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Clonality assay of IGH gene rearrangement in Iraqi patients with non hodgkin's lymphoma using FFPE tissue

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Abstract

Background: B cell Non Hodgkin lymphoma is a malignant that arose in B lymphocytes and dividing into several subtypes. A role of B-cell receptor (BCR) in lymphomagenesis has been inferred by studying immunoglobulin genes in human lymphoma. During maturation of b cell Ig gene undergoing to the process rearrangement. Ig gene rearrangement in B-cell lymphomas arise from monoclonal enlarged B-cells, this may be used as markers of neoplasm clonality.

Material and methods: Collected fifty six of FFPE tissue, 37 of NHL and 19 of reactive lymphoid hyperplasia that use as control for polyclonality, were collected from Baghdad medical city and used as source of DNA and the clonality of B-cell lymphoma determined by polymerase chain reaction (PCR) and heteroduplex analysis to separated monoclonal from polyclonal according to BIOMED-2 protocol used as diagnostic tool of B-NHL

Results: The positive clonality was detected in rate 70% (26/37) as monoclonal while 29% (11/37) the clonality showed a polyclonal pattern. in group with DLBCL clonality was observed in 66%(10/15) as monoclonal, in SLL monoclonality detected in 75%(3/4), in BL monoclonality showed in 66%(2/3), in MALT monoclonality showed in 50%(1/2) and in patients diagnosed with FL and NMZL 100% of case showed a monoclonal pattern. In reactive lymphoid hyperplasia 100%(19/19) the clonality showed a polyclonal pattern.

Conclusion: Use the complete IGH rearrangement to determination the clonality of B cell has provided a good tool for diagnostic the B cell lymphoma**.**

Keywords: Non Hodgkin's lymphoma, Immunoglobulin heavy chain rearrangement, Clonality assay, Heteroduplex analysis, FFPE tissue

INTRODUCTION

Non Hodgkin lymphoma is cancer that originates in our lymphatic system, the disease-fighting network spread throughout the body. In non Hodgkin's lymphoma , tumors develop from lymphocyte – type of white blood cell. The B cell lymphomas are " blood cancers " in lymph nodes. They develop more frequently in older adults and in immunocompromised individuals. [1].

Non-Hodgkin lymphomas (NHLs) are a heterogeneous group of neoplastic disorders in which malignant lymphocytes have arrested at various stages of differentiation and have acquired the ability to proliferate, evade the host's immune system, and avoid programmed cell death (cellular apoptosis) The malignant cell for many lymphomas can be traced to a specific stage in lymphoid maturation, with the majority derived from mature B cells or those of germinal center origin. The monoclonal nature of tumor cells is established by demonstrating immunoglobulin (Ig) gene rearrangement in B cells, T-cell receptor (TCR) rearrangement in T cells, or a reciprocal cytogenetic translocation or molecular alteration.[2]. There are many different types of non-Hodgkin lymphoma (NHL), About 90% of B cell lymphoma, 9% of T cell lymphoma and less than 1% of Natural killer cell (NK) lymphoma, B and T lymphoma also divided into several subtype [3].

Non-Hodgkin lymphoma (NHL) is one of the most common cancers in the United States in 2017, accounting for about 4% of all cancers and 6th most common cancer in the UK, accounting for 4% of all new cancer cases in (2015) [4] [5]. In Iraq NHL was the fourth of the commonest ten cancers in 1995-1999 after breast, lung and urinary bladder. [6] While in 2002 it was the fifth after breast, lung, urinary bladder and brain and CNS. [7]. In 2003-2013 NHL it was the fourth of the commonest ten cancers in Iraq after breast, lung, leukemia [8]. Many factors found that effects to persons to get non hodgkins lymphoma such as Age ,Gender ,Race, ethnicity, and geography ,Exposure to certain chemicals and drugs ,Radiation exposure ,Having a weakened immune system ,Autoimmune diseases, Certain infections, Body weight and diet,Breast implants, infection with virus and bacteria but the cause of most lymphomas is not known [9]. NHLare neoplasms that arise from lymphoid cells of either B-cell or T-cell lineage [10][11] and are generally diagnosed via histomorphology and immunohistochemistry. In 5-15% of cases of NHL the differential diagnosis between reactive lesions and malignant lymphomas is inconclusive, requiring complementary methods [11]. In these cases, molecular assessment of clonal immunoglobulin heavy chain (IgH) and T-cell receptor (TCR) gene rearrangement by PCR is an important diagnostic tool [12][13]. PCR-based tests are rapid, exquisitely sensitive and therefore applicable to very small quantities of DNA and can also be performed on formalin- fixed, paraffin-embedded (FFPE) tissues. However, the sensitivity and reproducibility of the PCR method can often be negatively influenced by a poor quality of extracted DNA [14][15]. In humans, B-cell differentiation is coordinated with the expression of particular antigen surface receptors called immunoglobulins (Ig) [16]. B Cell receptors on the cell

