Pathology - Research and Practice 215 (2019) 152615

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

The application of antigen receptor gene rearrangement of BIOMED-2 in the pathologic diagnosis of 348 cases with non-Hodgkin lymphoma in a single institution in Southwest of China

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ARTICLE INFO

Keywords:
Non-Hodgkin lymphoma
Ig/TCR gene rearrangement
Polymerase chain reaction
BIOMED-2

ABSTRACT

Objective: To explore the clinical value of immunoglobulin (Ig) and T cell receptor (TCR) gene rearrangement in the diagnosis of non-Hodgkin lymphoma.
Methods: Using the standardized BIOMED-2 multiplex PCR strategy to detect IgH, IgK and TCR in 272 cases of mature B-cell lymphoma, 55 cases of mature T-cell lymphoma, 21 cases of extranodal NK/ T-cell lymphoma, nasal type, and 20 cases of lymphoid tissue reactive hyperplasia.
Results: Among all mature B-cell lymphomas, the sensitivity of Ig gene rearrangement was 91.18% (248/272), IgH and IgK gene rearrangement was 76.47% (208/272) and 75.00% (204/272), respectively, meanwhile the sensitivity of TCRγ rearrangement was 3.68% (10/272). In the 55 cases of mature T-cell lymphoma, the sensitivity of the detection of TCRγ was 76.36% (44/55), at the same time the sensitivity of Ig gene rearrangement was 14.55% (8/55), IgH and IgK gene rearrangement was 7.27% (4/55) and 12.73% (7/55), respectively. In 21 cases of extranodal NK/T cell lymphoma, nasal type, and 20 cases of reactive lymphoid hyperplasia, no gene rearrangement was found in the samples of IgH, IgK and TCR. The sensitivity of gene rearrangement in Ig/TCR in B and T-cell lymphoma was significantly different from that in the control group ($P < 0.05$).
Conclusion: The Ig/TCR gene rearrangement of BIOMED-2 multiplex PCR strategy has important auxiliary value in the diagnosis of B/T-cell non-Hodgkin lymphoma respectively, however, a few B-cell lymphomas may company TCR gene rearrangement as well as a few T-cell lymphomas may accompany Ig gene rearrangement, it must be comprehensively judged with the combination of morphology, immunohistochemistry and clinical features.

1. Introduction

Malignant lymphoma (ML) is one of the commonest malignant tumors in China, and the incidence rate increases in recent decades. ML is divided into Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL), and the NHL is the majority. Due to its complexity and heterogeneity, NHL is regarded as one of the most difficult diseases in clinical pathologic diagnosis practice, leading to a high misdiagnosis rate in differential diagnosis. Moreover, only 70%–80% of NHLs can be diagnosed by histomorphology and immunophenotype.

In 1970s, the molecular immunogeneticist Susumu Tonegawa discovered the genetic principle for generation of antibody diversity and awarded the 1987 Nobel Prize for Physiology or Medicine [35]. Tak Wah Mak has done an excellent work in the T-cell receptors and their genes [22]. Soon afterwards, McCarthy established oligomeric primers in immunoglobulin (Ig) and T cell receptor (TCR) to amplify rearranged genes sequences by using polymerase chain reaction (PCR) [24–26]. It was with regret that the sensitivity of this detection was a little bit low and cannot provide much help in diagnosis. In 2003, BIOMED-2 standard primer system was designed and applied to the detection of antigen receptor gene rearrangements in NHLs [36].

Each lymphocyte has the same and unique rearrangement sequence, called "molecular fingerprint". Using the uniqueness of the "molecular fingerprint", the antigen receptor gene rearrangement analysis can detect whether proliferating lymphocytes are monoclonal or polyclonal. Therefore, the detection of gene rearrangements in Ig and TCR are revolutionary technology and the diagnosis rate of NHLs can be improved 10%–20%, especially in small samples [23]. The BIOMED-2 primer system is particularly suitable for small tissue of biopsy samples, mixed B-cell and T-cell hyperplastic lesions and those which the cell properties

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https://doi.org/10.1016/j.prp.2019.152615
Received 22 June 2019; Received in revised form 12 August 2019; Accepted 23 August 2019

cannot be determined by immunohistochemistry detection. With the development of molecular biology, detection of Ig/TCR gene rearrangement, a specific marker of B/T lymphocyte clones' gene analysis has become an important supplementary method in the diagnosis of NHLs [2,6,40]. However, some factors have been found to affect the sensitivity and specificity of the BIOMED-2 test in clinical diagnosis work. For instance, Ig gene rearrangement may occur in T-cell lymphoma, and TCR gene rearrangement in B-cell lymphoma.

The BIOMED-2 primers system to detect the Ig/TCR gene rearrangements has been applied to pathologic diagnosis of NHLs in clinical practice in a large number of institutions in China. However, there are more primer tubes applied in Europe and the United States in order to get more accurate results. In this study, we retrospectively investigated a large cohort of cases to explore the detection rate of antigen receptor gene rearrangements, the clinical value of BIOMED-2 multiplex PCR strategy and its shortcomings to avoid false negative and false positive results in practice.

