was regarded as pseudo-monoclonal and was included in the oligo-polyclonal group.

A clonal GR was detected in 107/109 of the samples from CTCL patients. A stable clonal pattern was demonstrated in 18/47 (38%) CTCL patients, whereas a clonal heterogeneity occurred in the remaining 29. In the latter group, one patient showed an unstable clonal pattern.

SS sample GS clonal assessment

Both skin and blood samples of the 33 erythrodermic patients with a diagnosis of SS were analyzed and compared with morphological and phenotypic features (Table 3). Clonality was tested on skin and blood at diagnosis in 30 patients and repeated on skin (12 patients had repeated biopsies) and blood at different followup times, whereas the PCR was not available on blood in 3 patients diagnosed before 1999.

All the patients showed a specific TCRγ-GR in the peripheral blood, also in the presence of a circulating Sézary cell count $<1{,}000\,\text{cells}\,\text{mm}^{-3}$ (8 patients; Table 3). Conversely, a TCRVβ restriction was detected by flow cytometry only in 19/33 (58%) subjects.

GS analysis allowed the detection of a clonal population in all the SS skin biopsies, whereas multiplex /HD PCR failed to detect the TCRγ-GR in at least one skin sample of six SS patients (patient no. 2, 5, 6, 8, 20, 27). All these patients showed a mild perivascular infiltrate, confirming that multiplex/HD PCR sensitivity is dependent on the extent of the lymphoid infiltration (Ponti *et al.*, 2005). In these cases, GS analysis showed the major clonal involvement on blood compared with skin infiltration (Figure 1).

In two patients who achieved a clinical complete remission after treatment with mAb anti-CD52, we could investigate the MRD by means of multiplex/HD PCR and GS: we noticed that multiplex/HD PCR became negative in one of these patients when the number of circulating TCR-Vβ+ atypical cells dropped below $50\,\text{cells}\,\text{mm}^{-3}$, whereas GS always remained positive. In the other patient, we obtained a GS negativity on blood and skin when circulating TCR-Vβ+ atypical cells were 0.5% or $3\,\text{cells}\,\text{mm}^{-3}$.

A skin clonal heterogeneity with additional reproducible TCRγ-GRs was noted in 16/20 SS patients with repeated skin biopsies available (80%), 11 of whom had a constant individual prevalence of common TCRγ-GRs in all the skin and blood samples tested. The identification of the TCRγ-GR was even more difficult in the remaining five patients, owing to a non-dominant pattern in both skin and blood at diagnosis. In these cases, identity of the pathological peak was confirmed by comparing skin and blood patterns during the followup. Repeated GS analysis performed in four patients on the sorted TCRγ-Vβ+ population further confirmed this result (Table 3; Figures 1 and 2). No cases of pseudomonoclonal pattern were observed in SS patients.

MF sample clonal assessment

Multiple samples were available in 27 MF patients. Three out of 27 patients with multiple biopsies achieved complete remission after a median followup of 8 years, whereas 10 showed clinical progression. Nineteen are alive with the disease in course (Table S1).

Clonal heterogeneity was documented in 13/27 (48%) MF patients. There was no statistically significant correlation between the TCRγ clonal heterogeneity and clinical progression. Furthermore, an occult but stable clonal TCRγ-GR was identified by comparing the multiple skin samples, confirming the presence of disease in eight early-stage MF patients with a non-diagnostic histological picture then reclassified as early MF samples; sequencing confirmed the clonal TCRγ-GR in these cases (Table S1). To better illustrate the diagnostic efficacy of the comparison between multiple biopsies, Figure 3 and Figures S1 and S2 report detailed patient data.

