

Investigation of T-cell receptor- γ gene rearrangement in gastrointestinal lymphomas by PCR-SSCP analysis

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Abstract

AIM: To analyze the characterization of T-cell receptor- γ (TCR- γ) gene rearrangement in the gastrointestinal lymphomas and evaluate the value of PCR-SSCP analysis in gastrointestinal lymphomas investigation.

METHODS: TCR- γ gene rearrangement segments of gastrointestinal lymphomas were cloned and sequenced. Single clone plasmid and mixed clone pIsamids were subsequently submitted to PCR-SSCP analysis to investigate the relationship between the number of amplified clones and band patterns of the amplified products. The PCR products of TCR- γ gene rearrangement of 40 gastrointestinal lymphomas were electrophoresed on agarose gels and the positive cases on agarose gels were studied by SSCP analysis.

RESULTS: The sequencing showed that TCR- γ gene rearrangement of the gastrointestinal lymphomas included functional gene and pseudogene with extensive variety in the junctional regions. In SSCP analysis, the number of the single-stranded bands was about two times of the number of amplified clones, and double-stranded band became broad with the increased number of the amplified clones. Thirteen of the 25 B-cell gastrointestinal lymphomas and 14 of the 15 gastrointestinal T-cell lymphomas were positive detected on agarose gel electrophoresis. Of the positive cases detected by SSCP analysis, 3 B-cell lymphomas and 13 T-cell lymphomas showed positive bands. The other cases showed only smears. The rearranged pattern included 13 monoallelic gene rearrangements and 3 biallelic or oligoclonal gene rearrangements.

CONCLUSION: The pattern of TCR- γ gene rearrangement in gastrointestinal lymphomas are similar to that of the nodular lymphomas. PCR-SSCP analysis for TCR- γ gene rearrangement can be applied both for adjuvant diagnosis of gastrointestinal lymphomas and analysis of the gene rearrangement pattern. The ratio of TCR- γ gene rearrangements occurred in T-cell gastrointestinal lymphomas is significantly higher than that in B-cell gastrointestinal lymphomas. The gene rearrangement pattern involves monoallelic and biallelic (or oligoclonal) gene rearrangement.

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INTRODUCTION

Primary gastrointestinal lymphomas are the most common extranodal lymphomas and are almost exclusively of non-Hodgkin types^[1-3]. These lymphomas are biologically different from their nodal counterparts. Distinguishing the two phenotypes is of importance as the survival rates are different between T-cell and B-cell gastrointestinal lymphomas^[4-6]. As cytological features of gastrointestinal lymphomas are usually not specific, the combination with clinical, histological, immunophenotypic and molecular genetic studies is necessary to characterize these neoplasms^[7].

T-cell receptor (TCR) gene rearrangement provides a convenient genetic marker for demonstrating clonality in T-cell malignant neoplasm. Among the four TCR gene types, the TCR- γ gene rearrangement occurs in the early stage of cell differentiation and the rearrangement retains in both α β and γ δ phenotypic T cells along with the cell differentiation^[8]. Analysis of TCR- γ gene rearrangement is of practical value in determining the clonality of T-cell populations and for diagnosis T-cell malignant neoplasms^[9-11]. But the junctional diversity in TCR- γ gene rearrangement is limited compared with the other TCR or IgH gene rearrangement because of the fewer inserted nucleotides during the rearrangement, this lead the PCR products of TCR- γ gene rearrangement amplified by a consensus primers pair to be similar in length. The similarity may result in false clonal bands, when analyzed by standard gel electrophoresis methods that separate DNA molecules based solely on size^[12,13]. A high-resolution for the amplified products of TCR γ gene rearrangement is required.

Single-strand conformational polymorphism analysis (SSCP) separates different DNA molecules based on their single-strand secondary structure conformations under certain electrophoretic condition. DNA molecules with different conformations migrate different rates on polyacrylamide gel and therefore can be separated from each other^[14]. Besides gene point mutation analysis, SSCP analysis is also suitable for TCR- γ gene rearrangement study^[15].