surface of the B cell are distinguished and associated with the antigen and mediate immune response [17]. The B cell receptors are immunoglobulin (IG) and consist of heavy chains (H) and light chains (L). the regions outside the immunoglobulin can be divided into variable regions (V) and constant regions (C). The variable region V with different composition by includes specific binding of antigen [18]. The diversity of the B receptor is the result of a process of IG gene rearrangement during the process of growth or formation of the B cells [19]. The gene of the heavy chains consists of a number of genes VH, DH, JH and CH, and the gene of the light chains consist of the genes κ, λ, and the κ gene is composed of the genes Vκ, Jκ, Cκ and λ gene consists of genes Vλ, Jλ, Cλ. These genes reproduce only after the process of rearrangement of IG gene is completed [20]. The process of rearrangement is a natural physiological process occurs during the maturity of lymphocytes and in the case of the fetal state, the B cell receptor gene consists of the variable region V, Diverse region D, Joining region J, and constant region C [21]. These areas are not sequentially any closer to each other in the chromosome and are separated by sequences of different lengths [21]. After many stages of lymphocyte growth, these regions will be rearranged to accumulate to form the structure of the IG gene and they will undergo to a process of rearrangement of the gene and this process is stimulated by the recombinase enzyme [22]. The gene arrangement for all lymphocytes is unique, and the B cells can distinguish millions from different antigens. If one cell can avoid physiological regulation because of certain factors, this cell will grow irregularly through a process called cloning hyperplasia and lymphoma cancer can arise in this case [23]. The process of cloning hyperplasia can lead to the process of special gene rearranging to become dominant, which can lead to the generation of a detection indicator of the cloning of the lymphoma malignant [24][25]. malignant lymphoma is monoclonal hyperplasia and theoretically represent all the cloned genes from the process of rearrangement.However, the inflation in the natural lymphatic organs or in the reactive lymphoid tissues is polyclonal in the process of regenerating the arrangement of IG gene.In the process of detecting the rearrangement of the gene, IG Can always help determine the nature of inflation that was benign or malignant by Know the source of reproduction is Monoclonal or Polyclonal [23]. In 2003, 47 organizations from 7 different European countries developed a system called BIOMED-2 multiplex PCR which included 107 primers divided into 18 strands PCR [13][26]. In this study, BIOMED-2 was used to clonality analysis of IGH gene rearrangements (framework I, II, III) in the diagnosis of NHL

MATERIALS AND METHODS

Samples collection of Formalin Paraffin-embedded tissue (FFPE) block samples

In this study, 56 samples of Iraqi patients that were collected, 37 for patients with non-Hodgkin's lymphoma before they take any treatment and 19 for reactive lymphoid hyperplasia that was used as control **,** The samples were collected from the Educational laboratories and the Martyr Ghazi Al-Hariri Hospital in Baghdad Medical City and from some private laboratories between July 2017 - February 2018 . All patients were diagnosed by pathologists with conventional methods, parameters, and clinical symptoms. Samples were included 20 men and 17 women their age rate 20 – 72. Specimens were classified by pathologists based on the World Health Organization (WHO) into 15 diffuse large B cell lymphoma (DLBCL) , 4 small lymphatic lymphoma (SLL), 3 follicular lymphomas (FL), 3 Burkitt's lymphoma (BL), 2 Nodal marginal zone lymphoma (NMZL) , 2 Mucosa-associated lymphoma tissue (MALT) and 8 case with an unclassifiable lymphoma, which was categorized as B cell lymphoma (BCL)