Table 3. Immunophenotype and TCRγ GR studies in 33 SS patients

No.	Immunophenotype	L per mm^3	SS per mm^3	SS%	GS TCRγ GR (bp), skin and blood	HD skin	GS on skin cell suspension[1] or blood-sorted vβ+ cells[2]
1	CD7+CD26−	1,066	234	22	VIIγJγ (261)	+	+ (2×10^6)[2]
2	TCRVβ21CD2+/− CD3+/−CD7+/−CD26−	1,991	358	18	VIII/IVγJp(267)VIγJp(271)	+	
3	CD3+dimCD7+/−CD26−	2,608	652	25	VIII/IVγJp (267)	+	
4	TCRVβ22+CD26−	4,326	692	16	VIγJγ(299)VIγJγ(296)	+	
5	TCRVβ22+CD3dimCD7−CD26−	4,095	737	18	VIγJγ (283)	OLIGO	+(19%)[1]
6	TCRVβ17+CD2dimCD7−CD26−	1,938	814	42	VIγJp(269)VIII/IVγJγ(304)	POLI	+(22%)[1]; +(2.7×10^6)[2]
7	TCRVβ5.1+CD2dimCD3dim CD7+CD26−	1,818	818	45	VIγJγ (291) VIγJp(258)	+	+(49%)[1]
8	TCR Vβ3.1+CD7+CD26−/+	3,659	878	24	VIγJγ (292) VIγJγ(305)	POLI	+(19%)[1]
9	TCRVβ6.7+CD2dimCD7−CD26−	2,134	1,003	47	VIγJp (266) VIγJγ (298)	+	
10	CD7+CD26−	2,026	1,134	56	VIγJp(259)	+	
11	CD3dimCD7−CD26−	3,429	1,269	37	VIII/IVγJp(263)	+	
12	CD2+dimCD3+CD4+dimCD7+CD26−	3,446	1,378	40	VIIγJγ(251)	+	
13	TCRVβ13.1+CD2dimCD7−CD26−	2,236	1,431	64	VIγJγ (296)VIγJγ (262)	+	
14	TCRVβ5.2/5.3+CD3dimCD7+CD26−	2,561	1,434	56	VIIγJp(263)	+	
15	TCRVβ6.7+CD3dimCD7−CD26−	3,518	1,583	45	VIII/IVγJγ(294)	+	
16	CD7+CD26−	3,255	1,660	51	VIγJγ(297)	+	+(1.7×10^6)
17	CD2+dimCD3+dimCD7+CD26−	3,801	2,395	63	VIγJγ (296)VIγJγ (299)	+	+
18	CD2dimCD7dimCD26−CD45RA+	3,600	2,598	65	VIJγ (283)VIIJγ (257)	+	+(76%)[1]; +(1.3×10^6)[2]
19	TCRVβ16+CD3+dimCD7−CD26−/+	4,661	3,030	65	VIγJγ (296)VIγJγ (299)	+	
20	TCRVβ17+CD2dimCD7−CD26−	4,708	3,625	77	VIγJγ (284)	OLIGO	
21	TCRVβ13.2+CD2dimCD4dimCD26−	4,966	3,973	80	VIIγJp(225)	+	
22	CD7+CD26−	5,569	4,455	80	VIII/IVγJp(264)	+	
23	CD4+dimCD7−CD26±	10,848	6,075	56	VIγJγ(297)	+	
24	CD7+CD26−	6,603	6,339	96	VIγJγ (297)VIγJp (265)	+	
25	CD7+CD26−	8,226	6,581	80	VIγ(300)VIγ (294)	+	
26	CD7+CD26−	8,505	7,144	84	VIpJγ(262) VIII/IVγJp(267)	+	
27	TCRVβ2+CD3+dimCD7−CD26−	9,053	7,514	83	VIγJp(263)VIγJp(264)	OLIGO	
28	TCRVβ6.7+CD3+brightCD26−	11,316	9,053	80	VIγJγ(298)	+	
29	TCRVβ6.7+CD2±CD7−CD26−	12,454	11,084	89	VIγJγ(293)	+	
30	TCRVβ20+CD3+dimCD7−CD26−	15,939	15,301	96	VIγJγ(290)	+	
31	CD3+dimCD7−CD26−	31,493	30,863	98	VIγJγ(287)VIγJγ(298)	+	
32	TCRVβ9+CD7±CD26−	53,036	50,384	95	VIγJp(263)VIγJp(270)	+	
33	CD3+dimCD7−CD26−	5,789	3,994	69	VIγJp(260)	+	

bp, base pair; HD skin, multiplex/HD PCR results from skin samples; L per mm^3, absolute lymphoid cells number per mm^3; SS per mm^3, absolute SS cells number per mm^3; % SS, Sézary cells percentage on lymphoid cell; TCRγ GR, TCR γ gene rearrangement.
[1]Values in parentheses denote the percentage of atypical clonal cells within the total extracted lymphoid cells (from 20,000 to 115,000).
[2]Values in parentheses denote the number of cells sorted.

