

# Employing PCR Technique in Assessment of Monoclonality in Large B-cell Non-Hodgkin's Lymphoma

Noushin Lotfi<sup>1</sup>, Maryam Rastin<sup>1</sup>, Parisa Shoaee<sup>2</sup>, Bahram Memar<sup>3</sup>, Nafiseh Sadat Tabasi<sup>1</sup>, Zohreh Mahmoudi<sup>4</sup>, Reza Alimohammadi<sup>1</sup>, Bahman Yousefi<sup>1</sup>, \*Mahmoud Mahmoudi<sup>1</sup>

<sup>1</sup>Immunology Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran;

<sup>2</sup>Nosocomial Infection Research Center, Isfahan University of Medical Sciences, Isfahan, Iran;

<sup>3</sup>Department of Pathology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran;

<sup>4</sup>School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.

Received Jun 23, 2015; accepted Oct 10, 2015

**Introduction:** Most B-cell malignancies are diagnosed based on morphologic and immunohistochemical criteria. Some cases, however, still present a challenge for the pathologist to discriminate between reactive hyperplasia and neoplastic disorders. Molecular techniques can be used as a helpful diagnostic tool in these cases. In this study, we assessed the value of polymerase chain reaction (PCR) technique in determination of monoclonality of immunoglobulin heavy chain gene rearrangements for the diagnosis of large B-cell non-Hodgkin's lymphoma (NHL) in paraffin embedded tissue samples. **Methods:** DNA was extracted from paraffin embedded tissues of 44 diffuse large B-cell lymphoma (DLBCL) cases and 20 samples of reactive lymphoid tissues from appendix and tonsils as control. Framework 3 and the joining region (FR3/JH) of the variable segment of the immunoglobulin heavy chain gene were amplified using degenerated primers. PCR products from each sample were analyzed on 8% polyacrylamide gels following AgNO<sub>3</sub> staining. **Results:** Monoclonal rearrangements were identified in 33 of 44 cases (75%) of DLBCL using FR3/JH primers. Monoclonal IgH gene rearrangements were not detected in any of the reactive lymphoid hyperplastic samples. All these control cases showed polyclonal pattern. **Conclusion:** Through PCR analysis, using degenerated primers, monoclonality was demonstrated in 75% of DLBCL cases. This technique can thus be used as a sensitive, reliable and valuable diagnostic supplement to conventional morphologic examination and immunohistochemical evaluation of lymphoproliferative disorders, particularly in cases with restrictions in amount or type of analytic material. *J Med Microbiol Infec Dis, 2014, 2 (3): 121-124.*

**Keywords:** Immunoglobulin Gene, PCR, Non-Hodgkin's Lymphoma.

## INTRODUCTION

High prevalence of non-Hodgkin's lymphoma (NHL) [1, 2, 3, 4] and the difficulty of distinguishing between non neoplastic lymphoproliferative lesion and malignant neoplasms is a diagnostic problem in the field of lymphatic system pathology [5]. The majority of these malignancies will be diagnosed by immunophenotyping and morphologic markers, however these methods are not always useful to establish the diagnosis. Therefore, other molecular methods, such as determination of clonality using PCR, can be helpful.

Lymphocytes are the only cells which physiologically undergo somatic rearrangement. In the early stages of B-cell development, heavy chains of immunoglobulins (Igs) are rearranged. During this process, a D segment is joined to a J segment, and then this complex recombines to a V segment. [6, 7]. In normal lymphoid populations, rearrangement is done randomly and leads to the production of antibody repertoire, however in neoplastic populations rearrangement occurs in one clone and leads to monoclonality.

There are various methods to evaluate the rearrangement of Ig genes in order to examine the monoclonality in B-cell neoplasms [8, 9, 10, 11, 12, 13]. Hybridization methods such as southern blot analysis, has a high validity, but it requires a lot of fresh tissue, as well as radioisotopes [14, 15].

Therefore using these methods in small samples like endoscopic, and in paraffin embedded specimens is not possible. These methods are also expensive, difficult to perform and time consuming.