DNA Extraction from FFPE Samples

In the beginning, all samples were cut from 4 to 5 slices of thickness 10 - 15 microns by microtome and placed in Eppendorf tubes 1.5 ml and the DNA was extracted according to Promega (ReliaPrep™ FFPE gDNA Miniprep System, USA) The extracted DNA from FFPE tissues was assessed using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel 1.5%

PCR-Amplification for IGH Gene Rearrangements

The gold standard multiplex PCR protocol was used (provided by European Biomedicine and Health (BIOMED- 2) Concerted Action Project BMH4-CT98-3936) for enhancement of diagnosis and analysis of clonality gene rearrangement in lymphoma tumor. Specific primers for immunoglobulin heavy chain VH-JH region from (integrated DNA Technologies IDT, USA) (Table 1). The gene amplification was carried out at a size of 25 ul with 5-5.5 DNA ul and 12.5 ul of the green master mix (Promega) and one microliter of each primer. The target reactions for *IGH* gene arrangement assays included three reactions targeting IGHA (VHFR1-JH), IGHB (VHFR2-JH) and IGHC (VHFR3-JH). Rearrangement assays of the target genes for three framework region (FRI, FRII, FRIII) there are amplified according to Biomed-2 program conditions: initial denaturation for 10 minutes at 95◦C, followed by 35 cycles of 95◦C for 30 seconds, 60 ◦C for 30 seconds and finally 72◦C for 90 seconds. Before the end of the amplification cycles, a final extension step was performed at 72◦C for 10 minutes. All samples were assessed two times to avoid pseudoclonality.

Heteroduplex analysis

For heteroduplex analysis, initially, the PCR products were denatured at 95ºC for 5minutes and subsequently incubated at 4ºC to generate homoduplex. the PCR products were loaded at 6% non-denaturing polyacrylamide gel, and 2% agarose gel, run at room temperature and visualized by redsafe staining (iNtRON, Korea). The observation of one or two bands within the expected size (between 310-360bp for FRI, between 250-295bp for FRII, and between 100–170 bp for FRIII) indicated clonality, while polyclonality patterns were revealed as a rough smear in the gel

Table 1: Specific multiplex primer mixes for *IGH* (Framework I , II, III) for clonality assay by BIOMED-2 **according to** [13]

Forward *(5′ - 3′)* VH-FRs	
VH1-FRI	GGCCTCAGTGAAGGTCTCCTGCAAG
VH2-FRI	GTCTGGTCCTACGCTGGTGAAACCC
VH3-FRI	CTGGGGGGTCCCTGAGACTCTCCTG
VH4-FRI	CTTCGGAGACCCTGTCCCTCACCTG
VH5-FRI	CGGGGAGTCTCTGAAGATCTCCTGT
VH6-FRI	TCGCAGACCCTCTCACTCACCTGTG
VH1-FRII	CTGGGTGCGACAGGCCCCTGGACAA
VH2-FRII	TGGATCCGTCAGCCCCCAGGGAAGG
VH3-FRII	GGTCCGCCAGGCTCCAGGGAA
VH4-FRII	TGGATCCGCCAGCCCCCAGGGAAGG
VH5-FRII	GGGTGCGCCAGATGCCCGGGAAAGG
VH6-FRII	TTGGGTGCGACAGGCCCCTGGACAA
VH7-FRII	TGGATCAGGCAGTCCCCATCGAGAG
VH1-FRIII	TGGAGCTGAGCAGCCTGAGATCTGA
VH2-FRIII	CAATGACCAACATGGACCCTGTGGA
VH3-FRIII	TCTGCAAATGAACAGCCTGAGAGCC
VH4-FRIII	GAGCTCTGTGACCGCCGCGGACACG
VH5-FRIII	CAGCACCGCCTACCTGCAGTGGAGC
VH6-FRIII	GTTCTCCCTGCAGCTGAACTCTGTG
VH7-FRIII	CAGCACGGCATATCTGCAGATCAG
Reverse *(5′- 3′)* JH-FRs	
JH-FRs	CTTACCTGAGGAGACGGTGACC