2. Materials and methods

2.1. Materials

Experimental specimens: we retrieved and reviewed pathology reports and material available from 348 NHLs collected in the Department of Pathology at the Basic Medical College (i.e., Pathology Department of the First Affiliated Hospital) of Chongqing Medical University between 2012 and 2018 in China, each case was diagnosed by at least two hemotapathologists to ensure the accuracy of diagnoses. There were 183 males and 165 females aged 9 to 92 years (average 58.1 years, median 61 years). Thereinto, 272 cases of B-cell non-Hodgkin lymphoma (B-NHL), 55 cases of T-cell non-Hodgkin lymphoma (T-NHL), 21 cases of extranodal NK/T-cell lymphoma, nasal type (ENKTCL-NT). According to the 2017 WHO Classification of Lymphoid Hematopoietic Tumors [33], the histological subtypes included 124 cases of diffuse large B-cell lymphomas (DLBCLs), 71 cases of marginal zone lymphomas (MZLs), 33 cases of follicular lymphomas (FLs), 10 cases of mantle cell lymphomas (MCLs), 7 cases of B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma (B-CLL/SLL) and 27 cases of undetermined type or other B-cell non-Hodgkin lymphoma (other B-NHL). There were 20 cases of peripheral T-cell lymphoma (PTCL-U), 24 cases of angioimmunoblastic T-cell lymphoma (AITL), 4 cases of anaplastic large-cell lymphoma (ALCL) and 7 cases of undetermined type or other T-cell non-Hodgkin lymphoma (other T-NHL). As shown in Table 1.

2.1.1. Control

20 cases of reactive lymphoid hyperplasia were collected as the control group.

Table 1
The information of specimens with each subtype of non-hodgkin lymphoma.

	Subtype	No. of specimens	Male: Female	Average age
B-NHL	DLBCL	124	57:67	58.4
	MZL	71	41:30	59.6
	FL	33	16:17	54.8
	MCL	10	6:4	65.1
	B-CLL/SLL	7	7:0	59.4
	Other B-NHL	27	15:12	58.6
T-NHL	PTCL-U	20	14:6	61.0
	AITL	24	10:14	64.5
	ALCL	4	3:1	26.0
	Other T-NHL	7	2:5	57.0
ENKTCL-NT		21	12:9	49.1
Total		348	183:165	58.1

2.1.2. Main reagent

BIOMED-2 primer sequence (Invivoscribe); 50 bp Marker 6 Loading buffer, 2.5 mmol /L dNTP Mixture (Tiangen biochemical co., LTD.); DNA extraction Kit QIAamp DNA FFPE Tissue Kit (QIAGEN, Germany); Taq Hotstar enzyme, 10 x PCR buffer (Takara).

2.2. Methods

The specimens were isolated, fixed with 4% neutral formalin, dehydrated and embedded in paraffin.

2.2.1. Nucleic acid extraction

The paraffin tissues were treated by conventional dewaxing procedures. DNA was extracted from the samples according to the instructions of the kit, and the purity and concentration were determined.

2.2.2. Primer design

This study used 37 primers in the BIOMED-2 primer system (as shown in Table 2), synthesized by invivoscribe for Ig heavy chain (IgH A + B + C), light chain (IgK A + B) and TCRγ A + B. The gene is rearranged and contains 10 internal reference primers.

2.2.3. PCR amplification

25 μl amplification system: primer concentration 5 μmol / L, dNTP mixture concentration 0.2 mmol / L, 10 × PCR buffer, 5 units of rTaq DNA polymerase. PCR amplification conditions: pre-denaturation at 95 °C for 7 min; denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, extension at 72 °C for 90 s, 35 cycles; finally extending at 72 °C for 10 min.

2.2.4. Heterologous double-stranded nucleic acid analysis and polyacrylamide gel electrophoresis detection

The PCR product was heated at 95 °C for 5 min and cooled at 4 °C for 1 h, allowing rapid random renaturation to generate heteroduplex nucleic acid molecules. The product was electrophoresed on a 10% polyacrylamide gel at 120 V for 50 min. After completion, the gel was taken out, stained with 0.2% silver nitrate for 5–10 min, then rinsed in the rinse solution for 3–5 min, photographed and analyzed.

2.2.5. Interpretation of results

a) The band width is no more than 1 mm and the edges are neat;
b) The band is within the desired base size range;
c) If the background is not diffuse, and no primer dimers indicate PCR amplification failure, not false negative;
d) There is a "main band" significantly different from other bands within the expected base size range, and the position of the "main band" is exactly the same as that of the normal positive band, which can be considered as a positive band, especially when it is in the form of dispersion.
e) If there is a band other than the expected size, it should be regarded as artificial illusion and cannot be used as a basis for judgment.

3. Results

3.1. Mature B-cell lymphoma (B-NHL)

In B-NHL, the sensitivity of IgH and IgK gene rearrangement detection was 91.18% (248/272), of which the rate of IgH and IgK gene rearrangement detection was 76.47% (208/272) and 75.00% (204/272) respectively. However, the rate of TCR gene rearrangement detection was 3.68% (10/272). Specific data are shown in Table 3. A typical case of diffuse large B-cell lymphoma (DLBCL) was selected and shown in Fig. 1.