Briefly, the patient described in Figure 3 had an oligoclonal pattern in the first non-diagnostic biopsy and two dominant TCRγ-GR peaks in the second (also non-diagnostic) biopsy. The true TCRγ-GR occult in the first biopsy was further confirmed on a third skin sample, which was diagnostic at histology. A common clonal TCRγ-GR was observed in seven MF patients and different clonal TCRγ-GRs in one case, in a total of eight patients with simultaneous skin biopsies, taken from different sites of involvement. Pseudo-monoclonal (false-positive) pattern occurred in two MF patients and these samples were than classified as poly/oligoclonal sample by repeated PCR runs and multiple sample comparison. The

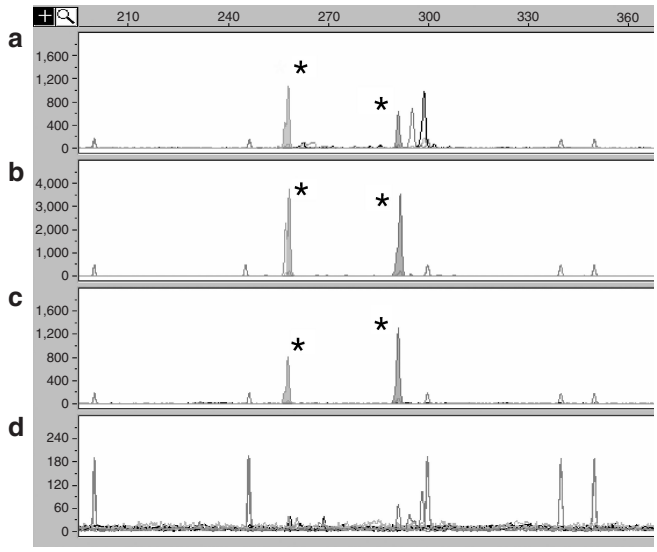

Figure 1. SS patient no. 7. (**a**) Ambiguous GS pattern in skin biopsy at diagnosis; (**b**) biallelic TCRγ-GR (*) confirmed by comparison with blood pattern at diagnosis; (**c**) GS on sorted CD4+CD26−Vβ5.1+ circulating lymphocytes; (**d**) GS on sorted CD4+CD26−TCR-Vβ5.1− circulating lymphocytes.

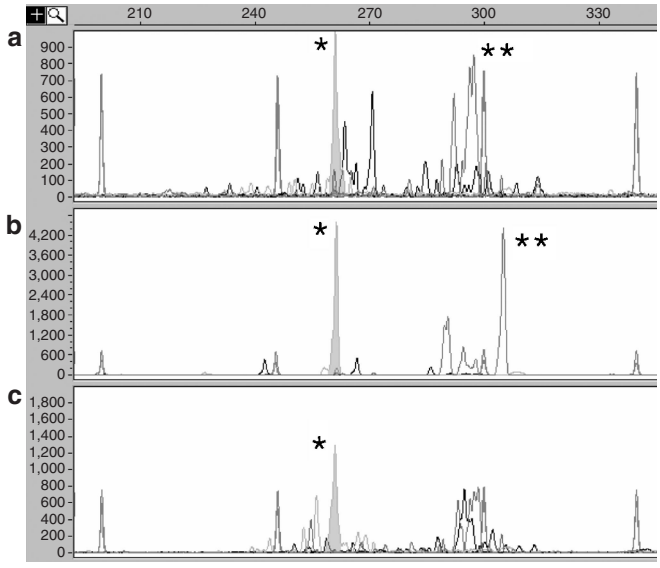

Figure 2. SS patient no. 1. (**a**) Ambiguous GS pattern in skin biopsy at diagnosis; TCRVγII GR (*) confirmed by comparison with blood pattern; (**b**) GS pattern in blood sample at diagnosis showing a pathological GR (*) and a concomitant transient GR (**); (**c**) TCRVγII clonal GR in the second skin sample.

rescue of a pseudo-monoclonal pattern is increased in blood samples (data not shown) leading to stress the importance of repetitive analysis and multiple sample approach.

