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Original Artice

Improved detection of monoclonality in B- and T-cell lymphomas by polymerase chain reaction (PCR) of formalin-fixed paraffin-embedded tissues with the use of deoxyribonucleic acid (DNA) purification and concentration: Efficacy of PCR-PAGE of Byori Center method for lymphoma, Ver. 2

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Summary

Polymerase chain reaction (PCR) method has been applied into formalin-fixed paraffin-embedded tissues for a pathological diagnosis of lymphoma in our laboratory for one year (5-7) , but their results were unstable in spite of an additional usage of deoxyribonucleic acids (DNA) extraction kit (DEXPAT, Takara, Japan) (7), which required the further reevaluation of PCR-processing. In this study to improve PCR specificity we made a crude DNA samples purified and concentrated before PCR procedures. The examined tissues were routinely-processed paraffin-embedded sections, and consisted of 4 cases of B-cell lymphoma, 4 cases of T-cell lymphoma, 1 case of normal lymph node, 1 case of normal tonsil, and 1 case of normal lung.

After usual retrieval of DNA by phenol/chloroform/isoamylalcohol, DNA samples were purified and concentrated by DNA Clean & Concentrator ™-5 (Zymo Research, USA) before PCR procedure.

On B-cell lymphoma the reasonable nested PCR condition was as follows: the first round PCR was done with the primers of an upperstream (sense) "Framework two (Fr2A) " and a downstream (anti-sense) "LJH", and the second round PCR was done with the primers of an uppersteam (sense) "Framework three (Fr3 A) " and a downstream (anti-sense) "VLJH". The annealing temperature in PCR was set up at 55℃. The amplification cycles consisted of 20 times and 23 times at the first round PCR and the second round PCR, respectively. A polyacrylamide gel electrophoresis (PAGE) followed. The monoclonality by PCR-PAGE could be confirmed in half cases of B-cell lymphomas and its specificity increased remarkably from 40% to 100%. The final diagnosis of B-cell lymphoma required comprehensive analysis by both clinical fingings and microscopic examinations with an immunohistochemical analysis.

On T-cell lymphoma the preferable PCR was performed with both TV γ as an upperstream primer of T-cell receptor-γ (TCR-γ) gene and TJ γ as a downstream one. Modified semi-nested PCR was performed. The first round PCR was done with primer TJ γ, and the second round PCR were done with primers of both TJ γ and TV γ. A reasonable annealing temperature was 55℃, and amplification cycles was 25 times and 28 times in the first round PCR and the second round PCR, respectively. PAGE analysis followed PCR. The monoclonality by PCR-PAGE could be identified 75% cases of T-cell lymphomas.

For a monoclonal detection with paraffin-embedded tissues the PCR-PAGE is very useful, and its specificity is improved by the purification and concentration of original DNA extracts in using routinely-biopsied formalin-fixed and paraffin-embedded tissues. And we call this improved method "PCR-PAGE of Byori Center method for lymphoma, Ver. 2."

Key Words : polymerase chain reaction (PCR), lymphoma, formalin-fixed paraffin-embedded tissue, purification and concentration of DNA retrieval, DNA Clean & Concentrator

Indroduction

Our previous monoclonal confirmation of lymphoma by PCR became routinely examined with an immunohistochemical method (5, 6, 7) . Even if long DNA chains were fragmented in the fixation process by acid-sided formalin, several specimens could be diagnosed as lymphoma by these formalin-fixed and paraffin-embedded pathological specimens. Whereas PCR was useful for a diagnosis of lymphoma, several suspicious cases of lymphomas confirmed by clinical findings and immunohistochemistry failed to be positive (60%, 3 cases / 5 cases) or were conversely false positive (60%, 3cases/ 5 cases) (Table 1).

