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Original Artice

Detection of monoclonality in B- and T-cell lymphoma by the use of polymerase chain reaction of formalin-fixed paraffin-embedded tissues

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Summary

The polymerase chain reaction (PCR) method was applied into formalin-fixed paraffin-embedded tissues for pathological diagnosis of lymphoma, but their results were unstable and required the reevaluation of PCR-processing. We approached the investigation of the most suitable condition in PCR procedures. The examined tissues were routinely processing paraffin-embedded sections and consisted of 7 cases of B-cell lymphoma, 2 cases of T-cell lymphoma, and one case of Hodgkin's disease.

In B-cell lymphoma the semi-nested PCR was very well done with the primers of Framework three (Fr3) within the variable (V) segment for an upperstream consensus primer and LJH within the joining (J) region for an outer downstream one and VLJH within the J region for inner downstream one. The annealing temperature in PCR was set up at 55℃ and a polyacrylamide gel electrophoresis (PAGE) was preferable to an agar gel because of their shortness of PCR products. The monoclonality by PCR-PAGE could be confirmed in B-cell lymphoma and also shown in T-cell lymphoma and Hodgkin's disease. The final diagnosis of B-cell lymphoma required comprehensive analysis with microscopic examinations by both a hematoxylin-eosin stain and an immunohistochemical one.

In T-cell lymphoma the ordinary PCR was performed with both TVγ as an upperstream consensus primer of T-cell receptor-γ (TCR-γ) gene and TJγ as a downstream one. It was desirable that the annealing temperature was 60℃ in PCR and PAGE analysis followed PCR. This monoclonality restricted in T-cell lymphoma.

This monoclonal detection in paraffin-embedded tissues by PCR-PAGE is very useful in cases with routinely-biopsied formalin-fixed and paraffin-embedded tissues.

Key Words: polymerase chain reaction, lymphoma, formalin-fixed paraffin-embedded tissue

Indroduction

The monoclonal confirmation of lymphoma by PCR became routinely examined with an immunohistochemical method (5). Demonstration of monoclonarlity got certain in using long DNA chains because they could include all points of DNA rearrangements. The shortness of examined DNA chains limited their searching areas, whick increased false negative results. This was why PCR analysis usually used in frozen tissues, which allowed an extraction of very long DNA chains. Even if long DNA chains were fragmented in the fixation process by acid-sided formalin, most pathologists should diagnose lymphoma by these formalin-fixed and paraffin-embedded path-

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ological specimens. In spite of their shortness the most valuable DNA areas were searched and several primers for PCR were recommended as follows: (1) in B-cell lymphomas; PCR was to research the rearrangement in VDJ regions (D: diversity) in immunoglobulin heavy chain gene (IgH) and consisted of both (i) Fr2A (Framework two A) or Fr3A (Framework three A) for annealing with IgH-V and (ii) LJH or VLJH for IgH-J annealing, (2) in T-cell lymphomas: TVγ and TJγ were used for the study of the rearrangement in VJ regions in TCR-γ genes (1,3).

In our institute of surgical pathology, more rapid and precise histopathological diagnosis was recommended. When only formalin-fixed paraffin-embedded tissues could be available, it was necessary that an accurate PCR technique should be established. In this study we reanalyzed previous methods and reported the most convenient one.

Materials and methods

Tissues

Speciments consisted of seven B-cell lymphoma, two T-cell lymphoma, and one Hodgkin's disease. The diagnosis of lymphoma was confirmed by immunohistochemistry against lymphocytic markers. All specimens were processed by formalin-fixation and paraffin-embedding.

DNA extraction from paraffin-embedded sections

Two or three pieces of 20μm-depth paraffin-embedded section were used for PCR analysis, and followed by routine extraction procedure as previous report (1,3,4): 1. deparaffinization, 2. dehydration, 3. digestion by 50% proteinase K 180μl and 10% SDS 20μl at 56℃ for one night, 4. deactivation of proteinase K at 95℃ for 10', 5. mixtured with same volume of mix liquid (phenol/chloroform/isoamyl alcohol=25:24:1), 6. retrieval of supernatant, 7. mixtured with same volume of 2-propanol at -20℃ for 6°, 8. retrieval of sediment, 9. rinsed by 70% alcohol, 10. dry, 11. dissolved in 100μl of Tris-EDTA buffer and adjusted into working concentration (50-70μg/μl of double strands DNA with spectrophotometer at 260nm).