ID GS clonality

A polyclonal/oligoclonal pattern was documented in 55/58 (95%) patients with skin IDs. Two erythrodermic patients showed a clonal TCRγ-GR coupled with discordant clinical and histopathological data. The first patient showed the persistence of a low and stable component of the clonal TCRγ-GR population both in skin and blood, and clinical examination evidenced the persistence of erythroderma. The second patient had persistent erythroderma and two non-diagnostic biopsies showed the onset of a small clonal TCRγ-GR in the second skin biopsy, whereas blood remained constantly polyclonal. Although there was an initial suspicion of clonal TCRγ-GR at multiplex/HD PCR analysis in another patient, GS analysis documented an oligoclonal pattern. Pseudo-monoclonal, false-positive results were obtained in 13 ID patients; repeated PCR runs and multiple sample comparison allowed reclassification of these cases as oligoclonal samples.

When all the non-diagnostic skin samples were considered as a whole, based on clinical outcome and all the available diagnostic techniques, there was a significant correlation between the finding of a clonal TCRγ-GR and the final diagnosis of CTCL ($P=0.02$).

DISCUSSION

PCR analysis for the detection of lymphoid clonality in clinical specimens has proven to be a useful and reliable technique in the diagnosis of CTCL. Moreover, the importance of a highly sensitive method to evaluate the clonality in these diseases has been stressed (Vega *et al.*, 2002; Rubben *et al.*, 2004), also to avoid the bias related to the frequent occurrence of oligo-/pseudo-clonal activation. However, only a few molecular studies on relatively low numbers of patients have reported clonal heterogeneity in CTCL (Bignon and Souteyrand, 1990; Dippel *et al.*, 2001; Vega *et al.*, 2002; Rubben *et al.*, 2004). Although the underlying mechanism of genetic instability in MF is still not yet fully clear, it is thought that it most probably evolves through a combination of clonal expansion (Dommann *et al.*, 1996; Muche *et al.*, 2003). Recently, the same authors stressed the importance of multiple PCR runs to avoid the problem of pseudo-monoclonality on single samples and the problematic biological interpretation of the rescue of T-cell clonality of undetermined significance over all blood samples (Dippel *et al.*, 2001; Klemke *et al.*, 2002; Vega *et al.*, 2002).

In this study, the PCR product detection was carried out by multiplex/HD PCR and GS analysis capillary electrophoresis on skin samples obtained from a large series of CTCLs (141 patients) in an attempt to combine the advantages of high sensitivity with accurate quantification and size determination. In the presence of a clinical suspicion of SS, GS was also carried out on blood samples.

The association between histological features and clonal evaluation was confirmed independently of cutaneous T-score with both methods. Indeed, there was no statistical difference in the percentage of clonal cases between the T1/T2 and T3/T4 specimens that shared a similar HS (HS5; $P=0.02$). Sensitivity in early-stage lesions appeared to be higher when the GS method was used than when the multiplex/HD PCR was used. In fact, considering all the diagnostic and non-diagnostic samples from the early-stage T1/T2 MF patients, multiplex/HD PCR revealed clonality in 60% of cases, whereas the percentage rose to 79% using GS.