Table 2
BIOMED-2 primer design.

	clonality detection targets	Heteroduplex PCR analysis and sequencing	Estimated size range(bp)
IgH (VH-JH)	IgH-A	VH1-FR1 + VH2-FR1 + VH3-FR1 + VH4-FR1 + VH5-FR1 + VH6-FR1 + JH	310-360
	IgH-B	VH1-FR2 + VH2-FR2 + VH3-FR2 + VH4-FR2 + VH5-FR2 + VH6-FR2 + VH7-FR2 + JH	250-295
	IgH-C	VH1-FR3 + VH2-FR3 + VH3-FR3 + VH4-FR3 + VH5-FR3 + VH6-FR3 + VH7-FR3 + JH	100-170
Igk	Igk-A	Vk1f6 + Vk2f + Vk3f + Vk4 + Vk5 + Vk7 + Jk1-4 + Jk5	120-160、190-210、260-300
	Igk-B	Vk1f6 + Vk2f + Vk3f + Vk4 + Vk5 + Vk7 + INTR + Kde	210-250、270-300、350-390
TCRγ (Vγ-Jγ)	TCRγ-A	Jγ1.1/2.1 + Vγ1f + Jγ1.3/2.3 + Vγ10	145-255
	TCRγ-B	Jγ1.1/2.1 + Vγ11 + Vγ9 + Jγ1.3/2.3	80-140、160-220

Table 3
The rate of Ig/TCR gene rearrangement detection for each subtype of B-NHL.

	IgH	IgK	IgH + IgK	TCRγ
DLBCL	67.740%	77.00%	89.51%	4.84%
(n = 124)	84/124	93/124	111/124	6/124
MZL	88.73%	73.24%	92.96%	0%
(n = 71)	63/71	52/71	66/71	0/71
FL	72.73%	75.76%	90.91%	9.09%
(n = 28)	24/33	25/33	30/33	3/33
MCL	100%	66.67%	100%	0%
(n = 10)	10/10	7/10	10/10	0/10
B-CLL/SLL	100%	100%	100%	0%
(n = 6)	7/7	7/7	7/7	0/7
Other	74.07%	74.07%	88.89%	3.70%
(n = 27)	20/27	20/27	24/27	1/27
Total	76.47%	75.00%	91.18%	3.68%
(n = 242)	208/272	204/272	248/272	10/272

3.2. Mature T-cell lymphoma (T-NHL)

In T-NHL, the sensitivity of TCR gene rearrangement detection was 76.36% (42/55), however, the sensitivity of Ig gene rearrangement detection was 14.55% (8/55), which IgH was 7.27% (4/55) and IgK was 12.73% (7/55). Specific data are shown in Table 4. A typical case of peripheral T-cell lymphoma (PTCL-U) was selected and shown in Fig. 2.

3.3. Extranodal NK/T cell lymphoma (ENKTCL-NT)

In 21 cases of extranodal NK/T cell lymphoma, nasal type (ENKTCL-NT), IgH, IgK and TCR were not detected. Specific data are shown in Table 5. A case of extranodal NK/T cell lymphoma was selected and shown in Fig. 3.

3.4. Lymphoid reactive hyperplasia

In 20 cases of lymphoid reactive hyperplasia, no gene rearrangement was detected in IgH, IgK and TCRγ.

3.5. Special cases

In the B-NHLs, 10 cases (10/272, 3.68%) was found simultaneous Ig and TCR gene rearrangements, including 6 cases (6/272, 2.21%) of diffuse large B-cell lymphoma, 3 cases (3/272, 1.10%) of follicular lymphoma, and 1 case (1/272, 0.37%) of small B-cell lymphoma (classified as undetermined or other B-NHL types). Specific data are shown in Table 6. A case of follicular lymphoma (Grade II) which detected simultaneous Ig and TCR gene rearrangements was selected and shown in Fig.4.

In the T-NHLs, 5 cases (5/55, 9.10%) was found simultaneous Ig and TCR gene rearrangements, 3 cases (3/55, 5.45%) of which were angioimmunoblastic T-cell lymphoma and 2 cases (2/55, 3.64%) were peripheral T-cell lymphoma. Moreover, there were 3 cases (3/55, 5.45%) only with Ig rearrangement and no TCR rearrangement, as shown in Table 7.

The 15 cases (10 B-NHLs and 5 T-NHLs) simultaneous Ig and TCR gene rearrangements and 3 cases of T-NHL with only Ig rearrangement were immunostained by more than two B-cell and T-cell markers.

4. Discussion

The pathological diagnosis of malignant lymphoma has always been a difficult problem in clinical pathological diagnosis. The combination of clinic, histopathology, immunophenotype and molecular genetics, is of very importance.

4.1. Gene rearrangement

Antigen receptor gene rearrangement is a normal physiological process during lymphocyte development process. Ig/TCR genes gradually rearranged the gene fragments of Variable (V), Diversity (D) and Joining (J), and nucleotides were deleted or randomly inserted at the joining site, resulting in the diversity and clonal specificity of the V-(D)-J gene sequence. In general, in normal lymphoid tissue and reactive hyperplasia, Ig/TCR gene rearrangement is polyclonal, but monoclonal in malignant lymphoma lesion [16]. We use specific primers of BIOMED-2 to amplify the genes. When the amplified product is subjected to polyacrylamide gel electrophoresis, the polyclonal lymphocytes will produce a diffuse band, while the monoclonal lymphocytes will show a distinct band. Therefore, by detecting whether the antigen receptor gene rearrangement is monoclonal, it is possible to determine whether lymphocytes are neoplastic proliferation [5,9]. As for ENKTCL-NT, approximately 70%–80% of cases originate from NK-cell lineage and without clonal rearrangement of TCR gene, but the remaining 20%–30% cases are cytotoxic T-cell phenotype, which have monoclonal TCR gene rearrangement [13,27,31].

4.2. BIOMED-2 primer system

In 2003, 90 pathologists, molecular biologists and other researchers from 47 institutes in 7 European countries collaborated to complete the standardized BIOMED-2 multiplex PCR clonality assays system [36], greatly reducing false positives and false negatives. BIOMED-2 is based on the combinatorial form of V-(D)-*J* gene rearrangement. According to the conserved sequence of V region and the common sequence of J region of different Ig/TCR families, a number of pairs of family primers and common primers were designed as the upper and lower primers respectively to cover as many rearrangements as possible.