PCR primers

For B-cell lymphoma a nested- or semi-nested PCR was performed with upperstream primers and downstream primers around VDJ region of IgH-gene: the former primers consisted of Fr2A (5'-TGG[A/G]TCCG[C/A]CAG[G/C]C[T/C][T/C]CNGG-3') placed in 5'-side of V region or Fr3A (5'-ACACGGC[C/T][G/C]TGTATTACTGT-3') placed in 3'-side of V region, and the latter consisted of LJH (5'-TGAGGAGACGGTGACC-3') placed in 3'-side of J region or VLJH (5'-GTG ACCAGGGTNCCTTGGCCCCAG-3') of 5'-side of J region (3).

For T-cell lymphoma a semi-nested or conventional PCR was done with one couple of upperstream and downstream primers in VJ region of TCR-γ gene: the former primer was TVγ (5'-AGGG TTGTGTTGGAATCAGG-3') and the latter primer was TJγ (5'-CGTCGACAACAAGTGTT GTTCCAC-3') (1).

Each working concentration of primer solution was adjusted to 20pmol/μl.

PCR amplification

Each 20 or 21 μl sample consisted of 14μl distilled water, 2μl 10xPCR buffer (Perkin Elmer), 2μl Gene Amp dNTP Mix (2mM/L), sense primer, antisense primer, 0.2μl Ampli Taq Gold (5U/μl), and 1μl DNA sample. In nested or semi-nested PCR for B-cell lymphoma each adding primer solution was 0.5μl at both first and second round PCR procedures. In semi-nested PCR for T-cell lymphoma the first round primer content was 0.2μl and, furthermore, second round primers were 0.8μl and 1μl, where total addition of each primers reached at 1μl respectively (4).

In B-cell lymphoma the first round PCR was as follows: 1. hot start at 95℃ for 10', 2. 30 cycles of DNA amplification, consisted of denaturation at 93℃ for 45", annealing at 50-62℃ for 45", and extension at 72℃ for 110", 3. last extension at 72℃ for 10' (3).

In T-cell lymphoma the first round PCR was as follows: 1. hot start at 95℃ for 10', 2. 25-45 cycles of DNA amplification, consisted of denaturation at 94℃ for 30", annealing at 55-63℃ for 45", and extension at 73℃ for 45", 3. last extension at 72℃ for 10'. Furthermore, the second round PCR followed: 1. hot start at 95℃ for 10', 2. 0-30 cycles of DNA amplification, consisted of denaturation at 94℃ for 30", annealing at 55-63℃ for 45", and extension at 73℃ for 45", 3. last extension at 72℃ for 10' (1).

Electrophoresis

Agarose gel was adjusted in 2% or 2.5% (1,3,4). PAGE concentration was 10% (5). Electronic voltage was 50V or 100V.

These mini-gels were stained with ethidium bromide and viewed under ultraviolet light and photographed.

Results

The conditions of PCR and electrophoresis were listed in the left side of figure (Fig. 1). Upper part listed B-cell lymphoma and lower part was T-cell lymphoma. The corresponding results were shown in the right side of figure.

B-cell lymphoma samples produced one or three discrete bands within the predicted size of 240-280bp with the usage of Fr2A primer or 80-120bp with Fr3A primer. The bands with Fr3A primer were more prominent than those with Fr2A. The most appropriate conditions consised of 55℃ of annealing temperature, 30 cycles of first round amplification, and 20 cycles of second round amplification. Short 100bp-DNA products by PCR recommended PAGE rather than Agarose gel in electrophoresis. Half samples showed multiple bands in PAGE. Several positive bands were also identified in T-cell lymphoma and Hodgkin's disease.

