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Original Artice

Detection of monoclonality in B-cell lymphoma by polymerase chain reaction (PCR) with the use of DNA extraction kit (Takara DEXPAT) for 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 B-cell lymphoma, but their DNA extractions were complex and time-comsuming. We approached the utility of one step procedure by the use of DNA extraction kit (Takara DEXPAT). The examined tissues were routinely processing paraffin-embedded sections and consisted of 7 cases of B-cell lymphoma, 3 cases of T-cell lymphoma, one case of Hodgkin's disease, and 8 cases of non-lymphomatous normal tissues for negative control.

DNA extraction procedure became easy by the use of DEXPAT kit: the operation was continuous in one microtube and required only 30 minutes.

The nested PCR was very well done with the following primers: 1. Framework two A (Fr2A) within the variable (V) segment as an outer upperstream consensus primer, 2. Framework three A (Fr3A) as inner upperstream one, 3. LJH within the joining (J) region as an outer downstream one, 4. VLJH within the J region as inner downstream one. The annealing temperature in PCR was set up at 55℃ and a concentration of polyacrylamide gel electrophoresis (PAGE) was 12.5% 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.

Key Words:polymerase chain reaction, B-cell lymphoma, formalin-fixed paraffin-embedded tissue, DNA extraction kit (Takara DEXPAT)

Indroduction

We reported the PCR-PAGE technique for the diagnosis of B-cell lymphoma by the use of these formalin-fixed and paraffin-embedded pathological specimens (5). In B-cell lymphomas, PCR should be to research the rearrangement in VDJ regions (D: diversity) in immunoglobulin heavy chain gene (IgH) and consisted of both (i) Framework two A (Fr2A) or Framework three A (Fr3A) for annealing with IgH-V and (ii) LJH or VLJH for IgH-J annealing (1-9).

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

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Materials and methods

Tissues

Speciments consisted of seven B-cell lymphoma, two T-cell lymphoma, and one Hodgkin's disease, and eight normal tissues for negative control. 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 20 μm-depth paraffin-embedded section were used for PCR analysis, and followed by DNA-extracting procedures: 1. tissue specimens drop into 1.5 ml-microtube, 2. pouring of 10 drops of DEXPAT (0.25ml), 3. vortex, 4. adding of another 10 drops of DEXPAT (0.25ml, total 0.5ml), 5. stirring with Vortex, 6. heating in 100℃ for 10' with several stirring, and with cap-roof piercing by a needle to avoid increasing pressure and sealing by alminium-sheet against vaporizaton, 7. centrifuging at 12000rpm, 10', 4℃, 8. collecting the DNA-solving water-layer. The length of retrieved DNA chains was less than 400bp (4,6).

PCR primers

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'-GTGACCAGGGTNCCTTGGCCCCAG-3') of 5'-side of J region (5,6).

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

PCR amplification

Each 20 or 21 μl sample consisted of 14 μl distilled water, 2 μl 10xPCR buffer (Perkin Elmer), 2 μl Gene Amp dNTP Mix (2mM/L), sense primer, antisense primer, 0.2 μl Ampli Taq Gold (5U/μl), and 1 μl DNA sample. In nested or semi-nested PCR for B-cell lymphoma each adding primer solution was 0.5 μl at both first and second round PCR procedures (3,5).

The first round PCR was as follows: 1. hot start at 95℃ for 10', 2. PCR by 30 cycles of DNA amplification, consisted of denaturation at 93℃ for 45", annealing at 52-65℃ for 45", and extension at 72℃ for 110", 3. last extension at 72℃ for 10'. Furthermore, the second round PCR was as follows: 1. hot start at 95℃ for 10', 2. 10-20 cycles of DNA amplification, consisted of denaturation at 93℃ for 45", annealing at 52-65℃ for 45", and extension at 72℃ for 110", 3. last extension at 72℃ for 10' (3).

Electrophoresis

PAGE concentration was 12.5%. Electronic voltage was 125V. 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 Table. Upper part listed specimens of lymphoma and normal negative controls. The corresponding results were shown in the middle of Table.

B-cell lymphoma samples produced one or two discrete bands around the predicted size of 100bp. The most appropriate conditions consisted of 1: primers: Fr2A and LJH for first round PCR, and Fr3A and VLJH for second round one, 2: 55℃ of annealing temperature, 3: 30 cycles of first round amplification, and 4: 15-18 cycles of second round amplification. Short 100bp-DNA products by PCR recommended 12.5% PAGE. In B-cell lymphoma, this nested PCR showed 30% of definitive positivity and 70% of total positivity. Several false positivity was found in T-cell lymphoma and Hodgkin's disease. No false positivity was confirmed in any normal tissues for negative controls.