As for the sensitivity and efficiency of BIOMED-2 primers design, several standard tubes were recommended, but TCRβ (Vβ-Jβ) primers cannot cover all non-functional Vβ fragments, while TCRγ gene primers do not cover Jγ1.2 fragments to avoid false positive results caused by normal rearrangement of Vβ9-Jγ1.2.

In the primer strategy, the BIOMED-2 primers used in our study were IgH (A + B + C), IgK (A + B), TCRγ (A + B). Compared with the same primer tubes adopted, the rate of Ig(IgH and/or IgK) gene rearrangement in our study (91.18%) is slightly higher than 81.1% which was reported by Ira et al. [12]. Meanwhile, the IgH rearrangement rate was similar to the report (77.0%) from Kros et al. [18] and to the report

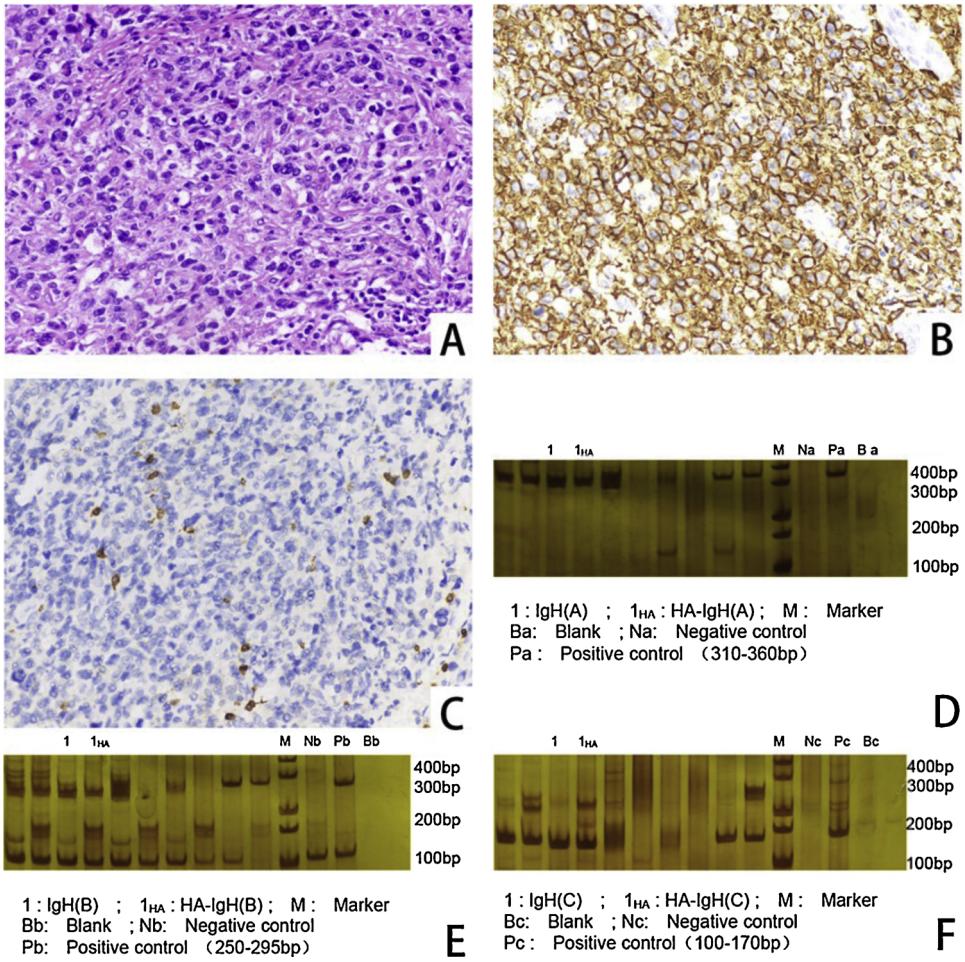

Fig. 1. Hematoxylin-eosin(HE), Immunohistochemical staining and IG gene rearrangement of diffuse large B-cell lymphoma.
(A) Diffuse large B-cell lymphoma showed diffuse distribution of tumor cells(HE)(x400); (B) The lymphoma cells were positive for CD20 protein in the cytomembrane (x400); (C) The lymphoma cells were negative for CD3 protein in the cytomembrane (x400); (D) IgH(A) gene rearrangements were detected. (E) IgH(B) gene rearrangements were detected. (F) IgH(C) gene rearrangements were detected.

Table 4
The rate of TCR/Ig gene rearrangement detection for each subtype of T-NHL.

	TCRγ	IgH	IgK	IgH + IgK
PTCL-U	80.00%	5.00%	10.00%	15.00%
(n = 16)	16/20	1/20	2/20	3/20
AITL	75.00%	12.50%	16.67%	16.67%
(n = 19)	18/24	3/24	4/24	4/24
ALCL	75.00%	0%	25.00%	25.00%
(n = 3)	3/4	0/4	1/4	1/4
Other	71.43%	0%	0%	0%
(n = 5)	5/7	0/7	0/7	0/7
Total	76.36%	7.27%	12.73%	14.55%
(n = 55)	42/55	4/55	7/55	8/55

(79.9%) by Can Lu et al. [21], but lower than 88% from the report of van krieken et al. [37]. Compared with the same primer tubes adopted, the rate of TCRγ gene rearrangement in mature T-cell lymphoma specimens (76.36%) is similar to the report (78.2%) from Diss et al. [8], but it is lower than 89% which was reported by van Krieken et al. [37].