In this study, we expected to analyze the characteristics of TCR- γ gene rearrangement and evaluate the practicability of PCR-SSCP for TCR- γ gene rearrangement in the diagnosis and analysis of gastrointestinal lymphomas.

MATERIALS AND METHODS

Clinical specimen

Specimens of the gastrointestinal lymphoma were obtained by biopsy and surgical resection between January 1997 to October 2002 in the department of pathology of the Nangfang Hospital of the First Military Medical University, the First Municipal Hospital of Guangzhou and the No. 157 Hospital of The People's

Liberation Army. Of the 40 patients, 9 had tumors located within stomach, 1 in duodenum, 1 in jejunum, 10 in ileum, 11 in ascending colon, 2 in transverse colon, 5 in descending colon and 1 in rectum. All cases were regarded gastrointestinal lymphomas because of absence of disease in other organs. The specimens were fixed in buffered formalin, embedded in paraffin and stained with haematoxylin-eosin (HE). Lineage was confirmed by staining with CD20 and CD79a for B-cells, and CD45RO and CD3 for T-cells. The 40 cases were retrospectively diagnosed according to the new WHO classification^[16]. Twenty-five of the 40 cases were confirmed B-cell lymphomas and 15 cases were confirmed T-cell lymphomas. A reactive lymph node and Jurkat cell line were considered as negative and positive control, respectively.

DNA extraction

The 7 μm thickness sections were cut with disposable blades, collected on glass slides, deparaffinized with xylene, washed with ethanol, and rehydrated in deionized water. The moist tissues of 0.5 cm \times 0.5 cm containing abundant malignant cells (according to HE) were scraped off the glass slides with a sterile blade, and collected in Eppendorf tube. Jurkat cells were collected and rinsed twice in PBS. A total of 50 μL digesting buffer containing 10 mmol/L Tris, 1 mmol/L EDTA, 10 g/L Tween 20, and 200 mg/mL proteinase K were added to the tubes. The digestion was performed at 37 $^{\circ}\text{C}$ for 16 h. Samples were then heated at 96 $^{\circ}\text{C}$ for 10 min to inactivate the enzyme, centrifuged, and the supernatant was used as template for PCR amplification.

PCR and agarose gel electrophoresis

The primers design was based on those used by Benhattar *et al.*^[13], spanning the rearranged V γ 1-8 and J1/2 region with the expected products ranging from 160 to 190 bp in length. The sequences of the primers were: TVG sense, 5' AGGGTTGTGTTGGAATCAGG3', and J1/2 antisense, 5' CCTGTGACAACAAGTGTGT3'. PCR amplification was performed in GeneAmp PCR system 9600 (Perkin Elmer, Norwalk, CT). The reaction mixture (30 μL) contained PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3), 200 mmol/L each of dNTP, 1.5 mmol/L MgCl₂, 0.5 mmol/L of each primer, and 1.25 U of AmpliTaq (Perkin Elmer). A total of 300 ng of samples and cell line DNA (or 20 ng of plasmids) were used as templates, respectively. The PCR amplification cycles were designed to a decreasing annealing temperature similar to a touch-down PCR^[17]: 5 cycles consist of 94 $^{\circ}\text{C}$ for 45 s, 60 $^{\circ}\text{C}$ for 45 s, 72 $^{\circ}\text{C}$ for 30 s; the next 5 cycles consisted of 94 $^{\circ}\text{C}$ for 45 s, 56 $^{\circ}\text{C}$ for 45 s, 72 $^{\circ}\text{C}$ for 30 s; and the last 25 cycles consisted of 94 $^{\circ}\text{C}$ for 45 s, 56 $^{\circ}\text{C}$ for 45 s, 72 $^{\circ}\text{C}$ for 30 s. The reaction mixture was first incubated at 95 $^{\circ}\text{C}$ for 5 min to denature double-stranded DNA. Finally, an additional incubation for 10 min at 72 $^{\circ}\text{C}$ was performed to ensure full extension of the products. In all experiments, monoclonal (Jurkat cell lines) and polyclonal (reactive lymph node) controls were run in parallel with the test samples. Products were analyzed by electrophoresis on 20 g/L agarose gels stained with ethidium bromide, visualized and photographed on an UV transilluminator. SSCP analysis was performed only if a single band of the expected size was detected on the gel. DNA from each sample was amplified at least twice.