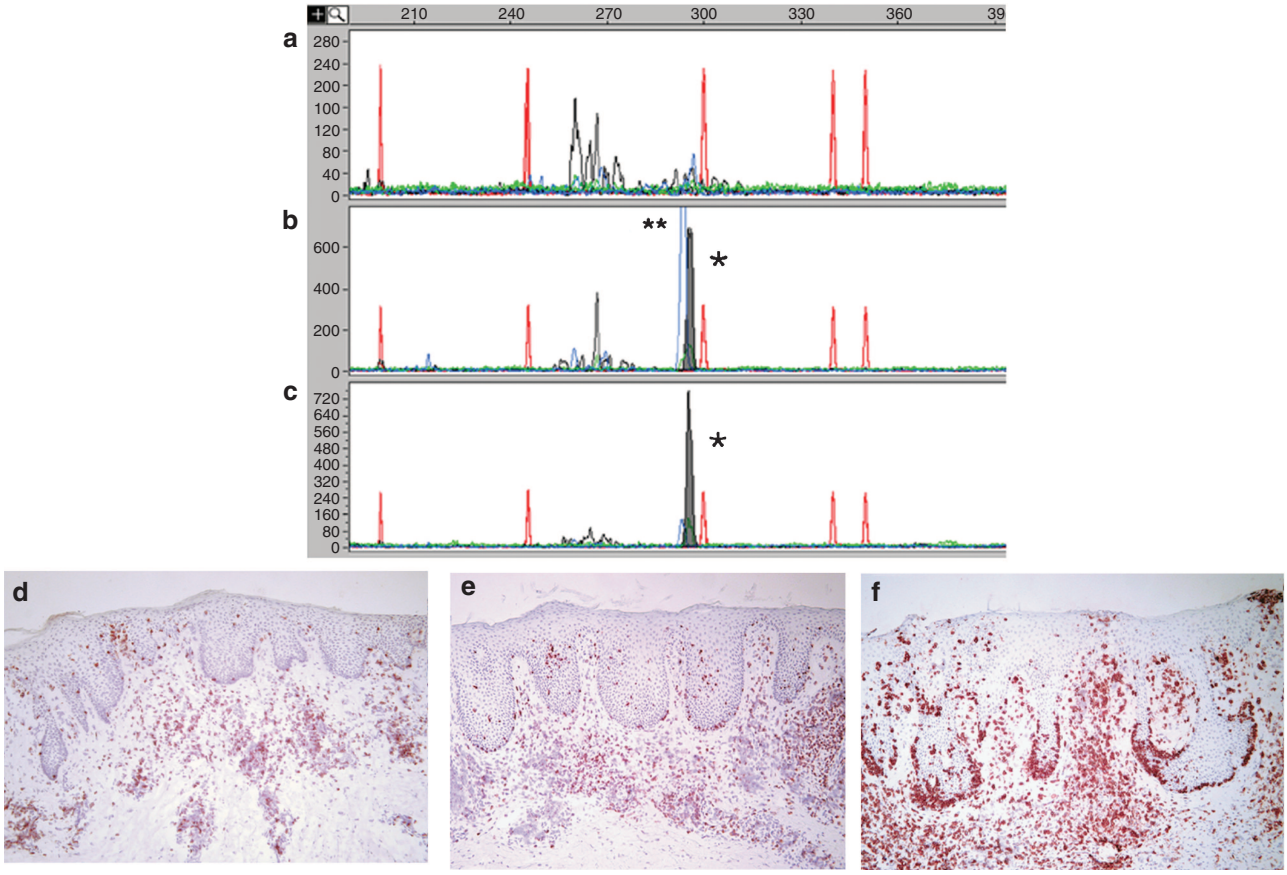

Figure 3. CD3 expression and corresponding GS analysis in MF patient no. 9. (**a**, **d**) The first non-diagnostic biopsy showing an oligoclonal pattern; (**b**, **e**) second biopsy with borderline histopathology showing the stable clonal TCRγ-GR 296VγIII/IV Jγ (*) and a dominant transient VγIJγ GR (**); (**c**, **f**) third biopsy with a massive infiltrate confirming the clonal VγIII/IV Jγ GR (sequence: Vγ10Jγ2); (**d**–**f**) CD3 staining showing a progressive increase in dermal and epidermal lymphoid infiltrate in the three consecutive samples.

