B Cell Clonality Detection By Immunoglobulin Light And Heavy Chain Primers In Paraffin Embedded Lymphoma Tissues*

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Abstract **Objectives** Clonality determination by using Polymerase chain reaction (PCR) amplification of the immunoglobulin heavy chain (IgH) has been a useful tool in diagnosis of B cell lymphoproliferative disorders. However, the biggest problem of this method is its high false negative rate, especially in paraffin embedded tissues. To improve this, several attempts have been used in our study, including the combination detection of IgH and immunoglobulin light chain (IgL) gene rearrangements. **Methods** two conventional pairs of primers for IgH and TCR γ, two newly designed primer paires for Igκ or Igλ were used to detect 55 cases of paraffin-embedded blocks, which had been diagnosed ″lymphoma″ in clinic. And 3 cases of reactive proliferative lymph node tissues and DG75 cell lines were used as negative and positive controls respectively. **Results** The positive rates of IgH primers (P1) and IgL primers (Pκ/Pλ) were 79.5% and 71.8% in 39 cases of B cell lymphomas, 6.3% and 12.5% in T cell lymphoma (16 cases) respectively. There is no significant difference between IgH and IgL primers in the detection of 39 cases of B cell lymphomas, while the positive rate was increased to 92.3% in combining the two pair of primers together. **Conclusion** Gene rearrangement detection rate can be greatly increased by combining Ig light and heavy chain in the B cell lymphoma on paraffin embedded tissue.

Key Words B cell clonality; Lymphoma; PCR; Paraffin tissues

Abbreviations:
ALCL: Anaplastic Large Cell Lymphoma; MCL: Mantle Cell Lymphoma; MALT: Extranodal marginal zone B-cell lymphoma of mucosa–associated lymphoid tissue type; PZBL: Marginal Zone B-Cell Lymphoma, LPL: Precursor T-lymphoblastic lymphoma; PTCL: Peripheral T cell lymphoma, LBL: Precursor T-lymphoblastic lymphoma, AITCL: Angioimmunoblastic T cell lymphoma; DLBCL: Diffuse large cell B-cell lymphoma; PLA: Peoples´ Liberation Army; IHC: Immunohischemistry. PET: paraffin embedded tissues; NHL: Non-Hogkin's lymphoma

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Assessment of clonality at the molecular genetic level has become an important and clinically useful test for differentiating benign and malignant lymphoproliferative disorders, comfirming a morphological diagnosis of lymphoma, establishing the presumptive lineage of a clonal lymphoid proliferation.

The immunoglobulin (Ig) gene rearrangement is main character of B cell lymphoid tumor, which is benefit for the diagnosis of non-Hodgkin's lymphoma (NHL) through the detection[1,2]. Its detection method can be categorized into Southern blot hybridization and polymerase chain reaction (PCR) methods. Now the former method has largely been replaced by the latter one for its several inconveniences: time consuming, requires relatively large amounts of pathological materials and its lower sensitivity (5-10%). As compared to southern blotting, PCR has the advantage of high sensitivity, low cost, fast turning around time, and technical simplicity. Furthermore, because small DNA fragments are enough for PCR, this approach has been successfully applied in the study of formalin-fixed and

paraffin-embedded tissue[3].

More methods for the detection of IgH rearrangement has been established [4–9] since McCarthy first reported in 1990 [1]. Most of them were designed either using oligonucleotides recognizing FR3 and FR2 fragments of variable regions of the IgH and expanding to the joint regions, or using heavy chain family-specific oligoprimers that recognize framework 1 regions (FR1) of Ig-heavy chains. However, even using multiple oligoprimers, the overall sensitivity ranges between 60% and 85%, and at least 15% of neoplasms remain undetected. And these false negative rates might be higher if the formalin-fixed and paraffin-embedded material is used.

In recent years, Ig light gene rearrangement analysis has been estimated as a powerful tool for establishing the clonal nature of B-cell disorders. It is reported that during B-cell differentiation, the Igκ gene locus undergoes rearrangement after IgH. In the case in which functional Igk chain products are not obtained, the Igλ locus subsequently undergoes rearrangement [10,11]. Studies have shown that in all Igκ+ and in a very large majority of Igλ+ B-cell malignancies, either productive or nonproductive Igκ products are formed. Therefore, the rearranged genomic products of Igκ and Igλ represent an excellent marker for B-cell clonal analysis[12].