RESULTS

Genotyping analysis

This study includes extracting DNA from FFPE of 56 subjects (males and females). The quality and integrity of DNA were checked on agarose gel electrophoresis before doing PCR. The extraction was made by Promega kit (USA) and the total DNA was shown in (Figure 1). The average of DNA samples concentration and purity in OD260/280 ratios 250 ng/µl (ranging between 75-1131 ng/µl) and 1.85 – 1.9 purity in using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) that were extracted by Promega kit.

Clonal detection of immunoglobulin heavy chain gene by multiplex PCR

Gene rearrangement analysis revealed clonality in 70%(26/37) of NHL, while 11(30%) case showed polyclonality as shown in (Table 2).

In the NHL two group, DLBCL and BCL showed clonality for complete IGH gene in all regions (FR1, FR2, FR3). Positive clonality was observed in 10 out of 15 (66%) case of DLBCL, while 5 cases (33%) clonality was not detected in any of the immunoglobulin gene family (FR1, FR2, FR3) and showed a polyclonality pattern. Also in BCL 5 out 8 (62%) showed clonality for IGH gene whereas in the rest of the 3 case (38%) clonality was not detected in any region of IGH family (FR1, FR2, FR3) and showed a polyclonality pattern.

In DLBCL group, the FR1 region clonality was detected in 1/15 (6%) case, FR2 5/15 (33%) case and 5/15 (33%) case for FR3.

In FL group positive clonality was observed in 3 out of 3 (100%) case, there was no case detected with clonality for FR1 0/3 (0%) whereas the result was 2/3 (66%) for FR1 & FR2 respectively.

In the BCL group, clonality was detected in 2/8(25%) for FR1, while for FR2&FR3 the result was 1/8 (12%), 2/8(25%) respectively

In SLL group, clonality was detected in 3 out of 4 (75%) case, there was no case detected with clonality for FR1 0/3 (0%) whereas the result was 1/4 (25%), 2/4(50%) for FR2 & FR3 respectively.

In NMZL group, positive clonality was detected in 2 out of 2(100%) case, 1/2(50%) for FR1,1/2(50%) for FR2 and no positive clonality was observed for FR3.

In the BL group, clonality was detected in 2 out of 3 (66%) case, 2/3 (66%) for FR3 and no positive clonality was detected for FR1 and FR2.

In MALT group, positive clonality was detected in 1 out of 2(50%) case for, 1/2(50%) for FR1 and no positive clonality was detected for FR2 and FR3(Table 3)(Figures 2-4)

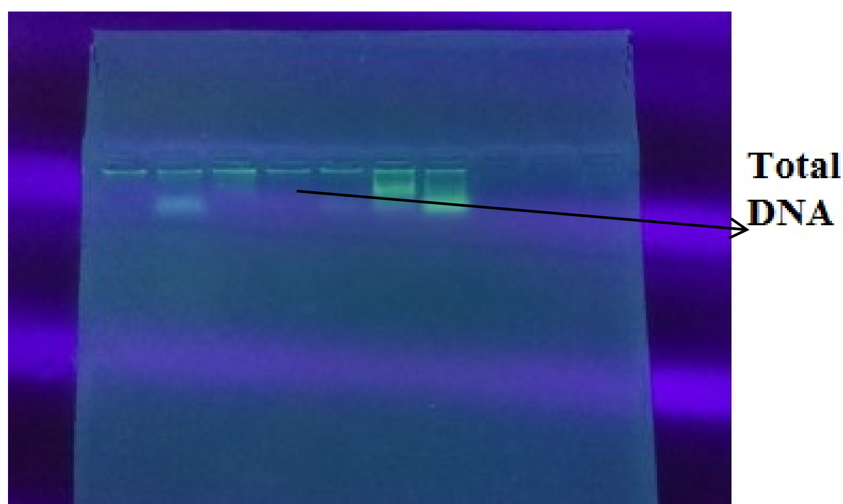

Figure 1: Agarose gel (1.5%) with redsafe staining and run for 1 hour shown the total DNA Extracted by Promega kit

