Original Article

PCR–based Clonality Analysis in Diffuse Large B-cell Lymphoma Using BIOMED-2 Primers of IgH (FR3) on Formalin-fixed Paraffin-embedded Tissue

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Abstract

Introduction: BIOMED–2 multiplex polymerase chain reaction (PCR) protocol is a widely accepted tool for evaluation of clonality in lymphoma diagnosis. Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin·s lymphoma and displays a special challenge for PCR-based clonality analysis due to a high frequency of somatic hypermutation in rearranged immunoglobulin (Ig) domains. In this study, we evaluated detection of B-cell clonality in DLBCL by using Ig heavy chain (IgH) framework region 3 (FR_3) primers in formalin- fixed paraffin-embedded (FFPE) tissue.

Method: FFPE samples from 100 cases diagnosed as DLBCL in the period of 2005 through 2011 were assessed in this study. Clonality of IgH (FR_3) was evaluated by PCR amplification method that was optimized for FFPE tissue.

Results: The clonal detection rate was (62.8 %) with IgH (FR_3) assay after modification, using filter for better DNA purification on negative cases. DNA quality in FFPE samples stored in recent five years were significantly better than older paraffin blocks ($P < 0.001$). Although a higher rate of clonality was observed in more recent group, it was not statistically significant.

Conclusion: By using IgH (FR_3) primers followed by one additional filter tube for better DNA purification, we could achieve a considerable rate of clonality with little adverse impact of DNA degradation.

Keywords: BIOMED-2, clonality detection rate, diffuse large B-cell lymphoma, formalin-fixed paraffin-embedded tissue

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Introduction

B-cell lymphoid malignancies comprise about 90 % – 95 % of all lymphoproliferative disorders.[1] The majority of them can be accurately diagnosed based on cyto- or histomorphology supplemented with immunohistochemistry or flowcytometric immunophenotyping. However, in about 5 % – 10 % of cases the diagnosis is more complicated.[1,2,3] Immunoglobulin heavy chain (IgH) gene rearrangement analysis by PCR has proved as an additional useful diagnostic tool to determine clonality in such cases.[2,3,4,5]

PCR- based methods have several limitations , the most relevant being its well- recognized false negativity rate , up to 30 % in some cases depending on the PCR strategy and lymphoma subtype.[6,7,8,9,10]

Presence of frequent somatic hypermutation in certain B-cell malignancies leads to insufficient annealing and is considered as a limiting factor in clonality detection rate.[4,9] Thus, tumors derived from germinal center (GC) or post-GC including diffuse large B-cell lymphoma (DLBCL) bearing somatically hypermutated VH region, show lower rate of clonality detection by PCR- based methods.[6,11]

Another well-known limiting factor is the use of formalin-fixed paraffin-embedded (FFPE) specimens. Fresh/frozen tissue is accepted as an ideal sample for extraction of DNA. However, fresh material is not always available and FFPE tissue constitutes the majority of diagnostic specimens submitted to pathology laboratories.[1,12] Formalin fixation hampers DNA integrity with irreversible changes to nucleic acid structure specially in older specimens.[1,13] Considering the mentioned factors, clonality analysis in some lymphoma subtypes such as DLBCL is more challenging.

In the present study, we focused on clonality detection rate in 100 cases of FFPE DLBCL, as the most common subtype of B-cell lymphoma, using IgH framework region 3 (FR_3) BIOMED-2 primers. Furthermore, we describe the impact of performing some modifications including application of One Step™ PCR inhibitor removal kit on the final recovery rate of clonality in DLBCL.

Materials and Methods

We retrieved paraffin blocks from 100 cases of DLBCL during the period of 2005 – 2011 from archives of pathology departments of Sina and Shariati hospitals, affiliated to Tehran University of Medical Sciences.

All cases were reviewed by a hematopathologist employing the WHO classification of lymphoid neoplasm. The diagnosis was based on clinical, histomorphologic, and immunophenotypical data. Three to five cut sections were obtained from each selected paraffin block. DNA extraction was performed mainly based on standard protocols directed by the manufacturer (Roche; high pure PCR template preparation kit).