With the completion of the human genome sequencing, almost all Ig-light and heavy chains have been published onlines (http://www.ncbi.nlm.nih.gov/igblast/showGermline.cgi). We collected and compared these sequences by using clustal W software, and designed Igκ and Igλ light chain primers from consensus fragments of the FR3 and J regions after analysis of all known V and J family members. These single pair of degenerate oligoprimers should be able to recognize the large majority of all Ig light chain variable region (V) members within the six different families of the genes. Combining with the detection of the traditional Ig-heavy chain primers, the detection rates of 58 cases the paraffin embedded tissue were increased to 92.3%.

MATERIALS AND METHODS

Archive blocks and lymphoma cell line

A panel of 58 cases of formalin-fixed and paraffin-embedded archival blocks (from 1996 to 2002) consist of 39 cases of B cell lymphomas (24 cases of diffuse large cell lymphomas, 6 cases of follicular lymphomas, 4 cases of small lymphocytic lymphoma, 1 case of B-ALCL, B-MCL, MALT, PZBL and LPL respectively), 16 cases of T cell lymphomas (9 cases of PTCL, 4 cases of AITCL and 3 case of LBL) and 3 cases of atypical reactive lymphoid proliferation. All of the samples were retrieved from the departments of pathology at Nanfang hospital of China (the affiliated hospital of Nannfang Medical University); Guangzhou No157 Hospital of PLA and the First people´s Hospital Guangzhou. They were diagnosed as ″lymphoma″ previously, and re-identified by two of our authors (Zhu Meigang and Zhao Tong), the diagnosis of NHL was established in accordance with the criteria of the latest WHO classification[13], basing on morphology (H&E section), and immunophenotypic criteria (expression of CD20, CD79a, CD3, CD45RO and TdT antigen). DG75 and Jurkat cell lines were used as positive controls of B and T lymphoma gene rearrangement detection respectively.

DNA preparations

Genomic DNA was extracted from cell line suspensions and paraffin-embedded archival tissue blocks. DNA from cell lines was extracted with phenol/chloroform, alcohol precipitated and TE buffer dissolved. DNA obtained from archival formalin-fixed, paraffin-embedded tissue, one to three 5-μm-thick tissue sections were cut and mounted on slides. Tissues were deparaffinization with xylene (three times) and then dehydrated with ethyl alcohol. Only tumor tissues on slide were selected as target tissues by using manual-dissections techniques. Selected samples were suspended in 50μl of digestion buffer (containing 250μg/ml of proteinase K (MERCK, Darmstadt, Germany), 50 mM Tris-HCl (pH8.5), 1mM EDTA (pH8.0), and 0.5% Tween-20), incubated at 55℃ for 3hrs, heated at 95℃ for 10 min to inactivate proteinase K, centrifuged at full speed for 10 min. The supernatant was used as a template for PCR amplification. When encountered negative amplification for β-globin (house keeping gene), the DNA was purified using Qiagen columes (QIAamp DNA mini Kit, USA), or re-extracted till the β-globin gene was showed positive.

Primer pairs for PCR

Primer design Ig-light chain includes Igκ and Igλ chain, each of them were composed of V and

J fragment, the primer of gene rearrangement was designed in these regions. All germline configuration gene segments of Vκ、Kλ、Jκ、Jλ were found out according to the following address: http://www.ncbi.nlm.nih.gov./igblast/showGermline.cgi and compared by the software of Clustal W/X, all the primers including newly designed primers were testified and analyzed by primer premier 5.0 software.

Five pairs of primers were used in this study (See table 1). Primer pair FR-JH (simply P1) is used for the amplification of IgH, and primer pair Vk-FRk-(Jk1+Jk2) (simply Pκ) was for Igκ, Primer pair VλFR3A-(Jλ1-7+Jλ4) (Pλ) was for Igλ; Primer pair TVG-TJX (simply PT) was for TCRγ.

PCR procedure All PCR primer-pair conditions were optimized on the T gradient PCR instrument (Whatman Biometra). The PCR reaction mixture consisted of: primers (2mM), dNTPs (200 μM), commercially prepared PCR buffer (Perkin Elmer), MgCl2 (25 mM), and Taq Polymerase (5 U/μl). The total reaction volume was 20μl. The procedure of PCR were: denaturation at 94°C, annealing at 55°C~61°C, which depend on the primer condition, and extension at 72°C, each for 30~40 seconds, with a total number of 35 cycles. All PCR products were electrophoresed in an 2% argrose (Spain) or 8% polyacrylamide gel (sigma)and visualized with ethidium bromide or $AgNO_3$.

