

INVESTIGATIVE REPORT

Comparative Analysis of TCR- γ Gene Rearrangements by Genescan and Polyacrylamide Gel-electrophoresis in Cutaneous T-cell Lymphoma

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Demonstrating T-cell clonality has become an important approach supporting a diagnosis of malignant T-cell neoplasms. A comparative study between Genescan analysis, polyacrylamide gel and agarose gel electrophoresis in visualizing T-cell receptor gamma gene rearrangement was performed on 25 biopsy specimens from 18 patients with different forms of cutaneous T-cell lymphomas. Clonality was detected in 17 biopsy specimens when PCR products were evaluated by Genescan analysis. Seventeen showed discrete bands when visualized in polyacrylamide gel and 14 cases were clonal when visualized with agarose gel. In five cases, a clonal population was seen in the gels, but not with Genescan. On sequencing the PCR products we demonstrated non-clonality of these five samples. Our results confirm that PCR-Genescan is a useful, reliable and specific screening method for detecting dominant clones in patients with T-cell lymphoma. Key words: Genescan; PCR; polyacrylamide gel electrophoresis and agarose gel electrophoresis; T-cell clonality.

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During recent years, several different polymerase chain reaction (PCR) assays have been claimed as specific and sensitive methods for assessing clonality in T-cell lymphoproliferative disorders (1). The different PCR methods usually vary in the selection of oligonucleotide primers for amplification of DNA fragments of the T-cell receptor- γ (TCR- γ) or β (TCR- β) subunits and in the screening techniques used to detect the amplicons.

PCR technology, in contrast to Southern blot, is a rapid and easy method that does not require radioactively labelled probes and permits detection of less than 1–5% of clonal cells. In addition, very small

amounts of DNA obtained from routinely processed, formalin-fixed, paraffin-embedded tissue samples can be analysed with good results (2).

Ethidium bromide-stained agarose gel (AGGE) and polyacrylamide gel electrophoresis (PAGE) are commonly used techniques for visualizing PCR products in routine diagnosis (3, 4). However, they have low separation capacity, especially agarose, which prevents reliable discrimination between clonal and non-clonal PCR products. Among techniques with a higher resolution for determining dominant T-cell clones are denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and automated fluorescent fragment analysis by Genescan (GSA), which are currently used by leading groups. GSA is a new method for determining dominant T-cell clones and is more accurate, specific and easy to interpret than AGGE and PAGE (5–8). Despite its unquestionable diagnostic interest, however, the definitive role of PCR-GSA amplification of the TCR- γ technique in the routine diagnostic evaluation of CTCL has not been clearly defined.

The aim of this study was to compare PCR-GSA with the conventional PCR-AGGE and PCR-PAGE in the detection of clonal T-cell predominance in cutaneous T-cell lymphoproliferative disorders (CTCL).

MATERIALS AND METHODS

Twenty-five skin biopsies from 18 patients with a proven diagnosis of CTCL were studied for genotypic analysis (PCR analysis of TCR γ) at the Laboratory of Cytogenetics and Molecular Biology, Pathology Department, Hospital del Mar in Barcelona, Spain.

The diagnosis was established on the basis of clinical data, routine histopathologic examination and immunophenotypic expression according to standard criteria for the diagnosis of primary cutaneous T-cell lymphoma of the EORTC classification (9).

Histopathologic features were diagnosed as advanced stage mycosis fungoides (MF) in six biopsies. Eight biopsies were obtained from small-medium sized CD30 negative T-cell pleomorphic lymphoma (SM-TCL), 10 biopsy specimens corresponding to plaque stage MF, 7 of them were from patients with extensive disease, and one patient had lymphomatoid

papulosis (LyP). In seven patients more than one biopsy from different lesions was analysed. All biopsy samples were obtained simultaneously for histopathologic and molecular diagnostic purposes and were stored at -70°C . In addition, peripheral blood samples from seven normal donors and a series of seven skin paraffin-embedded biopsies from patients affected by psoriasis were also studied as controls.

Immunohistochemical studies

Immunohistochemical studies were performed in all cases using formalin-fixed paraffin-embedded tissue sections with en-Vision technique in an automated immunostainer, using the antibodies CD3, CD8, CD20, CD30 (Dako, Glostrup, Denmark), CD4 and CD56 (Novocastra, Newcastle, UK).

DNA extraction

DNA from fresh tissue was isolated by cell lysis, phenol-chloroform extraction and ethanol precipitation according to standard procedures. For polyclonal skin controls, the DNA was extracted from paraffin-embedded biopsies using a Kit QIAamp Tissue Kit (QIAGEN GmbH, Hilden, Germany) and from peripheral blood lymphocytes of healthy donor samples using salt extraction and ethanol precipitation according to standard procedures.