However, as for the reason why we have a lower positive rate than some other institutions with the same tubes, this may result from reasons on tissue isolated and fixed, or experimental technique. The BIOMED-2 primer system requires a tumor cell load of 1%–5% [9], and false negative may occur if the proportion of tumor cells is too low. When the total nucleic acid content is less than 100 ng/ul, for example, the proportion of tumor components is too small, especially in mucosa, skin or puncture specimens, and false negative results may also occur. It has been pointed out in the literature that the dilution of DNA specimens can improve the detection rate of clonal rearrangement, which may be caused by the dilution of DNA as well as the dilution of PCR amplification inhibitors [30]. In our study, PCR heteroduplex analysis was applied to qualitatively determine the electrophoresis bands of PCR products according to the naked eye for qualitative judgment. The resolution and accuracy were relatively lower, which would affect the accuracy and sensitivity of detection to a certain extent. When paraffin tissue is used as the source of DNA, the semi-nested JH consensus can be used to improve the sensitivity and the detection rate of cloning can be increased from 63.9%–88.0% by using semi-nested BIOMED-2 compared with the standardized BIOMED-2 [28].

To improve the detection rate of Ig/TCR gene rearrangement, more primer tubes should be applied. As reported by van krieken et al. [37], when IgH (A + B + C) is used alone, the detection rate is 88%, while IgH (A + B + C + D + E) is applied, the detection rate raises to 91%, and when IgH (A + B + C + D + E) + IgK(A + B) are combined used, the detection rate raises to 99%. As reported by Liu et al. [11], when TCRγ (A + B) is used alone, the detection rate is 94%, but when TCRγ (A + B) + TCRβ (A + B + C) are used in combination, the detection rate is 98%. When Igλ and TCRδ are used alone, the detection rate is 28%

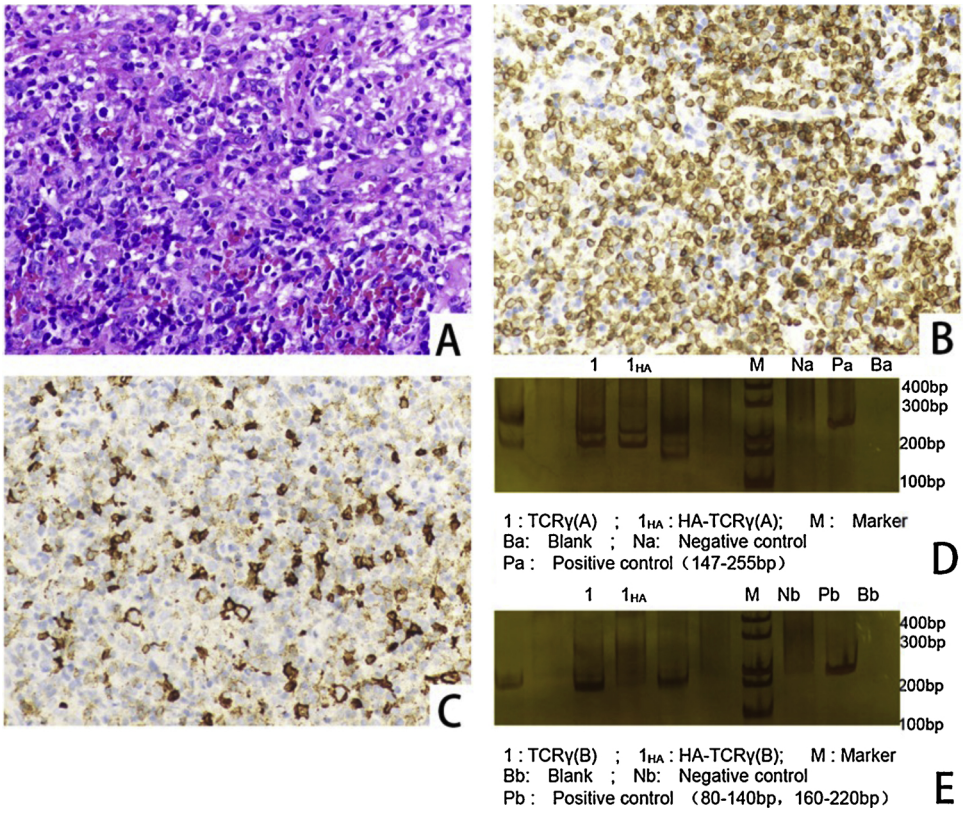

Fig. 2. Hematoxylin-eosin(HE), Immunohistochemical staining and IG gene rearrangement of peripheral T-cell lymphoma.
(A) peripheral T-cell lymphoma showed diffuse distribution of tumor cells(HE)(x400); (B) The lymphoma cells were positive for CD3 protein in the cytomembrane (x400); (C) The lymphoma cells were negative for CD20 protein in the cytomembrane (x400); (D) TCRγ(A) gene rearrangements were detected. (E) TCRγ(B) gene rearrangements were not detected.

Table 5
The rate of Ig/TCR gene rearrangement detection for ENKTCL-NT.