Control: DNA from DG75 and Jurkat cell lines were applied as positive controls of the gene rearrangement of B and T cell lymphoma respectively, and ddH_2O (no DNA) as negative control. A sample was considered positive when a clear band of expected size could be visualized after staining of the gel with ethidiun bromide (0.5ug/ml).

Statistics: McNemar test was used in the test by the software SPSS 8.0.

RESULTS

Primer analysis

Primer of VκFR3 region human Igκ genes locus are composed of 46 V gene fragment, all of them has been sequenced. The results have been compared by the Clastal W software and are listed in Table 2.

Most Ig light chain primers we collected are located in the conservation regions. VkFR3-1 and VkFR3-2 primer [14] of Vκ are similar to the Fk-FR3-3 primer[15]. We design a new primer VkRk(5′ CA(T/G) C(A/C/T/G) AGGTTCAGTGGCAGTG 3′) by comparison these primers, no dimmer and hairpins are found by the results of primer premier software. (Table 2)

Jλ region primer The locus of Igλ chain J genes are located in chromosome 22. Primer Vλ-FR3[14] and VλFR3A[15] are on the conservation regions,and their sequences are almost the same except the 5′ end. The primers of Jλ regions are slightly different from that of Vλ. Jλ1 and Jλ237 primers of Jλ region in the reference[14] are only fit to part of the Jλ sequences. It can be easily find that the other Jλ region genes are almost the same except Jλ4 gene sequence. So we designed two new primers Jλ1-7 and Jλ4, and no hairpin and dimmer are found. Primer analysis is showed in the table 3.

The detection of B and T cell lymphoma of paraffin embedded tissues The result of a diagnostic run is shown in fig.1 (a, b). A sharp dis-

Table 1 Nucleotide sequences of the primers used in the paper

Name of primers		sequences (5′ to 3′)	product Size (bp)
P1	FR	CTGTCGACACGGCCGTGTATTACTG	
	JH	AACTGCAGAGGAGACGGTGACC	100–120
Pκ	Vk-FRk	CA(T/G)C(A/C/T/G)AGGTTCAGTGGCAGTG	
	Jk1	TTGAT(A/T/C)TCCACCTTGGTCCC	
	Jk2	TTTAATCTCCAGTGGTGTCC C	130–150
Pλ	VλFR3A	GAGGA(C/T)GAGGCTGA(T/C)TATTACTG	
	Jλ1-7	GG(G/C)ACC(A/C)AG(C/G)TGACCGTCCT	
	Jλ4	AAAATGATCAGCTGGGTTCC	70–90
PT -TCRγ	TVG	AGGGTTGTGTTGGAATCAGG	
	TJX	CGTCGACAACAAGTGTTGTTCCAC	240–260
β-globin	PC04	CAACTTCATCCACGTTCACC	267
	GH20	GAAGAGCCAAGGACAGGTAC	

Table 2 the frequencies of nucleotides of Ig light chain conservative VKFR3 region

	VK FR3 region sequence
A	0 40 0 14 18 41 0 6 0 0 046 0 0 0 0 2 46 0 2 0 0 27 0 1 11 0 0 1
T	0 3 25 0 7 0 0 0 4646 0 0 0 310 0 0 0 0 0 40 0 0 0 43 0 35 0 0 0
C	46 3 1 32 17 5 0 0 0 0 46 0 0 150 0 44 0 0 5 0 0 0 1 45 0 0 0 7
G	0 0 20 0 4 046 40 0 0 0 0 0 46 04646 0 0 46 146 4619 2 0 0 46 46 38
Con	C A TG C AC A G G T T C A GTCG G C A G T G G AGT C TA G G GC

@: newly designed primer

Table 3 Jλ gene fragment multiple sequence alignment with Jλ primers

Jλ3	TTGGGTGTTCGGCGGAGGGACCAAGCTGACCGTCCTAG
Jλ6	_AATGTGTTCGGCAGTGGCACCAAGGTGACCGTCCTCG
Jλ1	TTATGTCTTCGGAACTGGGACCAAGGTCACCGTCCTAG
Jλ2	TGTGGTATTCGGCGGAGGGACCAAGCTGACCGTCCTAG
Jλ7	TGCTGTGTTCGGAGGAGGCACCCAGCTGACCGCCTCG
Jλ5	CTGGGTGTTTGGTGAGGGGACGGAGCTGACCGTCCTAG
pJλ1-7revc@	----------ACCAAGCTGACCGTCCTAG
pJλ4@	TTTTGTATTTGGTGGAGGAACCCAGCTGATCATTTTAG
pJλ1revc[14]	----------GGGACCAAGGTCACCGTCCT----
pJλ237revc[14]	----------GGGACCCAGCTGACCGTCCT----