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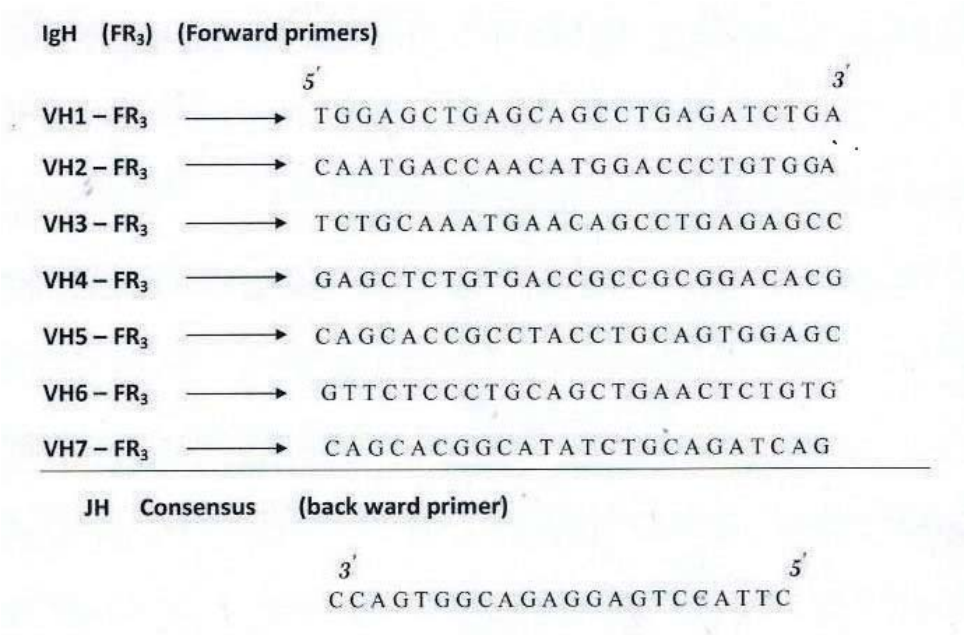

Figure 1. Sequences of primers used in PCR method.

Table 1. Amplification steps using BIOMED-2 IgH (FR_3) primers

PCR procedure	Temperature	Duration
Initial denaturation	94°c	10 min
Denaturation	94°c	30 s
Annealing	53°c	45 s
Extension	72°c	45 s
Final extension	72°c	5 min
Number of cycles:35.		

Table 2. Correlation between the quality of DNA and paraffin block age (five years considered as the cut-off)

PCR result	Age of paraffin blocks		Total	P-value
	2005–2006	2007–2011		
Negative control PCR product	17 (44.7 %)	5 (8.1 %)	22 (22 %)	
Positive control PCR product	21 (55.3 %)	57 (91.9 %)	78 (78 %)	0.001
Total	**38 (100 %)**	**62 (100 %)**	**100 (100 %)**	

Table 3. Correlation between the clonality detection rate and paraffin block age (five years as cut -off)

PCR result	Age of paraffin blocks		Total	P-value
	2005–2006	2007–2011		
Negative PCR product (FR_3 primers)	11 (52.4 %)	18 (31.6 %)	29 (37.2 %)	
Positive PCR product (FR_3 primers)	10 (47.6 %)	39 (68.4 %)	49 (62.8 %)	0.09
Total	**21 (100 %)**	**57 (100 %)**	**78 (100 %)**	

Table 4. Summary of clonality detection rate in B-cell lymphoma

Author	Control gene	Type of lymphoma	Colnality detection rate based on IgH (%) in paraffin blocks
Kummalue, et al.[19]	- - - - - - - - - - - - -	26 B-cell lymphoma (15 DLBCL , 3 FL)	FR_3 100 %
Halldorsdottir, et al.[13]	- - - - - - - - - - - - -	40 B-cell lymphoma	FR_3 13% (5/40) FR_1, FR_2, FR_3, 35 % (14/40)
Berget, et al.[12]	BIOMED-2	118 FL	FR_3 38.1 % FR_1, FR_2, FR_3 79.7 %
McClure, et al.[25]	- - - - - - - - - - - - -	DLBCL	IgH: 4/6 67 % (in paraffin blocks)
Amara, et al.[5]	β globin (268 bp)	DLBCL FL	34/43 79 % DLBCL 3/7 43 % FL FR_2 + FR_3 (IgH)
Nelson, et al.[2]	β globin (268 bp)	26 B- cell lymphoma	FR_1 77 %
Wang, et al.[26]	BIOMED-2	45 B-cell lymphoma (7DLBCL)	FR_3 56 % (25/45)
Wang, et al.[27]	β actin	63 ocular adnexal lymphoma	IgH: 38/48 (79.2 %)

