

Detection of clonal T cell populations by high resolution PCR using fluorescently labelled nucleotides; evaluation using conventional LIS-SSCP

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Abstract

Aims—To detect clonal T cell populations by high resolution polymerase chain reaction (PCR) using fluorescently labelled nucleotides and analysis on an ABI 377 DNA sequencer, and to evaluate this method using low ionic strength single strand conformation polymorphism (LIS-SSCP) analysis.

Methods—DNA samples from 11 patients diagnosed with a T cell disease and 15 with no known T cell disorder were amplified using four multiplex T cell receptor γ (TCR γ) PCR reactions containing fluorescently labelled nucleotides. PCR products were analysed using both LIS-SSCP electrophoresis and an ABI 377 DNA sequencer using Genescan™ software. A Jurkat T cell leukaemia cell line was used to determine the sensitivity of the two methods.

Results—Clonal TCR γ populations were detected in all 11 samples from patients with a T cell disease and no clonal populations were detected in samples from patients without a T cell disorder, using both LIS-SSCP and DNA sequencer analysis. Although the sensitivity of the two methods was comparable, the data generated by the sequencer were easier to interpret than the LIS-SSCP gels, and allowed accurate size determination of every product, which was not possible using LIS-SSCP.

Conclusions—The use of fluorescent labelled nucleotides provides a more flexible and economical alternative to end labelled fluorescent primers for the detection of clonal TCR γ gene rearrangements. This method allows clonal populations to be sized accurately and reproducibly, permitting the detection of identical clonal populations in different samples, and providing a method of monitoring disease progression and response to treatment.

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The value of polymerase chain reaction (PCR) amplification of T cell receptor (TCR) genes to distinguish neoplastic proliferations from reactive T cells is well established.¹⁻³ TCR γ is a useful marker of clonality because it is rearranged earlier during T cell development

than TCR β , and is therefore likely to be found in a wide range of T cell lesions, including high grade tumours. Detection of T cell clonality using TCR γ analysis is restricted by the relatively small number of possible rearrangements and the preferential use of certain gene segments in recombination; there are only 16 variable, five junctional, and no diversity region segments. The diversity arises predominantly from the insertion or deletion of random bases at the VJ junction. However, although as many as 29 bases can be added or deleted at this junction, the mean is much lower, and a normal distribution of rearranged gene size is generally observed within a reactive population. Therefore, the limited size diversity can lead to false positive results with apparent "clonal" products being found in patients with non-malignant conditions. TCR β gene rearrangement analysis is lineage specific and is therefore a superior method of detecting T cell clonality. However, the most reliable methods of detecting clonal TCR β populations rely on reverse transcription PCR (RT-PCR),^{4,5} which is not always possible if RNA is not available. TCR γ analysis is useful in the absence of RNA or when TCR β results are unclear. Therefore, a simple, reliable method for the detection of clonal TCR γ populations would be of great value to the diagnostic laboratory. Such a method must be relatively easy to perform, able to detect as many rearrangements as possible, allow clear separation of all PCR products, and generate data that can be interpreted with confidence.

Efficient size separation and subsequent interpretation using conventional polyacrylamide gel electrophoresis (PAGE) can be difficult owing to the limited number of similar sized products generated by PCR amplification of the TCR γ locus. Polyclonal populations are not always uniform in intensity and several products of a similar size may appear as a single bright band suggestive of a clonal population. Strategies to overcome this problem include the use of several different primer combinations,⁶ and enhanced gel techniques such as single strand conformation polymorphism (SSCP),⁷ temperature gradient electrophoresis (TGGE),⁸ and denaturing gradient electrophoresis (DGGE).⁶

SSCP analysis⁹ allows the separation of two or three products of the same size but with different sequences because the electrophoretic mobility of a single stranded nucleic acid in a non-denaturing polyacrylamide gel depends not only on size but also on sequence. Products

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may be rendered single stranded by heat denaturation in the presence of formamide or alkali, or using the low ionic strength method (LIS),¹⁰ and separated by electrophoresis on a conventional non-denaturing PAGE gel. The LIS-SSCP method provides a simple, rapid means of forming single stranded DNA using heat denaturation under conditions of low ionic strength, with no requirement for denaturing agents or cooling. The single stranded DNA generated using this method is stable at room temperature and provides clearer banding profiles upon electrophoresis.⁹ LIS-SSCP has been shown to improve the sensitivity of detection of clonal T cell receptor γ gene rearrangements⁷ compared with several other methods.