Table 2: Summary of patients characteristics and molecular finding

	patients characteristics					IG gene rearrengemente			
	Type	**Sex**	**Age**	**grade**	**site**	**FR1**	**FR2**	**FR3**	**Clonality**
1	DLBCL	F	58	high	nodal	-	-	-	-
2	DLBCL	M	75	high	nodal	-	-	-	-
3	DLBCL	M	42	high	extranodal	-	+	-	+
4	DLBCL	F	40	high	nodal	-	+	-	+
5	DLBCL	F	58	intermediate	extranodal	-	-	+	+
6	DLBCL	M	69	high	nodal	-	-	-	-
7	DLBCL	M	45	high	nodal	-	+	-	+
8	DLBCL	F	45	intermediate	nodal	-	-	+	+
9	DLBCL	M	50	high	nodal	-	-	+	+
10	DLBCL	M	53	high	nodal	-	+	-	+
11	DLBCL	M	70	high	extranodal	-	-	-	-
12	DLBCL	F	67	high	nodal	-	-	-	-
13	DLBCL	F	40	high	nodal	-	-	+	+
14	DLBCL	M	68	intermediate	nodal	-	-	+	+
15	DLBCL	F	48	intermediate	nodal	+	+	-	+
16	FL	F	55	low	extranodal	-	-	+	+
17	FL	M	53	low	nodal	-	+	-	+
18	FL	M	68	low	nodal	-	+	+	+
19	BCL	F	60	intermediate	nodal	-	-	-	-
20	BCL	M	60	intermediate	anodalextr	-	-	+	+
21	BCL	F	52	low	nodal	+	-	-	+
22	BCL	M	71	low	extranodal	-	-	+	+
23	BCL	F	28	high	nodal	-	+	-	+
24	BCL	F	40	high	nodal	-	-	-	-
25	BCL	M	60	low	nodal	-	-	-	-
26	BCL	M	50	low	nodal	+	-	-	+
27	SLL	F	55	low	nodal	-	-	+	+
28	SLL	M	70	low	nodal	-	-	-	-
29	SLL	M	59	low	nodal	-	-	+	+
30	SLL	F	72	low	nodal	-	+	-	+
31	NMZL	F	25	low	nodal	+	-	-	+
32	NMZL	M	62	low	nodal	-	+	-	+
33	BL	M	20	high	extranodal	-	-	+	+
34	BL	F	22	high	extranodal	-	-	+	+
35	BL	M	36	high	nodal	-	-	-	-
36	MALT	M	70	low	extranodal	-	-	-	-
37	MALT	F	68	low	extranodal	+	-	-	+

DLBCL: Diffuse large B cell lymphoma, FL: Follicular lymphoma, SLL: Small lymphatic lymphoma, BL: Burkitt's lymphoma, NMZL: Nodal marginal zone lymphoma, BCL: B cell lymphoma, MALT: Mucosa-associated lymphoma tissue, FR: Framework region, IG: Immunoglobulin