PCR				lymphoma												non-lymphoma							
primer	℃	PCR	%	B							T			H		negative control							
		II		A	B	C	D	E	F	G	H	I	J	K		stomach	instestine 1	intestine 2	colon	lung 1	lung 2	lung 3	lymph node
3+3	55	15	10	+												+	±	±	±	+	+	±	±
3+3	55	15	12.5	+	±	++										+				+	−		
3+3	55	15	15	+	±	+										+		+		+	−		
3+3	57	15	12.5	−	±	+										+		−		−	−		
3+3	57	15	15	±	±	+										+		−		−	−		
3+3	60	15	10	+												+	±	±	±	−	−	±	±
3+3	61.7	15	10	+												−	±	±	±	±	−	±	−
3+3	62	15	12.5	−	−	−	−	−	−	−							−	−	++	−		−	
3+3	62.2	15	10														±	±	±	±		±	
3+3	62.7	15	10	−													−	−	−	−		−	
3+3	63.4	15	10	−												−	−	−	−	−		−	
3+3	65	15	10	−												−	−	−	−	−		−	
2+3	52	15	12.5	−	−	−										−				−	−		
2+3	55	15	12.5	−	±	++										−				−	−		
2+3	55	16	12.5				+	±	+	−	+	−	+	+									
2+3	55	18	12.5	−	+	+										−				−	−		
2+3	57	15	12.5	−	−	±										−				−	−		
2+2	55	15	12.5	−	−	−										++				++	−		
2+2	57	15	12.5	−	−	−										++				++	−		

primer: 3+3=Fr3AxLJH+Fr3AxVLJH, 2+2=Fr2AxLJH+Fr2AxVLJH, 2+3=Fr2AxLJH+Fr3AxVLJH
℃: ℃ of DNA annealing in PCR
II: cycles of 2nd PCR
%: % of polyacrylamide in PAGE
H: Hodgkin's disease
A～K: # of lymphoma

Table. Results of PCR-PAGE positivity

Discussion

DNA-extracting kit (Takara DEXPAT) was very convenient method in the use of formalin-fixed paraffin-embedded sections in PCR (4,6). The time required shortened to 30' and the whole procedure could be performed in a day, which made PCR a reasonable technique for routine clinical examination.

In the PCR analysis of B-cell lymphoma, we have shown the nested PCR with primers Fr2A, Fr3A, LJH, and VLJH for the monoclonality of rearranged IgH gives efficient amplification in paraffin-embedded tissue samples. The detection of IgH monoclonal rearrangement was confirmed in 30% of B-cell lymphomas, and no false positivity was found in any normal tissues. Several bands of IgH gene arrangement also appeared in T-cell lymphoma samples, which required additional immunohistochemical examinations. The examination for a rearrangement of both IgH and TCR-γ (T cell receptor-γ) genes must be done simultaneously for each tissue, and the absence of any clonal rearrangement for genes of the opposite lineage provides strong evidence for B- or T-cell lineage (5). 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 becomes a valuable tool for diagnosis of lymphoma.

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原　著

ホルマリン固定・パラフィン包埋切片DNA抽出キット（Takara DEXPAT）使用による、B細胞リンパ腫単クローン性のpolymerase chain reaction（PCR）同定

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DNA抽出キット(Takara DEXPAT)によりホルマリン固定・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B細胞リンパ腫診断を検討した。今回の検討材料は、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、さらに、陰性対照8例で、いずれもホルマリン固定・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。

DNA抽出用簡易キット(Takara DEXPAT)の取り扱い上、単一のマイクロチューブで操作でき、DNAの消失が少なく、全行程が30分以内に終了し、簡便であった。

DNA産物が約100bpと短鎖の為、polyacrylamide濃度を12.5%とし、PAGE上で、明瞭にbandを確認できた。

B細胞リンパ腫は、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、初回PCRとして上流にFr2A（Framework 2A)と下流にLJH，二回目PCRとして上流にFr3Aと下流にVLJHとし、annealing温度を55℃とした。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。

キーワード：ＰＣＲ、Ｂ細胞リンパ腫、ホルマリン固定・パラフィン包埋組織、ＤＮＡ抽出キット

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