Polymerase chain reaction (PCR) is another method to evaluate B-cell monoclonality which requires small amounts of tissue, and has acceptable accuracy, high speed and the possibility to use paraffin embedded tissues [8, 9, 11, 12, 13, 16]. In this study, we assessed the value of PCR technique in determination of monoclonality of immunoglobulin heavy chain gene rearrangements for the diagnosis of large B-cell non-Hodgkin's lymphoma in paraffin embedded tissue samples.

**\*Correspondence:** Mahmoud Mahmoudi

Immunology Research Center, Bu Ali Research Institute, Mashhad University of Medical Sciences, Bu Ali Sq., Mashhad, Iran, 9196773113.

**Email:** mahmoudim@mums.ac.ir

**Tel:** +98 (51) 38022229 **Fax:** +98 (51) 37112596

## MATERIAL AND METHODS

**Patients.** 44 samples of paraffin-embedded tissues with the diagnosis of diffuse large B-cell lymphoma (DLBCL) from different body areas were chosen from the archive of the Pathology Department of Mashhad University of Medical Sciences, Mashhad, Iran. These samples were then evaluated by a pathologist using immunohistochemistry with default markers and H&E staining to confirm the diagnosis. Furthermore, 20 samples from benign reactive lymphoid tissues of tonsils and appendix were used as controls.

**DNA extraction.** 5 µm sections were cut from paraffin embedded samples and transferred to 1.5 ml microtubes. 1000 µl of Octane (Sigma, St. Louis, USA) was added and incubated at room temperature for 30 min, they were inverted 2 or 3 times. Then tubes were centrifuged for 5 min at 14000 rpm. The supernatant was removed and 500 µl of 95% ethanol was added and centrifuged for 1 min at 14000 rpm. Then 2 or 3 drops of acetone were added and allowed to evaporate for 10 min, afterwards, 100 µl of digestion buffer containing 100 µg Proteinase K (Sigma, St. Louis, USA) was added and incubated overnight at 37°C. Then proteinase K was inactivated by heating at 95°C for 10 min. After centrifugation at 8000 rpm for 1 min the supernatant, including DNA, was separated and stored at -20°C.

**PCR with β actin primers.** To confirm the quality of the extracted DNA, PCR was performed for all samples using β actin primers, and PCR products were electrophoresed in 2% agarose gel. Samples that did not have a distinct band of β actin primers were excluded from this study.

**Primer Design.** In this study, the primers were designed for FR3 and J regions of the variable portion of the Ig heavy chain. To design these primers, amino acid and nucleotide sequences of these regions were reviewed using gene bank database. Because of some differences among nucleotide sequences of these regions, degenerated primers were designed to increase the detection of monoclonality.

**Nucleotide sequence of primers.** Forward primers: FR31: 5'- ACA Cgg C(C/T) (g/C) TgT ATT ACT gT-3' FR32: 5'-ACA Cgg C(C/T) (g/C) (A/C/T) (A/C/g) T ATT ACT GT-3' Reverse primer: JH2: 5'-gTg ACC Agg GT (g/C/T) CC(C/A/T) Tgg CCC CAg-3'

**PCR.** PCR reaction to determine the monoclonality of rearranged genes of B lymphocytes heavy chain was performed as follows: 1 µg of extracted DNA from each

sample was amplified in a total volume of 25 µl, containing 1X PCR buffer, 0.4 mM MgCL<sub>2</sub>, 200 µM dNTP, 0.2 µM primer (TIB, Germany) and 1 unit taq DNA polymerase (Genet Bio, Korea). Thermal cycling conditions were as follows: 5 min at 94°C, followed by 44 cycles at 94°C for 50 s, 59°C for 50 s, and 72°C for 50 s, followed by a final extension at 72°C for 7 min (T3-Thermocycler-Biometra, Germany). After PCR reaction 15 µl of PCR product was electrophoresed on 8% polyacrylamide gel followed by AgNO<sub>3</sub> staining.