Primers and PCR method

All samples were assessed for DNA quality by evaluation of DNA/protein ratio (Actgene, 2680 Nanodrop Asp) and using the BIOMED-2 control gene primer set, which amplifies 100, 200, 300 bp DNA targets.[1] Only samples providing at least one clear 100 bp band were included in the study.

Eight primer pairs [seven forward primers, VH1-7 (FR_3) and one consensus JH reverse primer] according to BIOMED-2 protocol were used in a multiplex reaction tube (Figure 1). All amplification reactions were performed in an automated thermocycler (Techne electro) summarized in Table 1.

Finally, PCR products were run on a 12 % polyacrylamide gel electrophoresis (PAGE) (Bio RAD) at room temperature for four hours at 80 volts, stained with ethidium bromide, and visualized under UV light following Heteroduplex pretreatment.

Samples with dominant band between 100 – 170 bp were interpreted as monoclonal (positive) whereas absence of product or minimal smear pattern was considered as negative.

Positive (monoclonal) and negative (polyclonal) controls composed of one confirmed lymphoma case and reactive lymph node or tonsil in addition to blank (distilled water) were included in all experiments. SPSS software (SPSS Inc No: 17) was used for data analysis.

Results

One hundred known cases of DLBCL were included in the study. The mean age of the patients was 50.8 ± 15.9 years and female: male ratio was 41 : 59. Half of the cases (50 %) were of nodal and 50 % of extra-nodal origin.

After PCR amplification using BIOMED-2 PCR control gene, 77 cases (77 %) demonstrated at least 100 bp product, whereas 200 bp and 300 bp products were observed in 60 and 20 cases, respectively.

Duplicate test using filter tube (One Step™ inhibitor removal kit) was performed on negative cases for better DNA purification. Among them only one case yielded DNA of adequate quality.

All samples with product greater than 100 bp (78 cases) were considered suitable for further PCR analysis using IgH (FR_3) primer. Among 78 samples, 45 cases (57.7 %) revealed clonal band using IgH (FR_3) assay and 33 (42.3 %) cases demonstrated no or nonspecific bands.

Duplicate test with additional filter tube (One Step ™ inhibitor removal kit) on negative cases increased final clonality detection rate from 45 (57.7 %) to 49 (62.8 %).

We evaluated the relation between DNA quality (based on PCR product using BIOMED-2 PCR control gene) and paraffin block age and observed a significant decrease in DNA isolation rate from older paraffin blocks ($P = 0.006$).

Considering a five- year storage time as the cutoff, the whole paraffin blocks were divided into two categories. In respect to DNA quality, evaluated by the BIOMED-2 control PCR, a significant difference appeared between the two groups ($P < 0.001$) (Table 2). Clonality detection rate in cases with positive control product (78 cases) revealed a higher detection rate in more recent group. However, the difference was not statistically significant ($P = 0.09$), (Table 3).

In the 78 cases who had clonal bands in the control PCR assay, clonality detection rate was about 70 % in cases observed 300 bp product and decreased into 62.5 % and 61.5 % in cases revealed 200 bp and 100 bp products, respectively. So, there was no significant correlation between quality of control PCR product size and clonality detection rate ($P = 0.861$).