Cloning, sequencing and PCR amplifying for the plasmids inserted with TCR γ gene rearranged segment

A total of 100 μL PCR products of TCR- γ gene rearrangement from five T-cell gastrointestinal lymphomas was separately purified and ligated into pGEM-T Easy cloning vector (Promega Corporation, 2 800 Woods Hollow Road Madison WI 53711-

5399 USA). The recombinant vector DNA was then transformed into *Escherichia coli* JM109. The transformed bacteria solution was spread on selective LB-agar plate with X-gal, IPTG and ampicillin. After overnight incubation at 37 $^{\circ}\text{C}$, 2 to 3 white colonies (with DNA inserts in the plasmid) from each plate were selected randomly and cultured in LB medium containing ampicillin for 12 h at 37 $^{\circ}\text{C}$. Plasmid DNA was isolated from each culture following a standard protocol^[18] and dissolved in double distilled deionized water. Deoxyribonucleic acid sequencing of cloned PCR products was performed following the standard protocol^[19] involving the universal forward-sequencing primer M13 for pGEM-T vector. The concentration of the plasmids were adjusted to 20 ng/ μL . According to the sequencing results, 2, 3 and 4 plasmids with different inserted PCR products were mixed, respectively. Single and mixed plasmids were submitted as templates to PCR.

SSCP analysis

SSCP analysis was performed according to the Signoretti *et al.*^[15] with some alternation. Briefly, 6 μL of PCR products were mixed 1:1 with denaturing loading buffer (containing 95 g/L formamide, 0.5 g/L bromophenol blue, 2.5 g/L xylene cyanol and 20 mmol/L EDTA). The mixture was heated to 95 $^{\circ}\text{C}$ for 10 min, quickly chilled on ice for 1 min, and loaded on 120 g/L nondenaturing polyacrylamide (29:1 acrylamide/bisacrylamide) gel in Tris-borate- EDTA (1 \times TBE) buffer. The gel was electrophoresed at 120 (12 v/cm) volts for approximately 4 to 6 h at room temperature and subsequently stained with silver and photographed.

Statistical analysis

Comparisons between the 2 groups were performed by the chi-square test using SPSS 10.0 statistical software (SPSS Company, Chicago, Illinois, USA). *P* values < 0.05 in two-tailed were considered statistically significant.

RESULTS

Agarose gel electrophoresis analysis

PCR amplification for the TCR- γ rearranged gene showed single band of 160-190 bp in 93% (14/15) T-cell and 52% (13/25) B-cell gastrointestinal lymphomas on agarose gel electrophoresis. The positive rate was significantly higher in T-cell lymphomas (*P* < 0.05) than that in B-cell lymphomas. Both the reactive lymph node and the Jurkat cell line showed single band (Figure 1).

Analysis of the TCR- γ gene rearrangement by sequencing

Sequence of the clones were identified to the particular V γ (1-8) segment by internet (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>) assisted comparison with published TCR- γ sequence (gi 28436398 ref NG_001336.2. Homo sapiens T cell receptor gamma locus (TRG@) on chromosome 7, Length=140728). The sequenced V γ segments were assigned to the member of the V γ (1-8) family that, by comparison, showed the highest degree of homology (95% to 100%). Most of the V γ (1-8) -J γ 1/2 combinations had the functional V γ genes: V3, V4, V5, V8. Of the 14 sequenced cases, only one case was pseudogene V γ 7. In the VJ junctional N regions, the deleted nucleotides ranged from 2 to 23 nucleotides with an average length of 9.38 \pm 6.42 bp, and the size of the inserted nucleotides ranged from 3 to 13 bp (average length, 7.38 \pm 2.98 bp). The TCR γ junctional regions showed considerable diversity with no two clones showing the same sequence. The size of the PCR products amplified by this primers combination ranged between 160-190 bp. The letters with delete lines represent the deleted nucleotides and the capital letters represent the inserted nucleotides during the rearrangement.