The difference in sensitivity between the two methods was even more evident in the specimens with a low lymphoid infiltrate and an HS < 5 (from 53 to 76%). On the contrary, the difference was less evident in advanced-stage MF samples (from 85 to 94%, respectively, in samples with an HS > 5).

Our dilution experiments revealed a high *in vitro* sensitivity of TCRγ-PCR GS analysis. Percentages as low as 1–5%, or at times even lower (0.5–1%), using an MRD approach, were observed. However, few studies on CTCL carried out using GS report varying sensibility levels, ranging from 1 to 6%, depending on the amounts of oligo/polyclonal background and the Vγ family evaluated (Delfau-Larue *et al.*, 2000; Murphy *et al.*, 2000; Lukowsky *et al.*, 2002; Vega *et al.*, 2002; Klemke *et al.*, 2002). Indeed, there are a number of factors that may affect the sensitivity of the method. One such factor is the competition created during the aspiration phase in capillary electrophoresis by the electrostatic fields generated owing to the different size of the various PCR products. Another variability factor is the difference in primer sensitivity that may influence the yield of specific PCR products (Vega *et al.*, 2002). Moreover, only a few studies have taken into consideration the analysis of clonal variability of these pathologies by comparing multiple cutaneous lesions. Our long-term experience in MF/SS skin lesions showed that the absence of clonal dominance in oligoclonal background, coupled with the problem of suspicious pseudo-monoclonality in single sample, is not an uncommon finding, especially in the early stage of the disease. Therefore, whenever possible, a multiple sample approach was applied, confirming the clonal population by sequencing and by GS on cell sorting.

A high prevalence of oligoclonal TCRγ-GR pattern, rather than a polyclonal GR pattern was observed in skin lesions, as already reported in early MF lesions (Klemke *et al.*, 2002; Lukowsky *et al.*, 2002; Vega *et al.*, 2002). It has been suggested that the super-antigen stimulation has some role (e.g., bacterial proteins) in driving early proliferation of T cells to the skin in CTCL. Oligoclonal cytogenetic abnormalities have also been described in MF (Schlegelberger *et al.*, 1994). Clonal heterogeneity with adjunctive reproducible skin TCRγ-GRs was observed in multiple lesions in a large proportion of CTCL patients. Adjunctive TCRγ-GRs were also observed in peripheral blood during the followup of SS patients. The temporary dominance of transient clones and the problem of pseudo-monoclonality results on single sample could be misleading and could lead to incorrect

identification of the pathological population unless results are analyzed with repeated PCR runs on the same sample and compared with multiple skin and blood results.

Indeed, our results showed 80 and 48% of clonal heterogeneity in SS and MF patients, respectively. These figures are higher than those reported by Vega *et al.* (2002), who obtained about 30% heterogeneity in MF patients. The reasons for these discrepancies are still unknown, and further studies are needed to give more insights. Clonal heterogeneity has been found both in early and advanced MF lesions. The nature of the heterogeneity may differ in the following two conditions: genetic heterogeneity in early-stage MF may reflect a mixing of tumor clones and reactive cells in cutaneous lesions (Dommann *et al.*, 1996; Muche *et al.*, 2003; Delfau-Larue *et al.*, 2000), whereas the onset of a new stable GR in advanced lesions with a monoclonal dominant pattern and a low background could be due to the selection of a new clonal population. Noteworthy is the fact that, in many CTCL patients with clonal heterogeneity, only the availability of multiple biopsies allowed correct identification of the stable pathological clone. A few cases showed a clonal instability with different GRs in sequential MF/SS skin or SS blood samples from the same patients. Expanding clonal population on blood could be explained also by reactive anti-tumor populations or age-related expanding cells, as reported previously by Muche *et al.* (2003) Recently, the importance of multiple PCR runs has been stressed by Klemke *et al.* (2006) with the aim to get further insights into the biological problem of peripheral T-cell clonality of undetermined significance in the absence of a skin clonal rescue.

In conclusion, the high resolution and specificity obtained by the use of GS analysis for TCRγ-GR combined with the multiple sample approach enhance the early diagnosis in MF/SS patients. GS analysis could also be a useful tool for the detection of MRD in selected cases after therapy. In the future, the TCRγ-GR technique could be further improved by the use of standardized primers and protocols.