## RESULTS

Twenty six of cases in our study with DLBCL diagnosis were male (59.1%) and 18 cases (40.9%) were female. The most prevalent age group was 41 to 60 years followed by 61 to 90. Neck, nasopharynx and axilla were the most common sites of involvement in our study group. To confirm the diagnosis, all cases were studied by immunohistochemical method using CD20, CD3, LCA, cytokeratin markers and H&E staining. Cases that were CD20 and LCA positive, but CD3 and cytokeratin negative, were selected as definite DLBCL samples (Figures 1, 2). The criterion for monoclonality assessment of lymphocytes residing in tissues using PCR method is detecting a monoclonal band in electrophoresis. In polyclonal samples, because of rearrangements in different clones, the PCR product shows a smear pattern. In this study, paraffin embedded tissues were analyzed. In 33 out of 44 cases (75%) after PCR with designed primer for FR3/JH region, an obvious monoclonal band (125 bp) was seen, while in control samples from reactive hyperplastic tissues, no monoclonal band was detectable. All these cases had a smear pattern denoting a polyclonal rearrangement (Figures 3, 4, 5).

## DISCUSSION

Previous studies on monoclonality detection in lymphomas with B-cell origin have led to various results. Yanchi-Tai *et al.* reported the presence of monoclonality in 54.3% of cases with the use of designed primers for FR3/JH region in paraffin embedded tissues [17]. Adan Bagg *et al.* in 2002 reported monoclonality in 47% of paraffin embedded tissue samples with B-cell lymphoma [18]. Timep Gurbity *et al.* have reported monoclonality in 55% of cases [6].

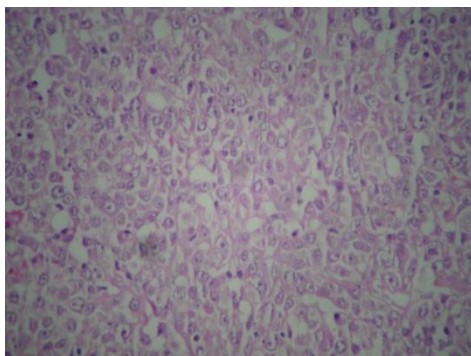


Fig. 1. Diffuse infiltration of large lymphoid cells

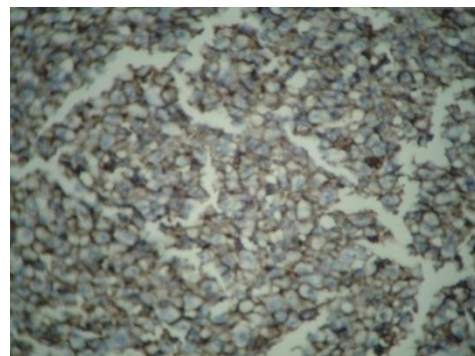
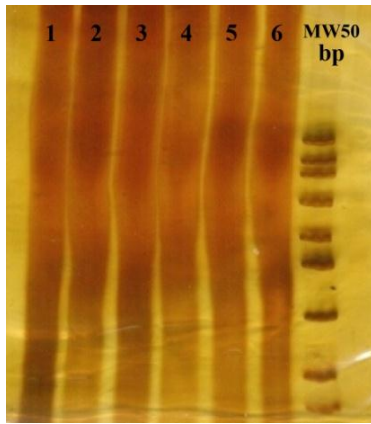
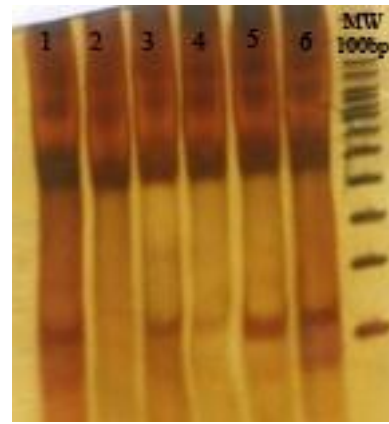


Fig. 2. CD20 in membrane of large lymphoid cells



**Fig. 3.** Lanes 1-6 control samples with smear pattern



**Fig. 4.** Lanes 1, 3, 4, 5, 6 patient samples with monoclonal band; lane 2 control sample with smear pattern



**Fig. 5.** Lanes 1-6 patient samples with monoclonal band and lanes 7-10 control samples

Poor results of previous studies might be related to chromosomal translocation between *bcl2* or *bcl6* genes and Ig heavy chain genes due to somatic hypermutation and poor primer design [19, 20]. Somatic hypermutation happens in the germinal center of differentiating B-cells. Target regions of hypermutation are framework (FR) and complementarity determining regions (CDR) of variable portions of Ig heavy chain, which may cause primer mismatch.