	V-region	N-region	J-region
(186 bp) V3.....	tctattactgtgccacctgggacaggCCTGGAgaattattataagaaactctttgg.....		J1/2
(185 bp)V4....	.attactgtgccacctgggatggGCCT gaattattataagaaactctttggc.....		I1/2
(185 bp) V5.....	.gtctattactgtgccacctgggaeaggTGGgaattattataagaaactcttt.....		I1/2
(188 bp)V5.....	.gtctattactgtgccacctgggacaggGGAGAGGgaattattataagaaactctt.....		I1/2
(164 bp) V7....	.ctgtgccacctgggaeaggTATTGGATTGgaattattataagaaactctttggca.....		J1/2
(164 bp) V8.....	tattactgtgccacctgggataggTCACGTTTTTGGGgaattattataagaaactctttg..		J1/2

PCR-SSCP analysis of plasmids inserted with TCR- γ gene rearranged segments

During SSCP analysis and gel electrophoresis, the PCR products of plasmids inserted with TCR- γ gene rearranged segments showed discrete bands in all lanes, except lane 7 (Figure 2) with faint band, which might be due to the insufficient DNA product in lane 7. The fastest migrating band was double-stranded DNA, located in the zone ranging from 160-190 bp. Running above the double-stranded DNA were single-stranded bands. PCR products of one cloned plasmid showed two single-stranded bands. The bands patterns of different clones were different (Figure 1 lane 5, 6 and 8). PCR products of 2 mixed cloned plasmids showed one double-stranded band and 3 to 4 single-stranded bands (Figure 2 lane 1, 2, 3 and 4). PCR Products of 3 to 4 plasmids mixed together showed one broad double-stranded band and 6 to 8 single-stranded bands (Figure 3). Some faint single-stranded bands presented in the lanes were disregarded or interpreted with caution. Based on the results of these studies, two single-stranded bands (sometimes only one) represented the monoallelic gene rearrangement, and those with more than two single-stranded bands might be the biallelic or oligoclonal gene rearrangement.

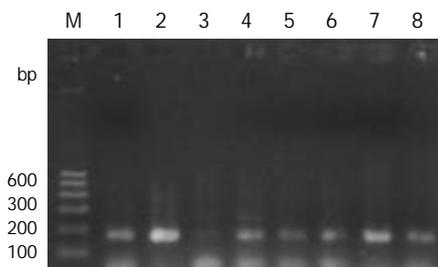


Figure 1 Agarose gel electrophoresis of the PCR products of TCR- γ gene rearrangement. M: DNA marker; Lane 1: reactive lymph node; lane 2: Jurkat cell line; lanes 3-8: gastrointestinal lymphomas.

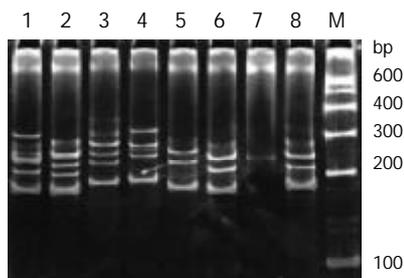


Figure 2 PCR-SSCP analysis of the plasmids inserted with TCR- γ gene rearranged segments. M: DNA marker; Lanes 1-4: two mixed plasmids amplified products showed one double-stranded band and three to four single-stranded bands; lanes 5-8: single plasmid PCR products showed one double-stranded band and two single-stranded bands (except lane 7).

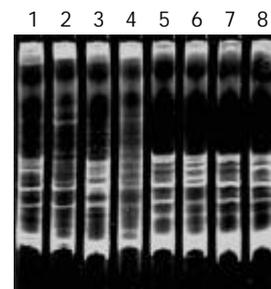


Figure 3 PCR-SSCP analysis of mixed plasmids inserted with TCR- γ gene rearranged segments. Lanes 1-4: PCR products of 3 mixed plasmids showed one double-stranded band and about five to six single-stranded bands; Lanes 5-8: PCR product of 4 mixed plasmids showed one double-stranded band and about six to eight single-stranded bands. The double-stranded bands were broader than that in the single or double plasmids amplified products.