T-cell lymphoma samples produced one prominent band within the predicted size of 160-190bp. There were many positive bands in B-cell lymphoma, which required elevated annealing temperature up to 60℃ and PAGE electrophoresis. The positivity by conventional single PCR was

Fig. 1. Conditions of PCR and their corresponding results.

lymphoma type							B lymphoma							T lymphoma		Hodgkin
# original							SN00-07	99-14620	99-10854	99-12206	99-12283	99-12709	99-13668	99-14844	99-9790	99-5487
organ							stomach	node	node	skin	stomach	node	node	intestine	node	node
PCR conditions																
1st.			2nd.			electrophoresis										
annealing	cycles	primers	annealing	cycles	primers											
B-cell lymphoma:																
50	30	Fr3A/LJ	50	20	Fr3A/VLJ	Agar. 2%			+		+	+	+			
52	30	Fr3A/LJ	52	20	Fr3A/VLJ	Agar. 2%			+	+		+	+	+	±	±
54	30	Fr3A/LJ	54	20	Fr3A/VLJ	Agar. 2%		+	+	+	+	+	+	+	±	±
60	30	Fr3A/LJ	60	20	Fr3A/VLJ	Agar. 2%			+	+	+		+	±	±	±
62	30	Fr3A/LJ	62	20	Fr3A/VLJ	Agar. 2%			±	±			+		±	+
65	30	Fr3A/LJ	55	15	Fr3A/VLJ	Agar. 2%	+	+	+	+		+	+	+	+	+
55	30	Fr3A/LJ	55	10	Fr3A/VLJ	Agar. 2%		±	+	+		+	++		+	+
55	30	Fr3A/LJ	55	20	Fr3A/VLJ	Agar. 2.5%	+	+	+	+	+	+	+	±	±	±
55	30	Fr2A/LJ	55	20	Fr2A/VLJ	Agar. 2%		+		+			++			±
55	30	Fr2A/LJ	55	20	Fr2A/VLJ	Agar. 2%							++			+
55	30	Fr3A/LJ	55	20	Fr3A/VLJ	PAGE 10%	++	++	±	3	2			±		+
55	30	Fr3A/LJ	55	20	Fr3A/VLJ	PAGE 10%	+	+	±~+	3	2			±		+
55	30	Fr3A/LJ	55	20	Fr3A/VLJ	PAGE 10%	++	++	2*	3	3			2		±
55	30	Fr3A/LJ	55	20	Fr3A/VLJ	PAGE 10%	++		−	2	3			2		+
T-cell lymphoma:																
55	25	TV.Y/TJ	55	30	TV.Y/TJ	Agar. 2%		+	+	+			+	+	+	+
57	25	TV.Y/TJ	57	30	TV.Y/TJ	Agar. 2%		+	+	+			+	+	+	+
59	25	TV.Y/TJ	59	30	TV.Y/TJ	Agar. 2%		+	+				+	+	+	
61	25	TV.Y/TJ	61	30	TV.Y/TJ	Agar. 2%		+	+				+	+	+	
63	25	TV.Y/TJ	63	30	TV.Y/TJ	Agar. 2%		+	+				+	+		
60	25	TV.Y/TJ	60	28	TV.Y/TJ	Agar. 2%		+							+	+
60	25	TV.Y/TJ	60	25	TV.Y/TJ	Agar. 2%										
60	25	TV.Y/TJ	60	20	TV.Y/TJ	Agar. 2.5%		+	+				+			
60	45	TV.Y/TJ	−	−	−	Agar. 2%		±	±	±			±	+	+	±
60	45	TV.Y/TJ	−	−	−	PAGE 10%	−	−	−					+	±	−

*: numbers of band in Arabic figures, +: single band

more reliable than that by semi-nested PCR.