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Table 1. B-cell lymphoma analyzed by PCR-PAGE for IgH with DNA retrieved by DEXPAT

DNA C/C	not performed										performed				
cases	B					lung		intestine		node	B				
nested PCR	○	○	×	×	×	○	×	○	○	×	×	×	×	×	×

DNA C/C: DNA Clean & Concentrator, B: B-cell lymphoma, T: T-cell lymphoma, lung: normal lung tissue, intestine: normal intestine tissue, node: normal lymph node, nested PCR: the second round PCR consisted of annealing 55℃ and 20 amplification cycles, result: ○: positive, ×: negative

Impurities in original DNA retrievals were thought to be one of the causes to block PCR and bring false positivity (5), and then DNA purification and additional concentration was recommended. But PCR sensitivity decreased from 40% (2 cases / 5 cases) to 0% (0 case / 5 cases), after the DNA extracted by DEXPAT was rinsed by DNA cleaning reactant (Table 1). For this reason we abandoned DEXPAT for our new DNA extraction and processing, and then decided that the first crude DNA solution had to be extracted by an ordinary phenol extraction method. In this study we examined if a removal of impurities was efficacious in improving PCR results.

Materials and methods

Tissues

Specimens consisted of four B-cell lymphomas, four T-cell lymphomas, one normal lymph node, one normal tonsil, and one normal lung. The latter three normal tissue specimens were for negative control in each PCR analysis. The diagnosis of lymphoma was confirmed by both clinical findings and an immunohistochemistries of lymphocytic markers. All specimens were processed on formalin-fixation and paraffin-embedding method.

DNA extraction from paraffin-embedded sections

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

DNA purification and concentration

DNA Clean & Concentrator™-5 (Zymo Research, USA, abbreviated to DNA C/C) was used in accordance with following protocol: 1. 150μl DNA extract was mixed with DNA Binding-Buffer, 2. centrifuged at 10000g for 10", 3. discarded the flow-through, 4. added Wash-Buffer, 5. centrifuged at 10000g for 10", 5. discarded the flow-through, 6. added another Wash-Buffer, 7. centrifuged at 10000g for 30", 8. discarded the flow-through, 9. added 8 μl water, 10. spinned and recovered, 11. added another 8 μl water, 12. spinned and recovered, and finally we got 16 μl of a purified and concentrated DNA sample.

PCR primers

For B-cell lymphoma a nested-PCR was performed with upperstream (sense) primers and downstream (anti-sense) primers around VDJ region of IgH-gene: (a) the uppersteam primers consisted of (i) "Fr2A" (5'-tgg [A/g] tc cg [c/A] cAg [g/c] c [t/c] [t/c] cn gg-3'), placed in 5'-side of V region and used for the first round nested-PCR, or (ii) "Fr3A" (5'-AcA cgg c [c/t] [g/c] tgt Att Act gt -3'), placed in 3'-side of V region and used for the second round nested-PCR, and (b) the downstream primers consisted of (i) "LJH" (5'-tgA ggA gAc ggt gAc c-3'), placed in 3'-side of J region and used for the first round nested-PCR, or (ii) "VLJH" (5'-gtg Acc Agg gtn cct tgg ccc cAg -3'), placed in 5'-side of J region and used for the second round nested-PCR (3, 6, 7).

For T-cell lymphoma a modified semi-nested PCR was done with one couple of upperstream (sense) primer and downstream (anti-sense) one in VJ region of TCR-γ gene: the former primer was TVγ (5'-Agg gtt gtg ttg gAA tcA gg 3'), which was used only in the second round PCR, and the latter primer was TJγ (5'-cgt cgA cAA cAA gtg ttg ttc cAc 3'), which was used for both the first round PCR and the second one (1, 5, 6).

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

PCR amplification

(1) PCR reactant mixtures:

In B-cell lymphoma, each 20 μl sample consisted of 14 μl distilled water, 2 μl 10xPCR buffer (Perkin Elmer), 2 μl GeneAmp dNTP Mix (2mM/L), an upperstream (sense) primer, a downstream (antisense) primer, 0.2 μl Ampli Taq Gold (5U/μl), and 1 μl DNA sample processed with DNA C/C. In a nested PCR for B-cell lymphoma, each adding primer solution was 0.5 μl, "mixtures of 0.5 μl Fr2A and 0.5 μl LJH" and "mixtures of 0.5 μl Fr3A and 0.5 μl VLJH" at the first round PCR procedure and the second round one, respectively.