It is now possible to perform semi-automated high resolution analysis of fluorescent PCR products using a DNA sequencing machine. PCR products are fluorescently labelled by using a primer with a fluorescent dye at the 5' end, separated by electrophoresis on a DNA sequencer, detected by a laser, and analysed using specific computer software. The resolution of this method allows the separation of PCR products differing in length by as little as one base pair. The accurate sizing of PCR products allows identical clonal populations to be detected within different samples, providing a means of monitoring disease progression, response to treatment, and minimal residual disease. Semiquantitative assessment of clonal populations within a background of polyclonal cells is also possible. This approach has been used for the detection of TCR γ ,¹¹ TCR β ,¹² and immunoglobulin heavy chain gene rearrangements.¹⁴ However, the cost of purchasing the large numbers of requisite PCR specific fluorescently labelled primers required for these techniques is high.

We describe an alternative technique in which TCR γ PCR products are internally labelled using fluorescently labelled nucleotides, separated by electrophoresis on an ABI 377 DNA sequencer, and analysed using GenescanTM and GenotyperTM software. DNA samples from 11 patients diagnosed with a T cell disease and 15 with no known T cell disorder were amplified using TCR γ PCR reactions containing fluorescently labelled nucleotides. The products were simultaneously analysed using LIS-SSCP electrophoresis and an ABI 377 DNA sequencer and the results compared.

Methods

SUBJECTS

Samples were obtained from 11 patients with a diagnosis of T cell disease, based on clinical findings (table 1). A range of samples was also obtained from 15 patients with no history of T cell disease. These included three reactive tonsils and samples from patients with disorders including slight lymphocytosis (two), lymphoproliferative disorder (one), neutropenia (two), tiredness and sweats (one), erythrodermic rash (one), B cell non-Hodgkin's lymphoma (four), and B cell chronic lymphocytic leukaemia (B-CLL) (one). DNA was extracted from blood and tissue samples using a range of different proprietary kits.

TCR γ PCR

Four multiplex PCR reactions were performed using one GV family primer with all three GJ primers, as described previously.⁷ Fluorescently labelled nucleotides R110-dCTP (Perkin Elmer, Warrington, Cheshire, UK) were added to each reaction to label the products internally. PCR reactions were performed in 25 μ l of 1 \times Amplitaq Gold reaction buffer II (Perkin Elmer) containing final concentrations of 2 mM MgCl₂, 200 μ M unlabelled dNTPs, 100 ng of each primer, 1 U Amplitaq Gold Taq polymerase (Perkin Elmer), 0.04 μ M R110-dCTP, and 40 ng of template. All reactions were heated to 95°C for 10 minutes to activate the Amplitaq Gold Taq polymerase and then subjected to 40 cycles of 94°C, 55°C, and 72°C for one minute each on a Hybaid Touchdown thermocycler. Clonal, polyclonal, and negative controls (water blank) were included for each multiplex PCR. Each clonal population generated by multiplex PCR was analysed to determine the exact gene rearrangement present.

ANALYSIS OF PCR PRODUCTS

Each PCR reaction was analysed using both LIS-SSCP analysis and an ABI 377 DNA sequencer. LIS-SSCP analysis was performed as described previously.⁷ Briefly, 10 μ l of PCR product was added to 35 μ l of LIS solution (10% sucrose, 0.01% bromophenol blue, and 0.01% xylene cyanol FF), denatured at 97°C, and separated by electrophoresis on a 5% non-denaturing polyacrylamide gel, which was stained using ethidium bromide and viewed

Table 1 Clonal TCR γ gene rearrangements detected in samples from patients with T cell disease using both LIS-SSCP PAGE and DNA sequencer analysis of products labelled using fluorescent nucleotides

Patient	Diagnosis	Clonal TCR γ gene rearrangements detected using LIS-SSCP PAGE and a DNA sequencer
1	Cutaneous T cell lymphoma	GV1J γ and GV2J γ
2	Cutaneous T cell lymphoma	GV1J γ P and GV1J γ P1/2
3	T cell non-Hodgkin's lymphoma	GV3J γ and GV4J γ
4	T cell non-Hodgkin's lymphoma	GV1J γ and GV1J γ P
5	T cell non-Hodgkin's lymphoma	GV1J γ P, GV2J γ , GV2J γ P1/2, GV3J γ P1/2, and GV4J γ
6	T cell non-Hodgkin's lymphoma	GV1J γ
7	T cell non-Hodgkin's lymphoma	GV1J γ and GV2J γ
8	Sezary syndrome	GV1J γ P, GV2J γ , GV2J γ P1/2, GV3J γ P1/2, GV4J γ , and GV4J γ P1/2
9	T cell chronic lymphocytic leukaemia	GV1J γ
10	T cell prolymphocytic leukaemia	GV2J γ and GV3J γ
11	T cell $\gamma\delta$ hepatosplenic lymphoma	GV1J γ