Discussion

The monoclonality of rearranged IgH or TCR-γ gene is of important diagnostic value in distinction between lymphoma and reactive lymphoid proliferation. PCR technique provides an accurate and simple methoid for the confirmation of monoclonality. Several method using PCR to formalin-fixed paraffin-embedded sections were reported, but their accuracies remained in dispute (1,3). Our results suggested that the PCR approach for IgH and TCR-γ genes was a reliable and easily reproducible adjunct method for the diagnosis of lymphomas. The examination for a rearrangement of both IgH and TCR-γ genes must be done simultaneously for each tissue, and the absence of any clonal rearangement for genes of the opposite lineage provides strong evidence for B- or T-cell lineage. This PCR approach can be applied to small routine biopsy specimens, such as endoscopic or skin biopsies as well as lymph nodes, and, furthermore, could have useful clinical implications, especially in the assessment of monoclonality in lymphoms without contributory immunohistochemical findings.

In the PCR analysis of B-cell lymphoma, we have found the Fr3 primers to give efficient amplification from all paraffin tissue samples. While the detection of IgH monoclonal rearrangement was confirmed in most lymphomas, their positivity could not be free from any false negative cases. A suitable strategy may involve initial screening with a Fr3 primers, and followed by a use of a Fr2 primers in negative cases. Several bands of IgH gene arrangement also appeared in T-cell lymphoma samples, which was unknown to date and required additional immunohistochemical examinations. It was easy to understand that Hodgkin's disease showed IgH gene rearrangement because Hodgkin's disease became regarded as B-cell lymphoma. There was no practical problem in the process of pathological diagnosis of Hodgkin's disease because Hodgkin's disease was easily differentiated by the presence of Reed-Sternberg cells in hematoxylin-eosin stained specimens. Despite the problems of false negatives and false positives, we propose that PCR detection of monoclonality represents a valuable tool in the distinction of a wide range of B-cell lymphoproliferative diseases with the combination of immunohistochemical examination.

In an application of PCR method for T-cell lymphoma, same rearrangement of TCR-γ genes was also reported in B-cell lymphoma (2,7,8). There were false positive bands of TCR-γ rearrengement in our B-cell lymphoma samples, which disappeared by the usage of PAGE electrophoresis istead of agarose gel. This suggested that PAGE was recommended because the length of DNA products became very short up to 100bp in formalin-fixed paraffin-embedded tissue samples.

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原　著

ホルマリン固定・パラフィン包埋切片のPCR手技によるB細胞性リンパ腫、T細胞性リンパ腫の単クローン性の同定

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ホルマリン固定・パラフィン包埋切片より抽出したDNAを使用したPCR・電気泳動法によるリンパ腫診断は不確実で、更に、その診断手法を発展する余地がある。今回、B細胞リンパ腫7例、T細胞リンパ腫3例、ホジキン病1例のホルマリン固定・パラフィン包埋切片を使って、PCRによるリンパ腫診断手技の適正化について検討した。

B細胞リンパ腫は、semi-nested PCR-PAGEで検討されるべきである。最適条件は、primerとして、IgH-遺伝子のVDJ部分を両端とし、上流にFr3（Framework 3)と、下流に初回LJH，二回目VLJHとし、annealing温度を55℃として、約100bpのDNA産物を得た。PAGE上、B細胞リンパ腫のみでなく、一部のT細胞リンパ腫並びにホジキン病にもbandが認められた。確診には、免疫組織化学的染色、並びに、通常の標本上でReed-Sternberg細胞の確認が必要であると考えられた。

T細胞リンパ腫も、PCR-PAGEで検討される必要がある。最適条件は、primerとして、TCR-γ遺伝子のVJ部分を両端とし、上流にTVγ、下流にTJγとし、annealing温度を60℃として、160-190bpのDNA産物を得た。PAGE上、T細胞リンパ腫を鑑別し得た。

われわれの施設のごとくに通常の外科病理診断施設においては、検索提供材料において制限がある。すなわち、（1）ホルマリン固定・パラフィン包埋標本に限局されており、（2）肺や胃などの反復生検が困難であり、また、（3）生検材料が極めて小さい。この様な状況において、PCR-PAGE手技は極めて貴重な診断手段となりうる。

キーワード：ＰＣＲ、T細胞性リンパ腫、ホルマリン固定・パラフィン包埋組織

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