PCR amplification

Genomic DNA (200 ng) was amplified in a semi-nested PCR as previously described (5). The initial amplification was performed using the following oligonucleotide primers: V γ 1₁₋₉ (5'-TGC AGC CAG TCA GAA ATC TTC C 3'), JGT₃ (5'AGT TAC TAT GAG C(CT) AGT CCC 3') and JGT_{1/2} (5'AAG TGT TGT TCC ACT GCC AAA 3'). The second amplification was performed using the following oligonucleotide primers: JGT₃, JGT_{1/2} and V γ 2₁₋₉ (5'ACG GCG TCT TC(AT) GTA CTA TGA C 3') labelled with FAM.

TCR- γ PCR-GSA analysis was performed in triplicate for each skin biopsy to avoid the false-positive interpretation of monoclonality (pseudomonoclonality).

Fluorescent fragments analysis (Genescan)

One microlitre of a 1:10 dilution of the PCR product was mixed with 9 μl of deionized formamide (Applied Biosystems, Foster City, CA) and 0.5 μl of molecular weight standard (Genescan 400-ROX, Applied Biosystems). Samples were analysed by automated fragment ABI 3100 (Genescan system).

A case was assigned as monoclonal when only one or two peaks with appropriate size range were seen and the size of the peaks was similar to 200 bp in all PCRs. Polyclonal cases showed multiple peaks of approximately 200 bp. In all cases a repeated determination was performed routinely.

Agarose gel electrophoresis analysis (AGGE)

Twenty microlitres of PCR products were simultaneously analysed in a 2% AGGE. Samples analysed were considered as monoclonal if one or two discrete narrow bands were observed within the appropriate size range expected for a particular primer pair. The result was considered correct if it was reproducible on duplicate runs. In contrast, polyclonal samples appeared as a broad smear.

Polyacrylamide gel electrophoresis analysis (PAGE)

Twenty microlitres of PCR products were analysed in 8% PAGE stained with ethidium bromide. The criteria for considering a sample monoclonal or polyclonal were the same as those described for analysis by agarose gel electrophoresis.

Sequencing of PCR products

When sequencing was performed, the second amplification was done with non-labelled V γ 2₁₋₉ primer. The PCR product was purified using a QIAquick PCR purification kit (QIAGEN). One microlitre of the purification product was subjected to fluorescent dye terminator cycle sequencing. The PCR product was purified with Centriscap, Priceton separations spin columns (Applied Biosystem) and analysed on DNA sequencing analysis software 3.7 (Applied Biosystem). In cases in which visualization of the PCR product with GSA, PAGE and AGGE was divergent, direct sequencing of the PCR products was routinely performed.

PCR sensitivity

To determine the sensitivity of the technique, DNA from a skin biopsy of a patient with a monoclonal pattern was serially diluted (1:1, 1:10, 1:50, 1:100, 1:500) with DNA from a normal donor with a polyclonal pattern.

Internal DNA controls

Amplification of exon 3 of the human β -actin gene was used as internal control of DNA integrity.

RESULTS

In 25 biopsies from the 18 CTCL diagnosed cases, TCR- γ GSA revealed a dominant clone in 17 biopsies (68%) (Table I). We failed to demonstrate a specific clone in eight biopsies (32%) with undoubtful histologic diagnostic and immunophenotypic expression being CD4 positive and CD56 negative (data not shown). The PCR product was simultaneously run onto AGGE and PAGE gels. The results of this study demonstrated that 17 out of 25 biopsies analysed (68%) showed discrete bands by PAGE. When the PCR products were visualized using AGGE, only 14 of 25 biopsies (56%) showed a distinctive band that was interpreted as monoclonal (Figs. 1–2).

Looking at different diagnosis, clonality was demonstrated in 5 out of 8 biopsies (62%) from SM-TCL, and in 11 out of 16 biopsies of MF (69%) using GSA. Analysing the same PCR product with PAGE and AGGE, 6 and 5 biopsies, respectively, showed distinct bands for the SM-TCL group. Among 16 biopsies of MF, 11 sharp bands were seen in PAGE (69%) and 9 biopsies were considered as clonal by AGGE (56%).