Revc: means reverse complementary sequence

@: newly designed primer

Table 4 The positive rates of different primers in detection paraffin lymphoma tissue

Primer pairs	B-NHL			T-NHL			LN		
	Positive	Total	%	Positive	Total	%	Positive	Total	%
P1	31	39	79.5	1	16	6.3	0	3	0
Pκ/Pλ	28	39	71.8	2	16	12.5	0	3	0
P1+Pκ/Pλ	36	39	92.3	2	16	12.5	0	3	0
PT	1	39	2.6	16	16	100.0	0	3	0

crete band is taken as a monoclonal response, a diffuse band as polyclonal response. The gels were interpreted visually, when the agrose gel could not distinguish the clear band and smear band, a PAGE gel will be used in this cases. DNA from DG75 and Jurket cell lines were as positive control of B and T cell lymphoma respectively.

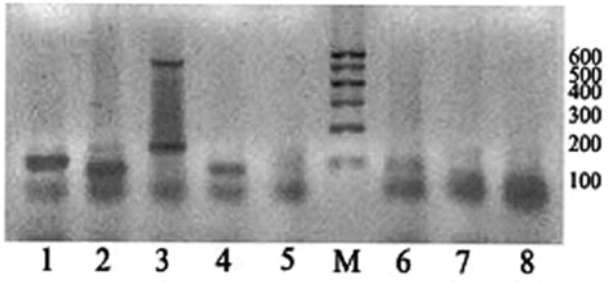

Fig.1a the agrose diagram of PCR analysis of Ig rearrangement from paraffin embedded tissue of lymphoma with the primer P1. Lane3: positive control; lane5: negative control; lane1, 2, 4, 6, positive results; lane7, 8 negative results; M: 100–600bp ladder marker

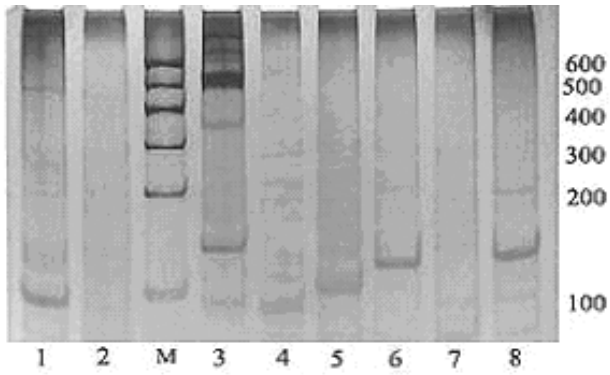

Fig.1b The PAGE diagram of PCR analysis of Ig rearrangement from paraffin embedded tissue of lymphoma with the primer P1. Lane4: positive Ctrl; lane2: negative Ctrl; lanes1,4,5,6,8 positive results; lanes 7:negative result; M:100–600bp ladder marker.

Among the 39 cases of B-NHL, clonal IgH gene rearrangement were detected positive in 31 cases (31/39, 79.5%) with FR-JH primer set, and clonal IgL chain gene rearrangement were detected in 28 cases (28/39, 71.8%) positive with Pκ/Pλ primer set, including 8 IgH negative cases. The detection rate was improved to 92.3%(36/39) for lymphomas using heavy and light chain PCR. In the meanwhile, there was one case showed positive in 39 cases of ´B cell lymphoma´ in TCR γ primer detection. Among 16 cases of T cell lymphoma, there were one or two positive cases in the IgH or IgL primer detections. All 3 reactive lymphoid samples showed polyclonal.

The detection rate of IgH and IgL in 39 cases B cell lymphoma According to the gene rearrangement theory of Ig light chains, the rearrangement of Igκ chain is happened at the start, if it failed, the Igλ chain rearrangement is followed. So either of Igκ or Igλ of gene rearrangement is positive, we regard it as positive cases. We analysis the gene rearrangement of IgH and IgL among the 39 cases of B cell lymphoma, the results showed that there were no significant differences between the detection rate with these two methods (McNemar tests, $P > 0.05$). (table 5)

Table 5 Comparison of the detection rate of IgL and IgH primers in B cell lymphoma tissues

Primer pairs		Pκ/Pλ		Total	McNemar
		+	−		P
P	P1	22	9	31	0.607
	+	6	2	8	
Total		28	11	39	

DISCUSSION

B cell Non-Hodgkin lymphoma is evolved from a malignant B cell that has completed IgH gene rearrangement. The detection of IgH rearrangement by PCR can supply a molecular marker of B-NHL, whereas, the widely application of this technique is blocked for its false negative results. Currently, some researchers have noticed the significance of the detection of Ig-light chain rearrangement by PCR method[14–15]. Theoretically, During the B cell differentiation, the Igκ gene locus undergoes rearrangement after IgH. In the case in which functional κ chain products are not obtained, the Igλ locus subsequently undergoes rearrangement[16].