LIS-SSCP PAGE, low ionic strength single strand conformation polymorphism polyacrylamide gel electrophoresis; TCR, T cell receptor.

under UV light. Samples for analysis on the DNA sequencer were prepared by adding 2 μ l of PCR product to 2 μ l of loading buffer containing a 5/1/2 mix of formamide, dextran blue, and the internal size standard TAMRA 500 (Perkin Elmer). Products were separated by electrophoresis on a 4% sequencing gel within an ABI 377 DNA sequencer and analysed using Genescan™ and Genotyper™ software.

SENSITIVITY

To determine the sensitivity of the two methods of analysis, Jurkat DNA was serially diluted in polyclonal DNA, amplified using the fluorescent GV1 multiplex PCR, and analysed using both LIS-SSCP and an ABI DNA sequencer. The Jurkat DNA was extracted from the T cell leukaemia cell line Jurkat E6.1

(ECCAC, CAMR, Porton Down, Salisbury, Wiltshire; reference number 88042803) and the polyclonal DNA from an individual with no known disease. Extractions were performed using a Qiagen QIAamp tissue kit (Qiagen Ltd, Crawley, West Sussex, UK) according to the manufacturer's instructions.

Results

LIS-SSCP analysis separates DNA on the basis of size and sequence, generating gel images in which a polyclonal population appears as a smear, and a clonal population can be identified by the presence of two distinct bands representing the individual strands of the clone (fig 1). Although the DNA sequencer separates products on size alone it has the capacity to resolve products that differ by a single nucleotide. The Genotyper electropherogram