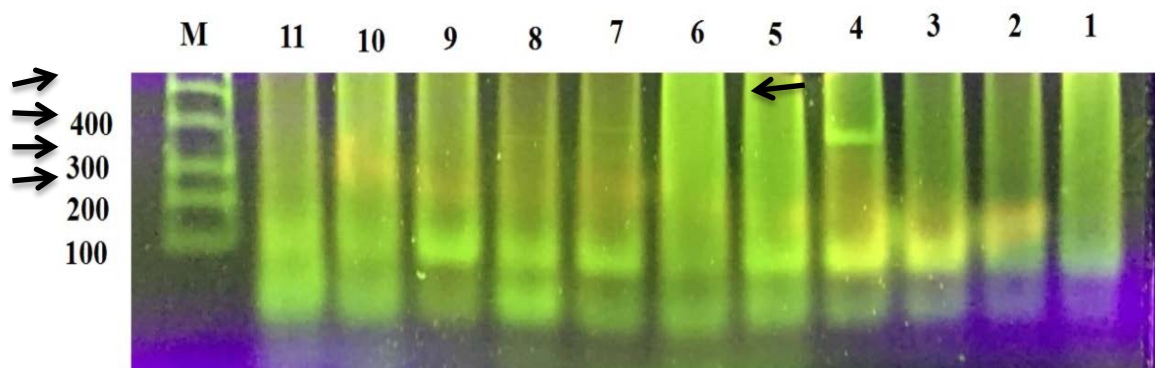

Figure 2: Gene rearrangement in FR1 region, polyacrylamide 6% gel in run 3 hours electrophoresis in 150v, Staining with redsafe in 20 min with 100 ml of 1x TBE from lain 1 to 10 non hodgkin lymphoma, monoclonal band (350bp) showed in line (4), lane 11: control reactive hyperplasia showed smear result M: Marker 100bp (Promega).

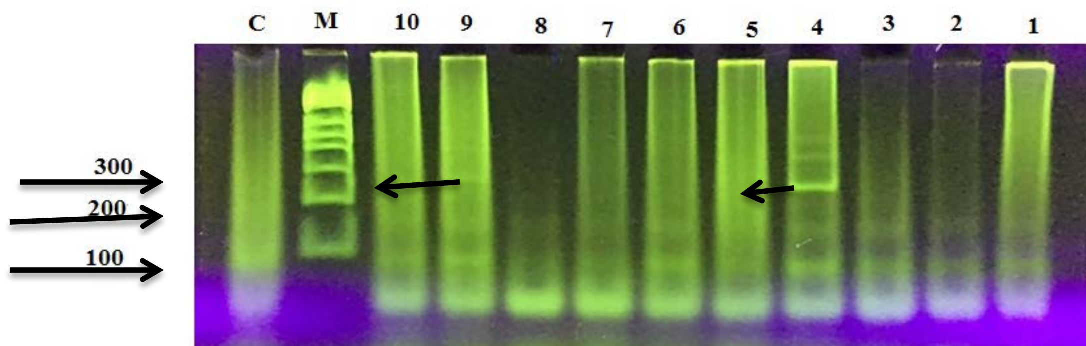

Figure 3: Gene rearrangement in FR2 region, polyacrylamide 6% gel in run 3 hours electrophoresis in 150v. Staining with redsafe in 20 min with 100 ml of 1x TBE, from lane 1 to 10 non hodgkin lymphoma, monoclonal band (280bp) showed in line (4,9,19) C: control reactive hyperplasia showed smear result M: Marker 100bp (Promega).

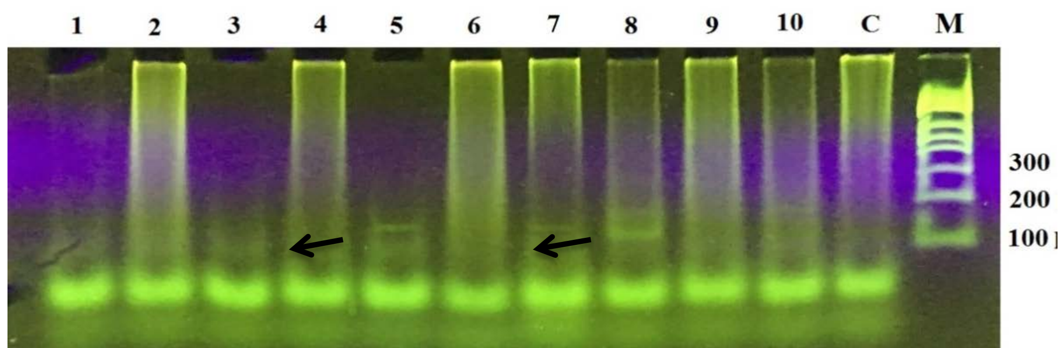

Figure 4: Gene rearrangement in FR3 region, polyacrylamide 6% gel in run 3 hours electrophoresis in 150v , Staining with redsafe in 20 min with 100 ml of 1x TBE from lain 1 to 11 non hodgkin lymphoma, monoclonal band (120bp) showed in line(5,7,8) M: Marker 100bp (Promega), C: Control negative (reactive lymphoid hyperplasia) showed smear result