We designed and modified two pairs of Ig light chain primers: primer Vk-FRk (Igκ variable region) and primer Jλ1-7 (Igλ Joint region), by comparison all published sequences on NCBI. These degenerate primer pairs were analysed by Primer Premiers Software, spanning the κ or λ CDR3 segment. As compared to the IgH locus, Igκ and Igλ does not contain a diversity(D) region resulting in short frag-

ment in VJ than the heavy chain VDJ segments. In order to clearly demonstrate the band, non-denaturing polyacrylamide gel(ndPAGE) were used to analysis Igκ and Igλ PCR products[15]. In the meantime, traditional Ig-heavy gene rearrangement primers FR-JH and T cell receptor γ (TCRγ) primers were also used in the detection.

We have studied a panel of 58 cases of lymphoproliferative disorders by using all these primers. Among 39 cases of B NHL tissues, the detection rate of Ig-light chain primers was 71.8%, which was slight lower than that of IgH primers 79.5%, it seems no statistics difference between them, and none of three cases of reactive proliferation cases showed clonal (see table 4). Whereas, by combining analysis of the results of the two methods, the detection rate did significantly increases to 92.9%. Compare to other investigators [14,15], we obtained higher results in paraffin embedded tissues. For example, Diss TC got 9 cases positive out of 12 (75%) in paraffin embedded tissues with the results of primers of Igκ/Igλ/IgH[14].

Besides the primer newly designed, other attempts have been made to improve the overall rate of detection of our paraffin embedded NHL tissues. One is manual micro-dissection technique as narrated in methods sections. This technique can benefit to remove the influence of non-tumor tissues in templates. The other is the quality of PCR template testing through the housekeeping genes amplification. Only when these genes had been successful amplified, the templates could be allowed the amplification of gene rearrangement products. It has been known that paraffin tissues are valuable sources of DNA for analysis, But the nucleotides maybe destroyed during the tissue disposal, so the extraction method from paraffin tissue is the most important[18,19].

Some researchers had reported that the combination of difference primer can improve the detection rate of PCR gene rearrangement[20,21]. Fodinger M[20] combined four IgH gene primer systems with the multiplex TCR γ gene PCR allowed detection of clonality in 84.2% of B-cell neoplasms, 92.1% of T-cell NHL, which was much more efficient than single PCR protocols. Catherwood MA et al[21] detected clonality in 93% of cases (27/29) with histological diagnosis of formaldehyde fixed, paraffin wax embedded B-NHL tissues, by combination of detection of IgH and IgL chain gene rearrangement.

In our studies, among 16 cases of T cell lymphomas tissues (by morphology and IHC), the IgH primer FR-JH and IgL primer Pκ/Pλ showed one positive case respectively; there are also one positive case among 39 cases of B-NHL (table 4). In these scenario, this ´fault positive´ maybe explained as: ①one possibility maybe re-check diagnosis of the morphology; ②another possibility, maybe the most important one, is the ´dual´ rearrangement or ´lineage infidelity´, whose detection rate may range from 5%~29%. Garcia MJ et al [22–23] analyzes the rearrangement pattern of IgH, TCR γ, and TCRβ genes in 80 cases of NHL (43 B-cell and 37 T-cell types), 9 of 43 (21%), B-cell types exhibited a monoclonal pattern in TCR genes, and 6 cases of 37 (16%) of T-cell neoplasms showed IgH gene rearrangements. The author also said that these dual rearrangements were detected in most precursor and immature B-and T-cell NHLs.

In conclusion, our study analyzed the primer of light κ and λ chains, combing the detection of IgH gene rearrangement in paraffin embedded tissues, and with a highly sensitive PAGE method, the detection rate can be increased to 92.3% in B cell NHL of PET. This approach is a sensitive, simple, and cost-effective method as an adjunct to IgH analysis. Owing to the short amplification products in FR3-J fragments of Ig light chains, it seems to be an ideal tool for analyzing poorly preserved formalin-fixed and paraffin-embedded materials, which is most frequently available in routine clinical laboratory practice.

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