Designing family specific primers can overcome this problem, and that is why in this study, designing new degenerate primers, increased the diagnosis of monoclonality up to 75% using FR3/JH primers.

Another factor which may affect the results is the type of samples [21, 22, 23]. In KJ Trainor study, which was performed on fresh tissues, the percentage of monoclonality detection with the use of FR3/JH primers was 83%. [24, 25, 26]. The probable cause of this higher level of monoclonality detection in their study was probably the absence of tissue fixatives, which are considered to be PCR inhibitors, on the other hand during tissue fixation steps, DNA might be degraded.

We have shown, PCR can be used as a reliable, sensitive and valuable method in the assessment of lymphoproliferation disorders next to conventional methods, particularly in the case of small and paraffin embedded tissue samples (such as needle aspirates and endoscopic biopsies). It must be also taken into consideration that designing suitable primers is crucial in order to achieve more reliable outcomes.

#### ACKNOWLEDGEMENT

The authors are grateful to research council of Mashhad university of Medical Science for financial support of this study.

#### CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

#### REFERENCES

1. Seow A, Lee J, Sng I, Fong CM, Lee HP. Non-Hodgkin's lymphoma in an Asian population: 1968-1992 time trends and ethnic differences in Singapore. *Cancer*. 1996; 77 (9): 1899-904.
2. Doglioni C, Wotherspoon AC, Moschini A, de BM, Isaacson PG. High incidence of primary gastric lymphoma in northeastern Italy. *Lancet*. 1992; 339 (8797): 834-5.
3. Chiu BC, Weisenburger DD. An Update of the Epidemiology of Non-Hodgkin's Lymphoma. *Clin Lymphoma*. 2003; 4 (3): 161-8.
4. Iqbal J, Joshi S, Patel KN, Javed SI, Kucuk C, Aabida A, d'Amore F, Fu K. Clinical implication of genome-wide profiling in diffuse large B-cell lymphoma and other subtypes of B-cell lymphoma. *Indian J Cancer*. 2007; 44 (2): 72-86.
5. Weirich G, Funk A, Hoepner I, Heider U, Noll S, PiJtz B, Fellbaum C, Höfler H. PCR-based assays for the detection of monoclonality in non-Hodgkin's lymphoma: application to formalin-fixed, paraffin-embedded tissue and decalcified bone marrow samples. *J Mol Med*. 1995; 73 (5): 235-41.