	IgH	IgK	IgH + IgK	TCRγ
ENKTCL-NT (n = 21)	0% 0/21	0% 0/21	0% 0/21	0% 0/21

and 28%, respectively [11]. Since the detection rate is too low, and have little help with diagnosis, therefore, we rarely use these two tubes. The above results indicate that add more primer tubes can improve the accuracy of detection, but the cost should be considered at the same time. The strategy in our study which is IgH (A + B + C), IgK (A + B) and TCRγ (A + B), can detect most B/T-cell lymphoma in practice and do have a great value in clinical diagnosis. Therefore, IgH(D + E) and TCRβ (A + B + C) could be recommended to add to our panel of tubes in our medical practice.

Recently, the EuroClonality-NGS Working Group has developed Ig/TCR assays, which include two types of quality controls and have been validated across expert European centers to allow for quality-controlled, streamlined, and comprehensive detection of clonal Ig/TCR rearrangements [15]. The next-generation sequencing (NGS) assays focusing on small amplicon sizes, highly suitable for analysis of formalin-fixed paraffin-embedded (FFPE) tissue specimens with low-quality DNA, and it indicate that only 10–40 ng of Qubit-quantified DNA is required for each multiplex PCR reaction. The NGS-based detection of Ig gene rearrangement shows improved performance compared with the current BIOMED-2 approach [29]. Meanwhile, the NGS-based assays also can be helpful in the quantification of minimal residual disease (MRD) in lymphoid neoplasms [4]. So far, the NGS-based detection is currently under intensive development for use in clinical diagnoses. When its sensitivity gets higher, we should be cautious whether the specificity may become a new challenge for EuroClonality-NGS Working Group.

4.3. Analysis of the causes of non-neoplastic proliferative diseases associated with antigen receptor gene rearrangement in lymphoid tissues

B/T-cell antigen receptor gene rearrangement and tumorigenesis are two relatively independent processes, therefore, monoclonal proliferation is not equivalent to malignant tumors. In viral infections (such as EBV, CMV, HIV, etc), reactive lymphoid hyperplasia of inflammatory infections (sarcoidosis, hashimoto thyroiditis, sjogren's syndrome, etc.), autoimmune disease, immunodeficiency and bone marrow transplantation or after chemotherapy, due to chronic antigen B and T cells drive selection, monoclonal rearrangement may be observed. For example, in infectious mononucleosis, the clonal proliferation of EBV-infected cells may be detected temporarily, and EBV-positive lymphoproliferative diseases should be carefully excluded [1]. In these diseases, the PCR band is usually weak and exists in the polyclonal background [20]. Normal elderly people may also have monoclonal rearrangements due to the depletion of the TCR library diversity [10]. In general, lymphoid reactive hyperplasia is a polyclonal antigen receptor gene rearrangement, but there are special cases [32], such as benign lymphoid epithelial lesions of salivary glands or myoepithelial salivary adenitis (MESA), Ig gene rearrangement can also occur.

4.4. Analysis of the causes of negative antigen receptor gene rearrangement in non-Hodgkin lymphoma

4.4.1. False negative of Ig gene rearrangement

In B-NHL, immunoglobulin heavy chain somatic hypermutation (SHM) of B cells in the lymphoid tissue follicle germinal center affects the detection rate of monoclonal in lymphoma which are originating from the germinal center [17]. If SHM occurs at the binding site of the primer, it will affect the effective binding of the gene to the primer, resulting in failure of PCR amplification. Since IgH rearrangement interferes with primer annealing during somatic hypermutation,