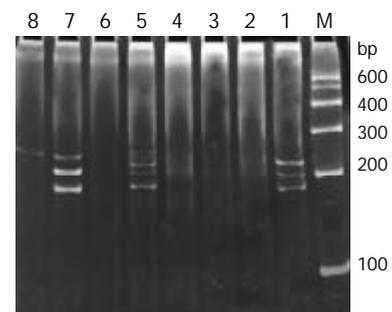


Figure 4 PCR-SSCP analysis of some gastrointestinal lymphomas. M: DNA marker; Lane 1: Jurkat cell line; lane 2: reactive lymph node; lanes 3-8: B-cell gastrointestinal lymphomas. Lane 5 had 4 single-stranded bands and lane 1, 7, 8 had one or two single-stranded bands. The other samples showed only smears with no predominant band.

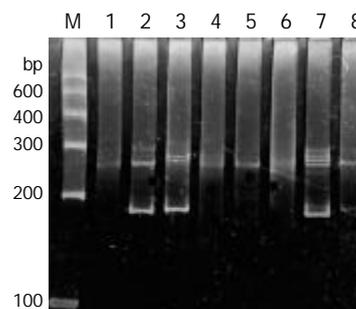


Figure 5 PCR-SSCP analysis of some T-cell gastrointestinal lymphomas. M: DNA marker; Lane 7 presented four single-stranded bands, and the others had one or two single-stranded bands. Four of the 8 samples were absent of the double-stranded bands probably due to insufficient PCR products.

PCR-SSCP analysis of the gastrointestinal lymphomas

Of the 29 samples submitted to SSCP analysis, 17 showed one or more discrete bands, including the Jurkat cell line, 3/13 B-cell gastrointestinal lymphomas (Figure 4) and 13/14 T-cell gastrointestinal lymphomas (Figure 5) confirmed by the HE and immunohistochemistry. The positive rate was significantly higher in T-cell lymphomas than that in B-cell lymphomas ($P < 0.01$). Less than half of the cases only showed the single-stranded bands, but not double-stranded bands. This might be due to insufficient quantity of PCR products. According to the numbers of the single-stranded bands of the 17 positive cases, 13 cases were monoallelic gene rearrangement, and 4 cases might be biallelic or oligoclonal gene rearrangements. The other 10 samples and reactive lymph node which were positive on the agarose gel electrophoresis yielded only smears with no obvious band. This patterns was the characteristic of rearranged TCR- γ V-J fragments amplified from polyclonal T-cell populations without a predominant.

DISCUSSION

Most T-cells express the $\alpha\beta$ T-cell phenotype and only a small population expresses $\gamma\delta$ TCR. The lineage is determined by the gene rearrangement and protein expression. γ -chain gene rearrangement generally takes place in the $\alpha\beta$ T-cell but not express a functional $\gamma\delta$ receptor^[12]. In addition, a fraction of B cells undergoes TCR- γ gene rearrangement, especially immature B cells (illegitimate gene rearrangement)^[20,21]. Furthermore, a T-cell lymphoma may aberrantly express the B-cell phenotype^[22]. In this study, the illegitimate TCR- γ gene rearrangement in B-cell gastrointestinal lymphomas was investigated, which showed about 12% (3/25) positive result. TCR-gamma gene rearrangement involves the recombination between one of the V region (V γ 1-8, V γ 9, V γ 10, V γ 11) and one of the J regions (J γ 1/2, J γ P1/2, J γ P)^[8]. In this study, we used only V γ 1-8 and J γ 1/2 consensus primers set to analyze the most common VJ gamma recombination. It is likely that using additional V and J region primers would have further increased the yield of positive results, but our study showed that this consensus primers combination seemed to be sufficient for the diagnosis of T-cell gastrointestinal lymphomas.