One patient had LyP coexisting with MF and the PCR revealed clonality when visualized with GSA. It was not possible to confirm this clonal population with AGGE or PAGE. It is remarkable that in this case an

Table I. Results of the PCR-TCR- γ gene rearrangement from 25 biopsies of primary cutaneous T-cell lymphoproliferative disorders visualized by Genescan analysis (GSA), polyacrylamide gel electrophoresis (PAGE) and ethidium bromide-stained agarose gel (AGGE)

Case	Diagnose	Staging	GSA Clonality	PAGE Clonality	AGGE Clonality
1	SM-TCL	T3N1M1	+	+	+
2	SM-TCL	T3N0M0	-	-	-
3	SM-TCL	T3N1M0	+	+	+
4	SM-TCL	T3N1M0	-	+	+
5	SM-TCL	T3N0M1	+	+	-
6	SM-TCL	T3N0M1	+	+	+
6	SM-TCL	T3N0M0	-	+	+
7	MF	T3N0M0	-	+	+
8	MF	T3N0M0	+	-	-
8	MF	T3N0M0	+	+	+
9	MF	T3N1M0	-	-	-
10	MF	T3N1M1	+	+	-
10	MF	T3N1M1	+	+	+
11	MF	T2N0M0	+	+	+
12	MF	T2N0M0	+	+	+
13	MF	T2N0M0	-	+	+
13	MF	T2N0M0	-	+	+
14	MF	T2N2M0	+	+	+
15	MF	T2N0M0	+	-	-
16	MF	T2N0M0	-	+	+
17	MF	T1N0M0	+	+	-
17	MF	T1N0M0	+	-	-
18	MF	T1N0M0	+	-	-
LyP		-	+	-	-

*To determine the possible false-positive results visualizing the PCR with AGGE and PAGE, direct sequencing was performed to demonstrate the non-clonality of the sample.

MF=mycosis fungoides; SM-TCL=small-medium sized CD30 negative T-cell pleomorphic lymphoma; LyP=lymphomatoid papulosis.

identical peak of rearranged TCR- γ genes was observed by GSA for the MF and LyP biopsies.

In cases 3, 6, 8, 10, 13, 17 and 18, two skin samples were obtained from different cutaneous lesions and an identical rearranged pattern was detected by GSA. In cases 6, 7, 13 and 16 we detected a discrete band by PAGE and AGGE; however, GSA revealed a polyclonal population. In these cases, sequencing of the PCR products was crucial to determining the non-clonality of the samples. Moreover, in cases 8, 15, 17 and 18, which had a clear clonal peak by GSA but not with PAGE and AGGE, clonality was verified by direct sequencing.

All samples from healthy donors and lesions of psoriasis showed clear polyclonal amplification patterns of TCR- γ with no difference in interpretation of GSA, PAGE and AGGE.

The methodological sensitivity of our PCR method for detecting a clonal TCR gene rearrangement on skin biopsies was approximately 2% (1:50 dilution) using GSA and 10% (1:10 dilution) using AGGE and PAGE (Fig. 3).