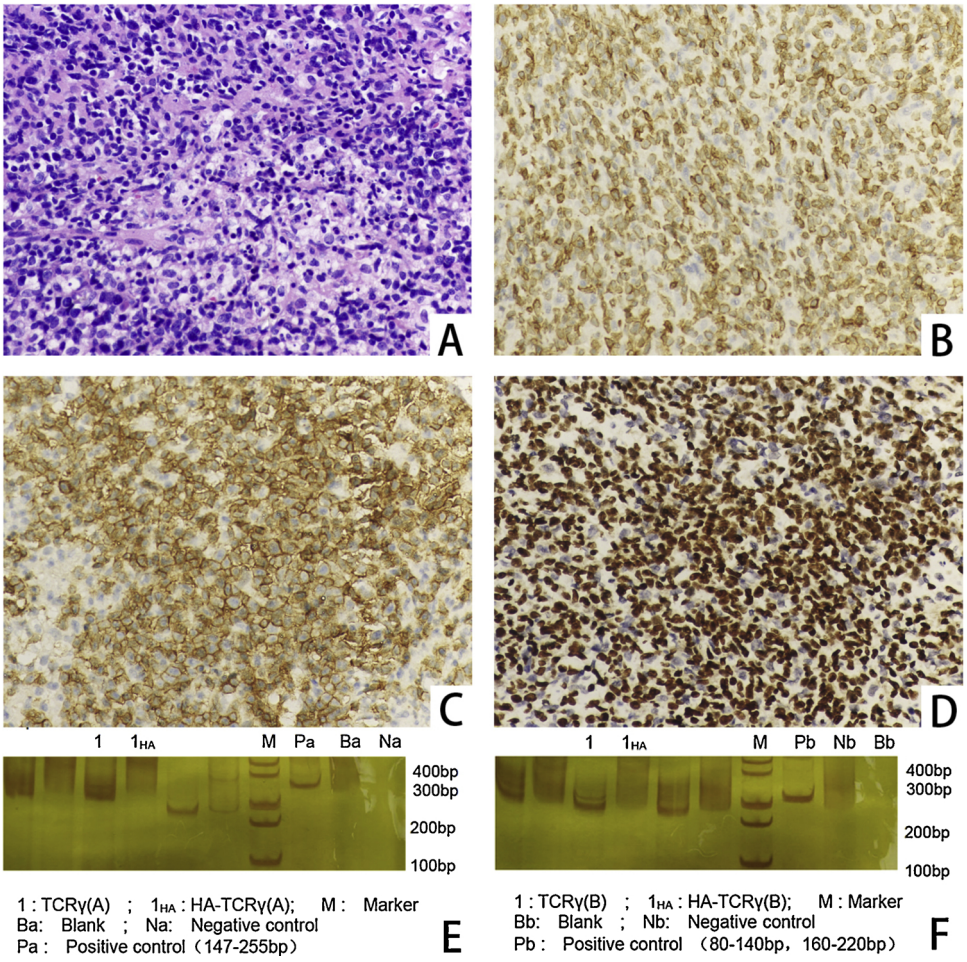

Fig. 3. Hematoxylin-eosin(HE), Immunohistochemical staining and IG gene rearrangement of extranodal NK/T-cell lymphoma, nasal type.
(A) extranodal NK/T-cell lymphoma, nasal type showed diffuse distribution of tumor cells(HE)(x400); (B) The lymphoma cells were positive for CD3(Ɛ) protein in the cytoplasm (x400); (C) The lymphoma cells were positive for CD56 protein in the cytomembrane (x400); (D) The lymphoma cells were positive for EBER-ISH in the tumor cell nucleus (x400);(E) TCRγ(A) gene rearrangements were not detected. (F) TCRγ(B) gene rearrangements were not detected.

Table 6
Special cases in B-NHL.

	Ig(+) and TCRγ(+)	Ig(-) and TCRγ(+)	TCRγ(+)
FL	3	0	3
DLBCL	6	0	6
Other	1	0	1
Total	10	0	10

detection of IgH rearrangement alone can lead to a higher false negative rate of B-cell clonal analysis for germinal center/post-germinal center B-cell lymphoma, especially in FL, as well as DLBCL and Burkitt lymphoma [14]. While IgK rearrangement is rarely affected by SHM, IgK detection is an important complementary detection item [18]. IgK detection is significantly more sensitive than IgH detection especially in FL.

4.4.2. False negative of TCR gene rearrangement

TCRγ in 25% of anaplastic large cell lymphoma (ALCL) and 4%–8% of the other mature T-cell lymphoma cannot be identified [5]. When TCRγ, TCRβ and TCRδ are applied jointly, they can detect gene rearrangement in the vast majority of T cell lymphoma, but 20% of ALCL is still negative [5]. Due to the small amount of T-NHL cases in this study, only 3 of 4 cases of ALCL found TCRγ gene rearrangement, it had little effect on the total positive rate (the rate of T-NHL clonal rearrangement was 76.47% after exclude ALCL).

4.5. Simultaneous Ig and TCR gene rearrangements

In this study, the TCR gene rearrangement rate of B-NHL cases was 3.68% (10/272), the Ig gene rearrangement rate of T-NHL was 14.55% (8/55), totally there were 15 special cases of simultaneous Ig and TCR gene rearrangements. According to the literature, 10%–20% of B-NHL may have TCR gene rearrangement [5,9]. The coexistence of B-cell and T-cell clones may due to the presence of a small amount of reactive T cells in small specimens or high-load B-cell lymphoma, so there are no enough cells produce a polyclonal background, and a small number of reactive cells produce surface-like clonal PCR products [19], or maybe there has cross-lineage gene rearrangement. Cross-lineage gene rearrangement is often considered to be a dominant PCR product rearranged at a single site [7]. It is also possible that the recombinase involved in Ig gene rearrangement at the early stage of differentiation of B lymphocytes is similar to that in TCR and has the same recombinant mechanism [39]. Similarly, Ig gene rearrangement can occur in more than 30% of AITL and 4%–9% of other mature T-cell lymphomas [5], the reason is similar to the TCR gene rearrangement in B-NHL. More importantly, the high frequency of Ig gene rearrangement in AITL may be associated with EBV infection, which leads to immune dysfunction and causes uncontrolled proliferation of B cells [34].

In addition, several cases of B lymphoblastic lymphoma and T lymphoblastic lymphoma were found to be simultaneous Ig and TCR gene rearrangements during the collection of cases (due to immature lymphoma, not included in the statistics of present study), the reason