MATERIALS AND METHODS

Patients and biopsy specimens

The present study was performed in compliance with the principles of good clinical practice and according to the Declaration of Helsinki Principles. The study protocol was approved by the responsible Institutional Board. All the patients were included after providing their written informed consent. The diagnosis of MF was made according to the standard clinical and immunopathological findings (Willemze *et al.*, 1997). MF patients were classified according to the modified tumor, node, metastasis (TNM) staging of the Mycosis Fungoides Cooperative Group (Kashani-Sabet *et al.*, 2001). The morphological and cytoarchitectural pattern in MF diagnostic samples was evaluated according to the nine-point HS proposed by Guitard *et al.* as reported previously (Ponti *et al.*, 2005). The SS diagnostic criteria included (i) erythroderma and peripheral lymphadenopathies, (ii) peripheral blood involvement by circulating Sézary cells, and (iii) cutaneous biopsy-proven CTCL. Peripheral blood involvement was defined on the basis of the criteria reported by the International Society of Cutaneous Lymphomas (Vonderheid and Bernengo, 2003). Peripheral blood immunophenotype and clonal TCR-Vβ expression were also assessed to characterize better the pathological population (Bernengo *et al.,* 2001). At initial diagnosis, absolute circulating Sézary cells at diagnosis were $<1{,}000\,\text{cells}\,\text{mm}^{-3}$ in eight patients; the percentage of atypical cells ranged from 16 to 95% of total lymphocytes.

The biopsy specimens were divided into two parts: one was used for histological evaluation on paraffin-embedded sections and the other was cryopreserved for immunohistochemical staining and later used for TCR-γ clonality studies. Immunohistochemical staining on 5 μm fresh frozen tissue sections was performed in all cases by the standard streptavidin–biotin–peroxidase method (LSAB2plus Kit, Dako, Glostrup, Denmark), using a wide panel of mAbs directed against the T-cell lineage, as well as activation and proliferation antigens. Immunohistochemical staining was used to define better the histological score and loss of antigens.

Moreover, tissue suspension for immunofluorescent analysis was performed by mechanical desegregation with enzymatic treatment as reported previously, in five SS patients (Novelli *et al.*, 2000).

Peripheral blood lymphocyte flow cytometry

Peripheral blood lymphocytes were analyzed, according to their immunofluorescence reactivity, using a FACSCalibur cytometer (Becton Dickinson, San José, CA) equipped with a 15 mW, 488 nm, air-cooled argon–ion laser and a second red diode laser at 635 nm for allophycocyanin-antibody detection. Three- or four-color immunofluorescence analyses were performed simultaneously using FITC-, phycoerythrin-, peridinin chlorophyll protein-, and allophycocyanin-conjugated antibodies as described previously (Bernengo *et al.,* 2001). A wide panel of mAbs directed against T-cell antigens was routinely tested. Clonal TCR rearrangement was identified using a panel of 28 mAbs directed against the variable regions of β-chain (Serotec Ltd, Oxford, UK; Immunotech Coulter Company, Marseille, France; Endogen, Cambridge, MA).

Identification of clonal TCRγ-GR

Genomic DNA was extracted from 15–20 OCT-cryopreserved tissue sections (20 μm) and from peripheral blood lymphocytes after centrifugation with Ficoll–Hypaque (Nycomed, Oslo, Norway) using a QIAgen tissue kit (QIAamp DNA Mini Kit; QIAGEn GmbH, Hilden, Germany) according to the manufacturer's instructions. TCRγ-GR was studied by multiplex/HD PCR on PAGE and GS capillary electrophoresis analysis. Both multiplex/HD PCR and GS-PCR were repeated by two separate runs in all CTCL and ID samples at diagnosis and during followup. Moreover, each sample had been processed by GS together with all the previous specimens of the same patient to allow semiquantitative comparison of clonal population.

