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Original Article

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

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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 AgNO3 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 hyperplasic 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 immunohistocytochemical 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 neoplasic 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.

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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 37ºC. Then proteinase K was inactivated by heating at 95ºC for 10 min. After centrifugation at 8000 rpm for 1 min the supernatant, including DNA, was separated and stored at -20ºC.

PCR with ß actin primers. To confirm the quality of the extracted DNA, PCR was performed for all samples using ß actin primers, and PCR products were electrophoresed in 2% agarose gel. Samples that did not have a distinct band of ß 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 $MgCL_2$, 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 94ºC, followed by 44 cycles at 94ºC for 50 s, 59ºC for 50 s, and 72ºC for 50 s, followed by a final extension at 72ºC for 7 min (T3-Thermocycler-Biometra, Germany). After PCR reaction 15 µl of PCR product was electrophoresed on 8% polyacrylamide gel followed by AgNO3 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 cytockeratin 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 hyperplasic 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].

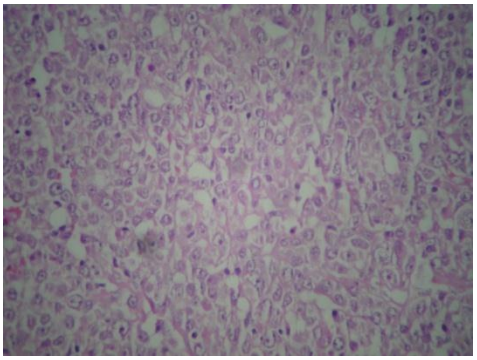


Fig. 1. Diffuse infiltration of large lymphoid cells

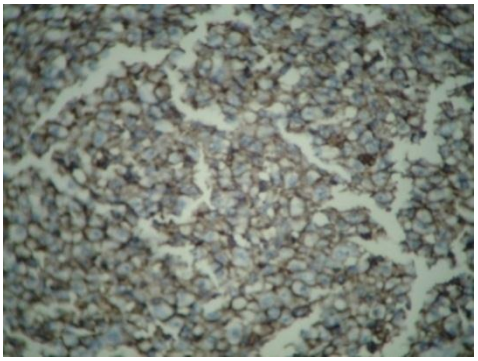


Fig. 2. CD20 in membrane of large lymphoid cells

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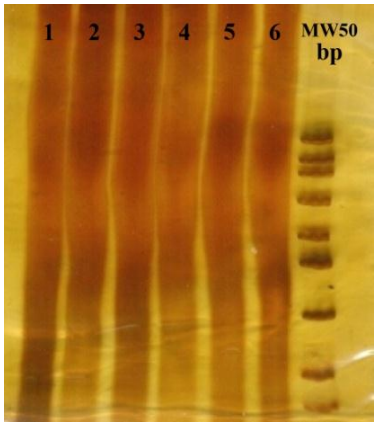


Fig. 3. Lanes 1-6 control samples with smear pattern



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

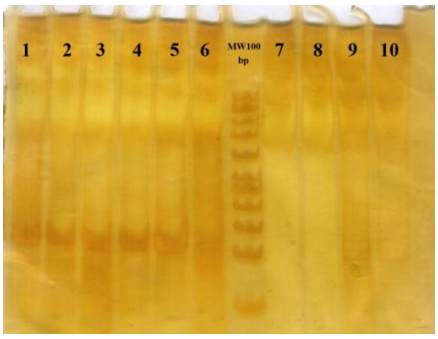


Fig. 5. Lanes 1-6 patient samples with monoclonal band and lanes 7-10 control samples

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.

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CONFLICT OF INTEREST

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

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