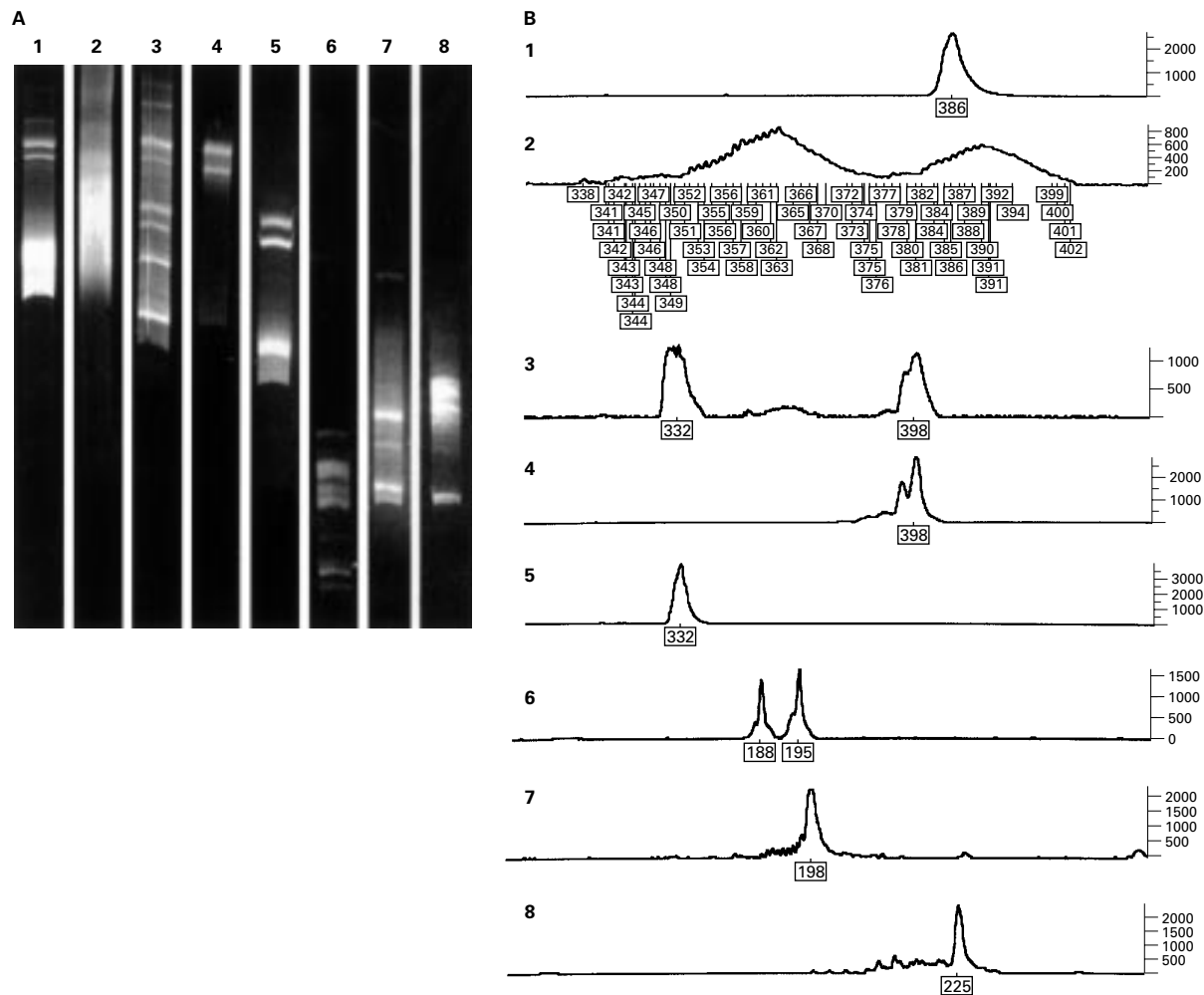


Figure 1 Comparison of low ionic strength single strand conformation polymorphism (LIS-SSCP) polyacrylamide gel electrophoresis (PAGE) images and DNA sequencer electropherograms for the identification of clonal T cell receptor γ (TCR γ) gene rearrangements. (A) Ethidium bromide stained 5% non-denaturing LIS-SSCP PAGE image. (B) Genotyper™ electropherogram from an ABI 377 DNA sequencer in which the size of each product (in base pairs) is displayed on the x axis with its relative fluorescence intensity on the y axis. Lane 1, Jurkat T cell line-GV1 multiplex; lane 2, normal polyclonal control-GV1 multiplex; lane 3, patient 4-GV1 γ ; lane 4, patient 4-GV1 γ ; lane 5, patient 4-GV1 γ ; lane 6, patient 6-GV2 γ P1/2; lane 7, patient 3-GV3 γ ; lane 8, patient 5-GV4 γ .

generated by the DNA sequencer displays the size of each PCR product (in base pairs) on the x axis and the relative fluorescence intensity on the y axis; a single peak is representative of a clonal population and many peaks of a polyclonal population (fig 1B, lanes 1 and 2).

Despite the differences in the two methods of analysis, identical clonal populations were detected using both LIS-SSCP and the DNA sequencer (table 1). Clonal populations were detected in all 11 samples from patients with T cell disease. The seven different primers (4GV and 3GJ) used in the multiplex PCRs were all used in the amplification of these clonal populations, demonstrating the ability of both analysis methods to detect rearrangements using all the different gene segments. The control reactions generated consistent results (fig 1, lanes 1 and 2). The 15 samples from patients without a T cell disorder generated polyclonal profiles. No clonal populations were detected.

Between one and six different clonal populations were amplified from each DNA sample (table 1). Both cutaneous T cell lymphoma samples (patients 1 and 2) generated two clonal populations. However, the same gene rearrangements were not detected in both samples. Clonal GV1J γ and GV2J γ rearrangements were amplified using DNA from patient 1 whereas GV1J γ P and GV1J γ P1/2 were detected in patient 2. The five cases of T cell non-Hodgkin's lymphoma each generated between one and five different clonal populations. These five samples alone contained nine different clonal populations and used all seven different primers. Amplification of DNA from patient 4 (T cell non-Hodgkin's lymphoma) using the GV1 multiplex PCR generated two clonal populations (fig 1, lane 3). Using the individual J γ primers these populations were identified as clonal GV1J γ (fig 1, lane 4) and GV1J γ P (fig 1, lane 5) gene rearrangements. The clonal GV3J γ and GV4J γ populations amplified using DNA from patients with T cell non-Hodgkin's lymphoma (patients 3 and 5) are shown in fig 1, lanes 7 and 8. Six different TCR γ gene rearrangements were amplified from the single case of Sezary syndrome investigated (table 1), including two GV2J γ P1/2 clonal populations (fig 1, lane 6). A single clonal population was amplified from the case of T cell chronic lymphocytic leukaemia examined. A T cell prolymphocytic leukaemia sample generated two clonal populations. A GV1J γ clonal population was amplified from a very rare case of T cell $\gamma\delta$ hepatosplenic lymphoma (patient 11).