The same primers, consensus and specific for each Vγ or Jγ segment family, were used in both the protocols (Bottaro *et al.*, 1994). The multiplex/HD analysis with two multiplex PCRs containing all J primers (J1/2, Jp1/Jp2, and Jp) plus VI (V2, 3, 4, 5, 7, and 8) or VII plus VIII/IV (V9 plus V10/11) respectively was performed and analyzed as reported previously (Ponti *et al.*, 2005).

To amplify the TCRγ-GRs by GS capillary electrophoresis, six simultaneous PCRs, resulting in PCR products of approximately 250–300 base pairs, were performed and contemporarily processed in a thermal cycle (model PCR Express, Hybaid, UK). The single PCR

containing one Vγ family fluorescent primer (VI–FAM or VII-HEX or VIII/IV-NED) plus a Jγ primer (Jγ1/2 or JγP1/2). PCR conditions were the same as those of multiplex/HD PCR, with the exception of the last 9 minute extension that was prolonged to 15 minutes to eliminate incomplete PCR product fragments; labelled PCR products were subjected to GS on the ABI 310 PRISM CE sequencer (PE Applied Biosystem, Weiterstadt, Germany). Before separation, two mixtures of PCR products (fluorescent V-family/Jγ1/2 products and fluorescent V-family/JγP1/2 products) were prepared with the addition of 15 μl of deionized formamide and 0.5 μl of GS 500 ROX internal lane standard (Applied Biosystems). The mixtures were denatured at 90°C for 5 minutes and chilled on ice. Each run was performed at 60°C and 15 KV with a 5 second injection and 36 minute separation time in high-resolution denaturing polyacrylamide gel (POP4 polymer; Applied Biosystem). The two runs were analyzed on the same plot using the GS Software (Applied Biosystems). To determine the clonality of a PCR product, peak-height ratio was calculated as reported previously (Luo *et al.*, 2001; Lukowsky *et al.*, 2002). GS analysis was assessed as clonal in the case of 1–2 prominent peaks yielding a peak-height ratio > 2. Oligoclonal pattern was defined as a non-Gaussian distribution of three or more prominent peaks. Multiple peaks with a Gaussian distribution indicated polyclonality. All these molecular patterns were confirmed if demonstrated in the same sample on two or more repeated runs. If the exact fragment size of a PCR TCRγ-GR product or its dominance (instable semi-quantitative product dimension) could not be confirmed on repeated analysis of the same sample, a multiple sample comparison is needed to distinguish between occult clonal population and pseudo-monoclonal pattern. The case was regarded as ''pseudo-monoclonal'' and was included in the oligo/polyclonal group when this same multiple approach failed to demonstrate a PCR product with a stable length or a stable dominance on background. The fragment lengths and V/J family rearrangement type corresponding to clonal population were recorded for the comparison between blood and skin samples, as well as multiple biopsies.

To determine the sensitivity of the GS technique, we employed DNA from blood samples of two patients with a detected TCR-Vβ+ population by flow cytometry. The first sample, with 98% of TCR-Vβ19+ monoclonal population and positive V-I/Jγp1/2 GR, and the second sample, with 95% of TCR-Vβ17+ monoclonal population and positive biallelic V-I/Jγ1/2/V-II/Jγ1/2 GR, were diluted with a pool of DNA from skin biopsies of reactive infiltrates at the following concentrations: 10, 5, 1, 0.5, 0.1, and 0.05%.

Sequence analysis of clonal TCRγ-GR

TCRγ-GRs were sequenced in eight cases where the common TCRγ-GR was not dominant in GS analysis in the consecutive biopsies. Each band was then excised from agarose gel and DNA was purified using the PCR clean-up gel extraction kit (Macherey-Nagel, Düren, Germany). Sequencing was carried out using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), and the analysis was performed on an ABI 310 automated capillary system (Applied Biosystems), following the manufacturer's instructions. All samples were sequenced in both directions. The sequences were compared with published germline sequences using the DNAplot on the Internet (http://imgt.cines.fr) to identify Vγ and Jγ rearrangements.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Table S1. Clinical and molecular data on 27 MF patients with multiple biopsies.

Figure S1. MF patient no. 27.

Figure S2. MF patient no. 7.

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