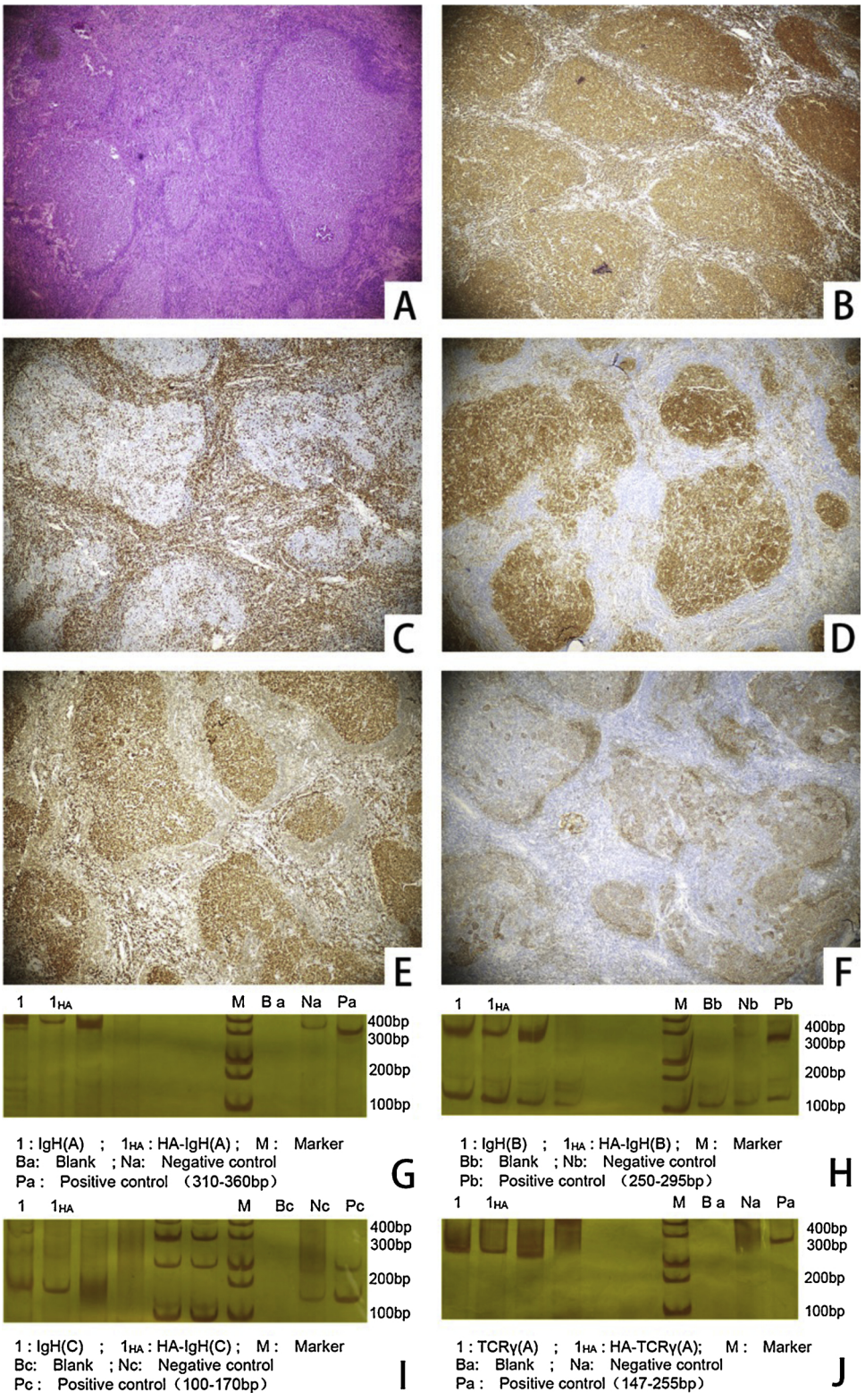

Fig. 4. Hematoxylin-eosin(HE), Immunohistochemical staining and Ig gene rearrangement of follicular lymphoma.
follicular lymphoma showed nodular/follicular distribution of tumor cells(HE)(x40); (B) The lymphoma cells were positive for CD20 protein in the cytomembrane (x40); (C) The lymphoma cells were positive for CD3 protein in the cytomembrane (x40); (D) The lymphoma cells were positive for CD10 protein in the cytomembrane (x40); (E) The lymphoma cells were positive for BCL-2 protein in the cytoplasm (x40); (F) The lymphoma cells were positive for CD21 protein in the cytomembrane (x40); (G)IgH(A) gene rearrangements were detected; (H) IgH(B) gene rearrangements were detected; (I) IgH(C) gene rearrangements were detected; (J) TCRγ(A) gene rearrangements were detected.

Table 7
Special cases in T-NHL.

	TCRγ(+) and Ig(+)	TCRγ(-) and Ig(+)	Ig(+)
PTCL-U	2	1	3
AITL	3	1	4
ALCL	0	1	1
Total	5	3	8

may be that the cells involved are in the early stage of differentiation, have two-way differentiation potential, and TCR rearrangement begins before lymphoblasts are not differentiated [36]. The dual genotype in tumor cells is described in up to 30% of cases of precursor lymphoid neoplasms, although it occurred rarely in mature lymphomas [38]. These results indicate that gene rearrangement results cannot be used alone for the identification of lymphoma lineages. More than two types of B-cell and T-cell marker immunostained should be recommended.

5. Conclusions

The standardized BIOMED-2 multiplex PCR clonality assays has been routinely used for detection of antigen receptor gene rearrangement in clinicopathological diagnosis, which plays an important auxiliary role in the diagnosis, differential diagnosis and post-treatment efficacy evaluation of suspected lymphocyte proliferative diseases [3]. However, in practical application, antigen receptor gene rearrangement cannot be used as an independent golden standard for the diagnosis of non-Hodgkin lymphoma, a few B-cell lymphomas may company TCR gene rearrangement as well as a few T-cell lymphomas may accompany Ig gene rearrangement, and false positive and false negative results cannot be completely avoided. This emphasizes that the diagnosis of non-Hodgkin lymphoma requires the cooperation of clinicians, pathologists and molecular biologists, and to use a more complete PCR primer system to reduce missed diagnosis and misdiagnosis. Meanwhile, we are looking forward to applying NGS-based detection in clinical diagnosis which is currently under further study by EuroClonality-NGS Working Group to improve the inadequacies of BIOMED-2 assays.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Acknowledgements

This research was supported by National Key R&D Program of China (No. 2016YFC1307205). The authors would like to thank you to all those who work in Department of Pathology, Chongqing Medical University for their support.

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