Cloning and sequencing are the precise but time and labour-consuming methods in the study of clonal gene rearrangement. Because of the admixture of reactive T-cells, at least 8 to 10 clones from each patient must be sequenced for the purpose of diagnosis, to confirm whether there are any two clones showing the same sequence^[21]. In this study, we intended to know the general aspects of the TCR- γ gene rearrangement in gastrointestinal lymphomas and to obtain the clonal rearranged gene segments. So, only 2 to 3 clones from each patient were sequenced, which were obviously inadequate for clonality judgement. The sequencing result showed that the TCR- γ junctional region (N region) presented considerable diversity with no two clones showing the same sequence. The junctional region differed in both sequence and size with the deleted and inserted nucleotides. The PCR products obtained by the consensus V $\gamma_{1-8}/J_{1/2}$ primers ranged from 160 to 190 base pairs. All these characteristics were similar to that of nodal lymphomas^[23]. The narrow size of the PCR products between different clones did not provide a means for distinguishing between monoclonal and polyclonal TCR junction by the conventional agarose gel electrophoresis^[13].

PCR-SSCP has been introduced to detect DNA mutation because it can resolve DNA molecules differing as little as a single base pairs substitution. The high sensitivity of PCR-SSCP makes it fit to tell apart the clonal from polyclonal gene rearrangement, and its simplicity makes it a good candidate for application in clinical laboratory tests^[15]. In the present study,

we would like to study whether the different band numbers observed on SSCP gel tell the different rearranged patterns apart in gastrointestinal lymphomas. The PCR products of single clone and mixed clones submitted to PCR-SSCP analysis showed that a consistent relationship existed between the number of single-stranded bands twice the numbers of clones amplified, which suggested that the number of single-stranded bands might estimate whether the samples were monoallelic or biallelic (oligoclonal) gene rearrangements. Two single-stranded bands (sometimes only one band) could definitely be regarded as monoallelic gene rearrangement. Three to four bands could be regarded as a clonal T-cell population with biallelic gene rearrangements or two clonal T-cells populations both with monoallelic gene rearrangements. Those with more than four single-stranded bands were most probably oligoclonal gene rearrangements^[24]. Owing to the high sensitivity and resolution of PCR-SSCP, multiple single-stranded bands must be interpreted with caution, and the PCR-SSCP analysis conditions must be optimized individually for a special pair of primers. The condition for the V $\gamma_{1-8}/J_{1/2}$ primers we used was optimized thoroughly before introducing to the study of gastrointestinal lymphomas.

Gastrointestinal lymphomas differ from their nodal counterpart in many aspects and are more likely to be misdiagnosed before surgical resections^[25]. The histological and cytological features may be more complex, with most having pleomorphic small, medium, large or anaplastic cell cytologic features and generally with a mixture of reactive cells. The immunophenotype is especially important for gastrointestinal lymphomas, as the prognosis for T-cell gastrointestinal lymphomas is poorer than that for B-cell type and more incline to perforation^[3]. It should be emphasized that gastrointestinal lymphomas must be defined by a combination of factors as not a single parameter is entirely specific.

The sensitivity and validity of TCR- γ gene rearrangement investigated by PCR-SSCP were compared with conventional histology and immunohistology. Of the 40 gastrointestinal lymphomas, 13 of the 15 T-cell lymphomas and 3 of the 25 B-cell lymphomas showed positive results. The high consistency indicates that PCR-SSCP analysis for the TCR- γ gene rearrangement study is an additional powerful tool for the diagnosis of T-cell gastrointestinal lymphomas.

Furthermore, the gene rearrangement pattern, which may be a prognostic factor, can be revealed by PCR-SSCP^[26]. Our study showed that 23.5% (4/17) of the positive cases were biallelic or oligoclonal gene rearrangements. PCR-SSCP gives reproducible band migration patterns for the same DNA molecules, therefore, this technique potentially can be used to compare multifocal lesion from a single patient or to compare a recurrent lesion with a primary one in a patient previously diagnosed with T-cell gastrointestinal lymphomas^[27].

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