Discussion

Despite well-established criteria, the definite diagnosis of malignant B-cell lymphoma cannot be provided in all cases.[13,14] Using PCR- based methods on the fact that all tumor cells are the progeny of a single malignant transformed cell is thought to produce valuable additional information in such cases.[1,3,14,15]

The PCR relies on the amplification of rearranged IgH gene to evaluate the status of clonality in B-cell disorders using conserved sequences within the FR of IgH gene to design primers.[14]

However, the overall sensitivity of these approaches is not optimal, especially in lymphomas originating from germinal center (GC) or post-GC ranging from 50 % to 76 %.[5,9,11,16,17,18] A major explanation can be due to improper annealing of rearranged VH segment caused by more frequent somatic hypermutations in this category of lymphomas including DLBCL.[5] High- grade lymphomas such as DLBCL are more prone to develop mutations in FR_1 region during clonal evolution of the disease and many investigations can demonstrate superior detection rate of high- grade lymphomas when using FR_2 and /or FR_3 primers on FFPE tissue.[2,5] Moreover, it is believed that FR_3 even shows higher positive rate in clonality detection in comparison with FR_2.[5]

We evaluated clonality detection rate in 78 cases of FFPE DLBCL which, dealing with another limiting factor, interferes with DNA recovery. So, we validated the cases by using BIOMED-2 control PCR gene.

Final amplification of 100 bp was possible in 78 cases (by using one additional step including One Step™ inhibitor removal kit). Regarding the fact that FR_3 with product size of 70 – 110 bp is proposed as a suitable choice in clonality detection rate in high-grade lymphomas, such as DLBCL, the presence of at least 100bp product was considered as including criteria.

Among 78 cases appeared suitable for PCR analysis, 45 (57.7 %) revealed clonal band. It is rather similar to clonality detection rate in GC /post-GC lymphomas in FFPE samples, reported in other studies, except for one reported by Tanawan Kummalue, et al.[15] that could reach to the 100 % detection rate by using rather the same method. A summary of clonality detection rate using different sets of primers in various types of lymphoma cases is shown in Table 4.

Considering the high false- negative rate, some strategies have been advised to enhance the sensitivity of PCR method. In this study, we employed one additional filter tube (One Step™ inhibitor removal kit) in duplicate testing on negative cases both in DNA extraction and amplification steps. This yielded positive results in one and four cases, respectively. Thus, by this method, the final clonality detection rate increased up to 49 cases (62.8 %). In another study by Nelson, et al.[2] they developed a sensitive monoplex PCR protocol using FR_1 primer with extended cycling (42 cycles versus 33 – 40 cycles in most studies). They concluded that by this approach the diagnostic sensitivity of PCR–based clonality analysis on FFPET will be optimized with minimal problem of specificity.

It is believed that the best results on FFPE samples usually obtained from blocks less than two years old, while blocks older than 15 years old tend to yield very degraded fragments.[1,20]

Most assays are based on analysis of samples fixed in buffered formalin. However, some laboratories use other fixatives such as unbuffered formalin or Bouin's. The use of 10 % unbuffered formalin permits amplification of wide range sizes of DNA fragments, whereas Bouin's appears to be the least amenable for use in PCR analysis.[1,21,22,23,24]

In this study, we evaluated the relationship between the quality of DNA with age of unbuffered fixed paraffin blocks and it was shown that the paraffin blocks aged less than five years had a significantly more qualified DNA ($P < 0.001$). Also, clonality detection rate was compared on blocks, which showed a higher rate of clonality detection rate on more recent paraffin blocks. However, the difference was not significant ($P = 0.09$).

In another study by Berget, et al.[12] on 118 cases of follicular lymphoma using IgH and Igκ rearrarngement assay with modified BIOMED-2 protocol, they found a tendency to a higher rate of clonality detection on blocks from 2004 through 2008 in comparison to blocks from 1998 through 2003 for the VH $FR_{1,2}$ but not for the VH FR_3 JH assay.

According to our findings we recommend that five years as the cut-off, can significantly affect DNA in unbuffered formalin-fixed paraffin blocks.

With the final clonality detection rate of 62.8 % in unbuffered FFPE DLBCL samples and comparison with other studies, we concluded that by using IgH (FR_3) primers with small product size followed by one additional filter tube modification (One Step™ inhibitor removal kit) we can achieve a considerable clonality detection rate with little adverse impact of DNA degradation.

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