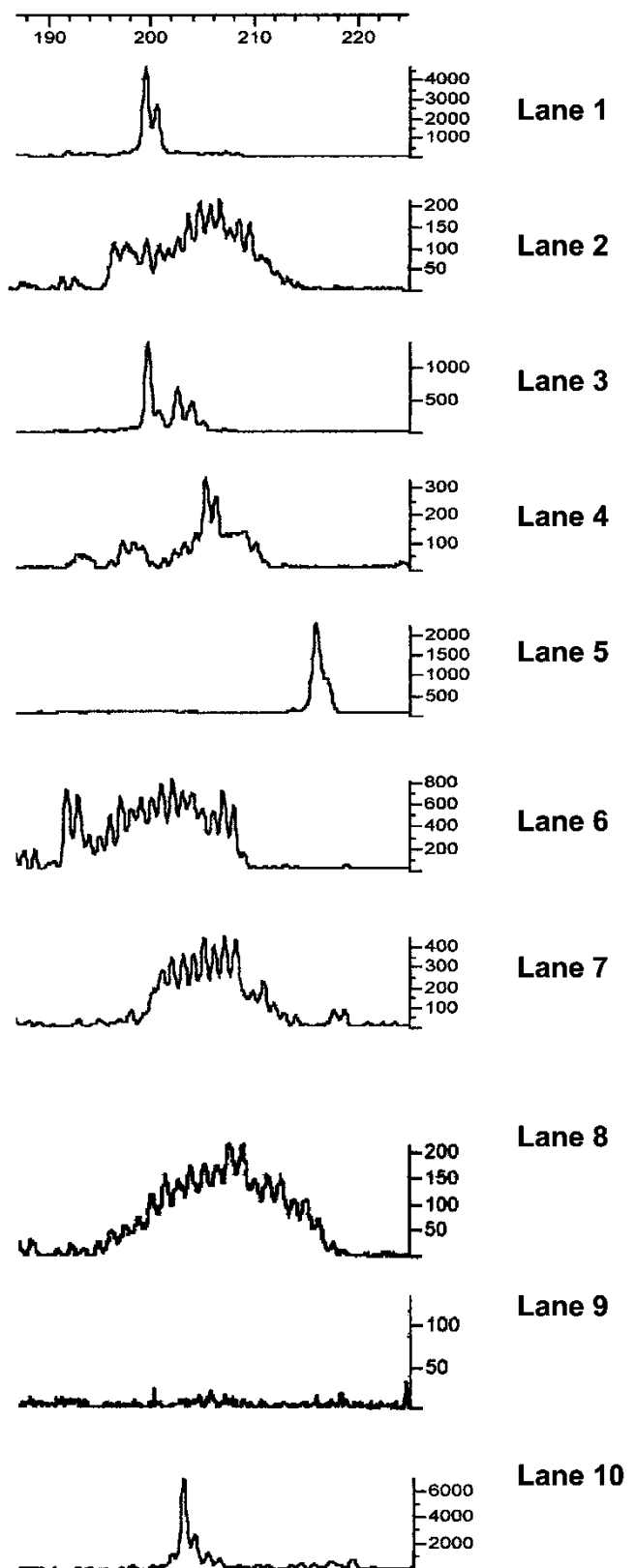


Fig. 1. Study of the TCR- γ gene rearrangement using PCR-GSA. A predominant clone is observed in lanes 1, 3 and 5 (patients no. 1, 3 (first biopsy), 5). Lanes 2, 6 and 7 (patients no. 2, 6 (first biopsy), 7) show a polyclonal pattern. Lane 8 is the polyclonal control, lane 9 is the negative control and lane 10 the positive control. The X-axis shows the size in bp (base pairs) of the PCR product and Y-axis the intensity of fluorescence (arbitrary units).

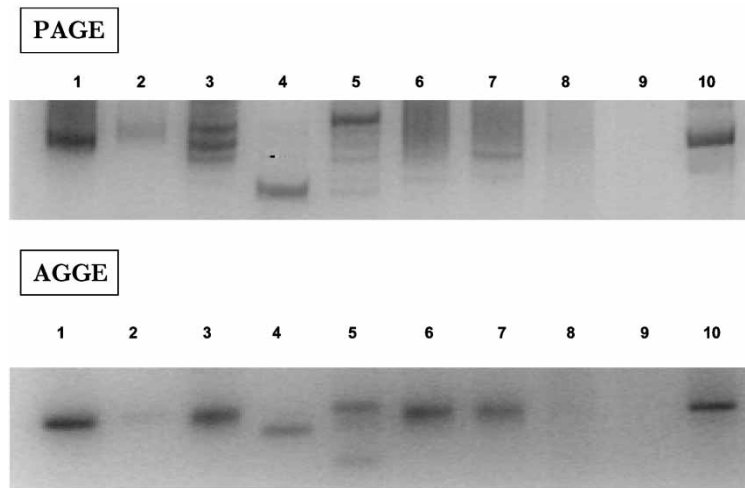


Fig. 2. Study of the TCR- γ gene rearrangement using PAGE and AGGE to visualize the PCR product (see Table I and Fig. 1 for abbreviations). The results are the same except for lane 7, which was polyclonal using GSA, see Fig. 1, and clonal with PAGE and AGGE. In this case it was necessary to sequence the PCR product to determine the non-clonality of the sample.