Although diagnostically the results generated by the two methods were 100% concordant, the Genotyper electropherograms were easier to interpret than the LIS-SSCP gel images (fig 1). The Genotyper software enabled exact size determination of each clonal TCR γ PCR product, which could only be estimated using LIS-SSCP gel analysis. The Jurkat cell line DNA used as a clonal control for both the TCR γ 1 and TCR γ 4 multiplex reactions reproducibly generated products of 386 bp (TCR γ 1) and 219 bp (TCR γ 4). Patient 5 was

used as a clonal control for both the TCR γ 2 and TCR γ 3 multiplex reactions and reproducibly generated products of 199 bp (TCR γ 2) and 175 bp (TCR γ 3). These control reactions were performed in excess of 20 times. In addition, seven different samples from patient number 11 were amplified using the TCR γ 1 multiplex reaction. All samples generated two peaks (387 and 393 bp). Therefore, this method can reproducibly detect a clonal T cell population in multiple samples from the same patient.

The sensitivity study revealed that Jurkat DNA could be detected to a dilution of 4% in normal DNA using both methods of analysis. However, identification of a peak of known size with a fluorescence intensity raised with respect to the surrounding peaks on a Genotyper electropherogram was considerably easier than detection of a slight increase in the ethidium bromide staining of one band within the polyclonal smear seen on the LIS-SSCP gel (data not shown).

Discussion

Our study demonstrates the use of fluorescent nucleotides to label TCR γ PCR products internally, allowing semi-automated analysis using an ABI 377 DNA sequencer. The results generated using this method were consistent with those achieved using an established LIS-SSCP technique. A wide variety of different TCR γ gene rearrangements were detected using this fluorescent method, including at least one clonal population using each of the seven gene segment families. Although the number of clonal populations detected was not increased using the DNA sequencer, the Genotyper profiles were much easier to interpret than the LIS-SSCP gel images. A great deal of experience is required before confident, accurate interpretation of these gel images is possible, which can only be achieved by supervised practice. Ease of interpretation is a major consideration when introducing a new technique into a diagnostic laboratory with multiple operators.

Each TCR γ PCR product can be sized accurately and reproducibly using the DNA sequencer, which allows identical clonal populations to be detected in different samples, providing a method of monitoring disease progression and response to treatment. The size of a product can only be estimated using LIS-SSCP analysis, making the comparison of different samples more complex. Semiquantitative assessment of a clonal population within a background of polyclonal cells can also be achieved using the DNA sequencer and this is not possible using LIS-SSCP.

TCR γ gene rearrangement analysis using fluorescently labelled primers in conjunction with a DNA sequencer has been described.^{11 12} Fluorescently labelled primers are specific for a single PCR. In contrast, fluorescent nucleotides can be incorporated into all PCR methods and the availability of different fluorescent dye labelled nucleotides (R110, RG6, and TAMRA) allows pooling of PCR products for analysis on the DNA sequencer.

Fluorescent nucleotides can considerably cut the cost of converting a number of different PCRs to fluorescent PCR. We have used fluorescent nucleotides in a further nine PCRs. In each case, labelled nucleotides at a concentration of 0.04 μM were simply added to the original reaction mixture. No changes to the PCR reaction mixture or cycling conditions were required.

LIS-SSCP and DNA sequencer analysis provided comparable sensitivity, the limit of detection being 4% Jurkat diluted in polyclonal DNA. However, a clonal peak (of known size) could be identified with greater confidence on a Genotyper electropherogram than could a slightly overexpressed band within a smear on an LIS-SSCP gel. These results are consistent with the finding of Simon *et al* that the use of fluorescently labelled primers in conjunction with a DNA sequencer failed to increase the sensitivity of detection above that of PAGE.¹² The sensitivity reported by Simon *et al* was 9 ng of Jurkat diluted in polyclonal DNA using both sequencer and PAGE analysis. The percentage of Jurkat in this DNA mix is not stated. Our detection limit of 4% is equivalent to 1.6 ng of Jurkat, much less than the 9 ng detected by Simon *et al*. Therefore, the use of fluorescently labelled nucleotides in place of labelled primers does not appear to compromise sensitivity.

We conclude that the use of fluorescently labelled nucleotides provides a more flexible and economical alternative to end labelled fluorescent primers for the detection and monitoring of clonal TCR γ gene rearrangements and other lymphoproliferative disorders.

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