In a modified semi-nested PCR for T-cell lymphoma, the first round 20 μl sample consisted of 15.4 μl distilled water, 2 μl 10xPCR buffer, 1.2 μl dNTP, 0.2 μl downstream primer TJγ, 0.2 μl Taq, and 1 μl DNA sample after DNA C/C. The second round 20 μl sample consisted of 5.9 μl distilled water, 1 μl 10xPCR buffer, 1.8 μl dNTP, 0.65 μl upperstream primer TVγ, 0.55 μl downstream primer TJγ, 0.1μl Taq, and 10μl of the first round PCR

products (4, 6).

(2) PCR thermal cycling conditions:

In B-cell lymphoma the first round PCR was as follows: 1. hot start at 95℃ for 10', 2. 30 cycles of DNA amplification, each cycle of which consisted of denaturation at 93℃ for 45", annealing at 55℃ for 45", and extension at 72℃ for 110", 3. last extension at 72℃ for 10'. The second round PCR was, furthermore, as follows: 1. hot start at 95℃ for 10', 2. 20-27 cycles of DNA amplification, each cycle of which consisted of denaturation at 93℃ for 45", annealing at 50-58℃ for 45", and extension at 72℃ for 110", 3. last extension at 72℃ for 10' (3, 6, 7).

In T-cell lymphoma the first round PCR was as follows: 1. hot start at 95℃ for 10', 2. 25 cycles of DNA amplification, each of which consisted of denaturation at 94℃ for 30", annealing at 55℃ for 45", and extension at 73℃ for 45", 3. last extension at 72℃ for 10'. The second round PCR, furthermore, followed: 1. hot start at 95℃ for 10', 2. 24-30 cycles of DNA amplification, each of which consisted of denaturation at 94℃ for 30", annealing at 55℃ for 45", and extension at 73℃ for 45", 3. last extension at 72℃ for 10' (1, 6).

Electrophoresis

PAGE concentration was 12.5% (5, 6, 7). Electronic voltage was 200V.

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

Results

Every B-cell lymphoma samples produced one discrete

Table 2. B-cell ymphoma analyzed by PCR-PAGE for IgH with the use of usual DNA retrieved after treated with DNA C/C

Cases				B				T	node
PCR									
1st		2nd							
℃	cycles	℃	cycles						
55	30	50	20	○	×	×	○	×	×
			23	○	○	×	×	×	×
			24						
			25						
			26						
			27						
		52	20	○	×	×	×	×	×
			23	○	○	×	×	×	×
			24						
			25						
			26	○	○	○	△	○	△
			27						
		55	20	○	×	×	×	×	×
			23	○	○	×	△	×	△
			24	○	○	△	△	△	○
			25	○	○	△	△	○	○
			26	○	○	○	△	○	○
			27						
		58	20	△	×	×	△	×	×
			23	○	△	×	△	×	×
			24	○	○	×	△	×	△
			25	○	○	×	△	×	△
			26						
			27	○	○	×	△	×	△

1st: the first round nested PCR, 2nd: the second round PCR, B: B-cell lymphoma, T: T-cell lymphoma, node: normal lymph, ℃, cycles: PCR annealing ℃ and amplification times, result: ○: positive, △: ambiguous/smear, ×: negative, blank: not examined because of its invalidity on the analogy out of a part

Table 3. T-cell lymphoma analyzed by PCR-PAGE for TCR γ with the use of usual DNA retrieval after treated with DNA C/C

Cases					T				B	tonsil	lung
PCR											
	1st		2nd								
	℃	cycles	℃	cycles							
previous	55	45		0	○	×	×	×	×	×	×
modified nested		25	55	24	△	△	△	×	×	×	×
				26	△	○	△	×	×	×	×
				27	○	○	○	×	△	△	×
				28	○	○	○	×	△	△	×
				30	○	○	○	△	△	△	×

1st: the first round nested PCR, 2nd: the second round PCR, B: B-cell lymphoma, T: T-cell lymphoma, tonsil: normal tonsil tissue, lung: normal lung tissue, ℃, cycles: PCR annealing ℃ and amplificaton times, result: ○: positive, △: ambiguous/smear, ×: negative

band at the predicted size of 80-120bp. The most appropriate conditions consised of 55℃ of annealing temperature, 30 cycles of the first round amplification, and 23 cycles of the second round amplification. The sensitivity increased slightly from 40% to 50% (Table 1) and the false positivity was significantly decreased from 60% to 0% (Table 2).