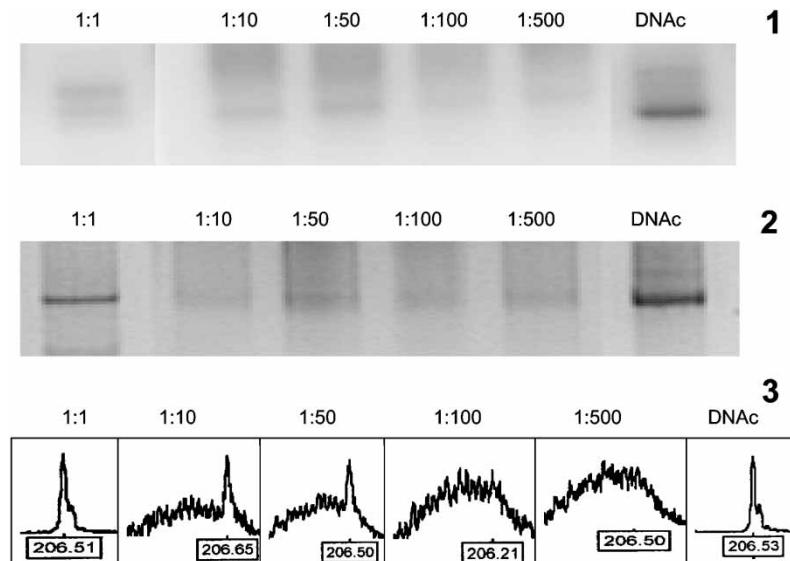


Fig. 3. Sensitivity of the technique using AGGE (1), PAGE (2) or GSA (3) to visualize the PCR products (see Table I for abbreviations). Serial dilutions (1:1, 1:10, 1:50, 1:100, 1:500) of DNA from a skin biopsy of a patient with a monoclonal pattern (DNAc) into DNA from a normal donor with a polyclonal pattern were performed.

DISCUSSION

Detection of lymphoid clonality in clinical specimens by PCR analysis has become a useful and reliable technique in the diagnosis of cutaneous malignant lymphomas. Some studies have reported the use of PCR for amplification of a conserved sequence of the variable and joining region segments of the TCR γ gene (3, 4, 10–19). PCR products can be separated based on PAGE (3, 4), on DGGE (20) or TGGE (6, 7, 21), by analysis of single-stranded conformational polymorphisms (15, 22), lightcycler-PCR (23), by heteroduplex

analysis (6, 7, 10, 14, 24) or by fragment analysis (5–8, 25). With these techniques, a monoclonal T-cell population can be detected in between 59% and 100% of CTCLs (23).

PCR-GSA is a novel, simple and rapid test with an overall low cost and theoretical major advantage over other methods for detecting TCR- γ and IgH chain gene rearrangements (5, 25, 26). PCR products are visualized as monoallelic peaks of fluorescence with high resolution of fragment size. Accuracy in determining the size of the PCR products and the ability to distinguish between polyclonal and monoclonal DNA patterns

makes this technique a reliable routine diagnostic procedure (5, 25).

Using an identical protocol, Dippel et al. (5) detected a dominant TCR- γ clone in 76% of samples of advanced stage CTCL. In our study, we detected a dominant clone in 68% of the samples. Eight of the biopsies were diagnosed as SM-TCL. In this group, 62% of analysed samples presented a monoclonal peak when the PCR product was visualized with GSA. In the MF group ($n=16$) the presence of mono clonal a peak was determined in 69% of the samples. These similar results can probably be explained by the difference in the number of cases of the two entities analysed (8 cases of SM-TCL and 16 cases of MF).

Dippel et al. (5) estimated that the *in vitro* sensitivity of the method for detecting a monoclonal population admixed in a polyclonal population of human tonsillar cells was 6%. However, additional dilution assays of tumoral DNA with germinal DNA increased the sensitivity to 1%. Our dilution assays with this PCR-GSA method obtained a sensitivity of 2% when clonal DNA was diluted with a polyclonal DNA sample (Fig. 3). These results confirm the greater methodological sensitivity of GSA compared with techniques such as PAGE, AGGE or SSCP (7, 23). Consequently, when this technique is used to visualize PCR products from samples with relatively few contaminating lymphocytes (for example skin biopsies), an increase in sensitivity can be expected.

Direct sequencing was performed in cases showing discrete bands by PCR-AGGE or PCR-PAGE and negative results with PCR-GSA. All cases with a "positive result" by PCR-AGGE or PCR-PAGE and negative with PCR-GSA turned out to be false-positive results after performing sequencing. Only in five cases was a monoclonal T-cell proliferation detected by PCR-GSA that was not evident by PCR-PAGE. In these cases PCR-GSA analysis was repeated three times and direct sequencing confirmed the presence of a clonal T-cell proliferation. All these results confirm that GSA is a simpler, faster and more specific technique than PAGE in detecting dominant clones. Other authors confirm that the study of clonality using fluorescence systems, e.g. fragment analysis, lightcycler or GSA, is the most sensible, accurate and specific way for detection of a monoclonal peak in samples with little lymphoid infiltration (6, 7, 8, 23, 25, 26).

In our opinion, PCR-GSA is one of the most effective methods for determining the clonality in skin biopsies. Nevertheless, only continued studies performed in parallel with a different PCR technique will permit the exact role of the different method variants to be clarified in the diagnosis of cutaneous T-cell lymphoproliferative disorders (6, 7, 22, 25, 26). Taking into account our results, we consider that TCR gene rearrangement detected by PCR-GSA

amplification could in the near future become a routine primary screening test for the detection of dominant clones in the evaluation of cutaneous lymphoproliferative disorders.

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