6. Gurbity TP, Bagdi E, Groen NA, Budel LM, Abbou M, Krenacs L, Dinjens WN. Increased sensitivity of B-cell clonality analysis in formalin-fixed and paraffin-embedded B-cell lymphoma samples using an enzyme blend with both 5'→3' DNA polymerase and 3'→5' exonuclease activity. *Virchows Arch.* 2003; 443 (5): 643-8.
7. Abul KA, Andrew HL, Shiv P. *Rearrangements of Immunoglobulins, Cellular and molecular immunology.* 7th ed. Philadelphia: W.B. Saunders Publishers; 2012; 159-70.
8. Diss TC, Liu HX, Du MQ, Isaacson PG. Improvements to B cell clonality analysis using PCR amplification of immunoglobulin light chain genes. *Mol Pathol.* 2002; 55 (2): 98-101.
9. Gonzalez M, Gonzalez D, Lopez-Perez R, Garcia-Sanz R, Chillón MC, Balanzategui A, Mateos MV, Alaejos I, Langerak AW, Orfão A, Van Dongen JJ, San Miguel JF. Heteroduplex analysis of VDJ amplified segments from rearranged IgH genes for clonality assessments in B-cell non-Hodgkin's lymphoma. A comparison between different strategies. *Haematologica.* 1999; 84 (9): 779-84.
10. Ling FC, Clarke CE, Corbett WE, Lillcrap DP. Sensitivity of PCR in detecting monoclonal B cell proliferations. *J Clin Pathol.* 1993; 46 (7): 624-7.
11. Koletsa TD, Beretouli EA, Mavropoulou SD, Kostopoulos IS. Primary cutaneous marginal zone lymphoma (PCMZL) presenting with heterochronous biclonal lesions. *Indian J Pathol Microbiol.* 2013; 56 (3): 325-7.
12. Parimal S, Pai R, Manipadam MT, Nair S. Lennert's lymphoma: Clinicopathological profile of five cases. *Indian J Pathol Microbiol.* 2013; 56 (3): 248-51.
13. Cardona DM, Layne A, Lagoo AS. Lymphomas of the gastrointestinal tract - Pathophysiology, pathology, and differential diagnosis. *Indian J Pathol Microbiol.* 2012; 55 (1): 1-16.
14. Hoeve MA, Krol AD, Philippo K, Derksen PW, Veenendaal RA, Schuurung E, Kluin PM, van Krieken JH. Limitations of clonality analysis of B cell proliferations using CDR3 polymerase chain reaction. *Mol Pathol.* 2000; 53 (4): 194-200.
15. Lehman CM, Sarago C, Nasim S, Comerford J, Karcher DS, Garrett CT. Comparison of PCR with Southern Hybridization for the routine detection of immunoglobulin heavy chain gene rearrangements. *Am J Clin Pathol.* 1995; 103 (2): 171-6.
16. Diss TC, Pan L, Peng H, Wotherspoon AC, Isaacson PG. Sources of DNA for detecting B cell monoclonality using PCR. *J Clin Pathol.* 1994; 47 (6): 493-6.
17. Tai YC, Peh SC. Immunoglobulin gene rearrangement of Lymphoid malignancies: application of the PCR technique to Formalin-Fixed, Paraffin embedded tissues. *J Clin Exp Hematopathol.* 2003; 43 (1): 1-9.
18. Bagg A, Brazier RM, Arber DA, Bijwaard KE, Chu AY. Immunoglobulin heavy chain gene analysis in lymphomas: a multi-center study demonstrating the heterogeneity of performance of polymerase chain reaction assays. *J Mol Diagn.* 2002; 4(2): 81-9.
19. Amara K, Trimeche M, Ziadi S, Sriha B, Mokni M, Korbi S. PCR-based clonality analysis of B-cell lymphomas in paraffin-embedded tissues: diagnostic value of immunoglobulin kappa and lambda light chain gene rearrangement investigation. *Pathol Res Pract.* 2006; 202 (6): 425-31.
20. Storbio U. The molecular basis of somatic hypermutation immunoglobulin genes. *Curr Opin Immunol.* 1996; 8 (2): 206-14.
21. Zhou XG, Sandvej K, Gregersen N, Hamilton-Dutoit SJ. Detection of clonal B cells in microdissected reactive lymphoproliferations: possible diagnostic pitfalls in PCR analysis of immunoglobulin heavy chain gene rearrangement. *Mol Pathol.* 1999; 52 (2): 104-10.
22. Camilleri-BroEt S, Devez F, Tissier F, Ducruit V, Diebold J, Audouin J, Molina T. Quality Control and Sensitivity of Polymerase Chain Reaction Techniques for the Assessment of Immunoglobulin Heavy Chain Gene Rearrangements from Fixed and Paraffin-Embedded Samples. *Ann Diagn Pathol.* 2000; 4 (2): 71-6.
23. Melotti CZ, Amary MF, Sotto MN, Diss T, Sanches JA. Polymerase chain reaction-based clonality analysis of cutaneous B-cell lymphoproliferative processes. *Clinics (Sao Paulo).* 2010; 65(1): 53-60.
24. Trainor KJ, Brisco MJ, Story CJ, Morley AA. Monoclonality in B-lymphoproliferative disorders detected at the DNA level. *Blood.* 1990; 75 (11): 2220-2.
25. Mies C. Molecular Pathology of paraffin embedded tissue. Current clinical applications. *Diagn Mol Pathol.* 1992; 1 (3): 206-11.
26. Guzmán LM, Castillo D, Aguilera SO. Polymerase chain reaction (PCR) detection of B cell clonality in Sjögren's syndrome patients: a diagnostic tool of clonal expansion. *Clin Exp Immunol.* 2010; 161 (1): 57-64.