Each T-cell lymphoma samples produced one prominent band within the predicted size of 160-190bp. The sensitivity increased from 25% to 75%. The suitable PCR condition consisted of 55℃ of annealing temperature, 25 cycles of the first round amplification, and 27 or 28 cycles of the second round amplification (Table 3).

Discussion

In the PCR analysis of B-cell lymphoma, we had reported the original PCR method using the commercial kit (DEXPAT, Takara, Japan) for retrieval of DNA from formalin-fixed paraffin-embedded sections, but their accuracies had remained in dispute (6, 7). While the detection of IgH monoclonal rearrangement had been confirmed in 40% of B-cell lymphomas, their positivity could not have been free from a possibility of false positivity. Despite the problems of low sensitivity we propose that this new improved PCR detection method of monoclonality increases its specificity from 40% to 100%, and represents a valuable tool in the distinction of a wide range of B-cell lymphoproliferative diseases with the combination of immunohistochemical examination.

In a diagnostic application of PCR method for T-cell lymphoma, our PCR-sensitivity of T-cell lymphoma was 75%. Even if there were false positive bands of TCR-γ rearrengement in our B-cell lymphoma samples, which became decreased by the usage of DNA purification and concentration before routine PCR. Because the same rearrangements of TCR-γ genes were also reported in B-cell lymphoma (2, 9, 10), these biclonal lymphomas could be distinguished by our new method.

The monoclonality of rearranged IgH or TCR-γ gene is of important diagnostic value in distinction between lym-

phoma and reactive lymphoid proliferation. PCR technique provides an accurate and simple method for the confirmation of monoclonality.

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原　著

ホルマリン固定・パラフィン包埋病理組織切片より回収したDNAを更に純化・濃縮する改良型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の改善に関する検討：病理センター方式PCR-PAGEによるリンパ腫診断，Ver. 2の意義

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われわれは、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を発表し、実際に1年間実施してきたが、その結果に関しては、臨床所見並びに免疫組織化学検査と比較してその整合性において未だ不確実で、更に、その診断手法を発展する余地があると判断された。今回、抽出されたDNA溶液をあらかじめ純化・濃縮する操作を実施することにより、PCR感受性と特異性を改善することに関して討した。DNAの純化・濃縮には、DNA Clean & Concentrator™-5（Zymo Research, USA）を使用した。B細胞リンパ腫4例、T細胞リンパ腫4例、また、正常陰性対照例として、正常リンパ節1例、正常扁桃1例、正常肺1例の各ホルマリン固定・パラフィン包埋切片に関して、PCRによるリンパ腫診断手技の適正化について再検討した。

B細胞リンパ腫は、nested PCR-PAGEで検討した。PCR最適条件は、annealing温度を55℃として、約100bpのDNA産物を得た。PCRによる診断陽性率は50%であったが、その特異性は40%より100%へ向上できた。偽陰性例に対しては、補助診断としての免疫組織化学的染色、並びに、通常の標本上での検討が必要であると考えられた。

T細胞リンパ腫においては、変法nested PCR-PAGEが検討された。最適条件は、annealing温度を55℃として、160-190bpのDNA産物を得た。PCR診断によるT細胞リンパ腫の感受性は75%であった。

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定されており、(2)特に、呼吸器、消化器などの反復生検が困難であり、また、(3)生検材料は、しばしば、極めて小さく、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。その為にも、偽陽性率を低下させること（すなわち、特異性を高めること）が最重要であり、抽出されたDNAを純化・濃縮してPCRに使用することが有意義であると確認できた。

キーワード：polymerase chain reaction (PCR), lymphoma, formalin-fixed paraffin-embedded tissue, purification and concentration of DNA retrieval, DNA Clean & Concentrator

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