Table 3: Gene rearrangement analysis on patients with lymphoid malignancies to detect clonality and polyclonality.

Type	FR1	FR2	FR3	FR1+FR2	FR2+FR3	FR1+FR3	FR1+FR2+FR3
DLBCL	1/15(6%)	5/15(33%)	5/15(33%)	6/15(40%)	10/15(66%)	6/15(40%)	11/15(73%)
FL	0/3	2/3(66%)	2/3(66%)	2/3(66%)	3/3(100%)	2/3(66%)	3/3(100%)
BCL	2/8(25%)	1/8(12%)	2/8(25%)	3/8(37%)	3/8(37%)	4/8(50%)	5/8(62%)
SLL	0/4	1/4(25%)	2/4(50%)	1/4(25%)	3/4(75%)	2/4(50%)	3/4(75%)
NMZL	1/2(50%)	1/2(50%)	0/2	2/2(100%)	1/2(50%)	1/2(50%)	2/2(100%)
MALT	1/2(50%)	0/2	0/2	1/2(50%)	0/2	1/2(50%)	1/2(50%)
BL	0/3	0/3	2/3(66%)	0/3	2/3(66%)	2/3(66%)	2/3(66%)
Total	5/37(13%)	10/37(27%)	13/37(35%)	15/37(40%)	23/37(62%)	18/37(48%)	28/37(75%)
Control	0/19	0/19	0/19	0/19	0/19	0/19	0/19

Discussion

The Ig gene rearrangement in each B-cell is thus special or since B-cell lymphomas and stability leukemias arise from monoclonal extended B-cells, this performs remain old namely markers concerning tumor clonality [27]. Analysis of somatic hypermutation on the immunoglobulin variable heavy chain (IgVH) genes has supplied precious perception between the starting place of B-cell lymphomas [28][29]. As mentioned above, lymphoblastic cells contain identical Ig gene rearrangement patterns. Several routine diagnostic criteria for lymphoma detection exist in histopathology laboratories; however, these are not suitable for all cases with B-cell lymphoma. Use of the molecular genetics approach clonality testing in assumed B-cell malignancies has been proposed to be a reliable method for atypical subjects PCR [13][26].

The BIOMED-2 system can increase the sensitivity and specificity of the detection os lymphocytes clone hyperplasia with the rearrangement of the IG gene. The use of PCR to detect the gene of the IG receptor has relatively low requirements in terms of quality and DNA concentration of the template but showed a high sensitivity of 1% -5% to detect the rearrangement of the tumor cells [12]. However, this technique can be important in early detection of lymphoma. With this technique using BIOMED-2 multiplex, PCR the rate of early diagnosis of lymphoma increased significantly [23]. In this study, BIOMED-2 was used to clonality analysis of IGH gene rearrangements (framework I, II, III) to avoid false negative samples, in 55 samples of paraffin-embedded lymphoid tissue, divided into 36 lymphoid lymphoma and 19 samples of reactive lymphoid hyperplasia. Twenty-six of the non-Hodgkin's from 37 case showed a positive result for IG gene monoclonal rearrangement (70%), which is less than proportion 72% for study [30] and 77%, 80%, 96% [31][23][32] and higher than [33] by 63%. No positive

result was shown in 10 samples. In this study, a positive result was obtained 13/37 by (35%) for FR3, less than the previous study 36% [32], 44% [30], 42% [33], and 10/37 – (27%) for the FR2 area, which is lower than the previous studies 36% [33], 32% [32], 5 / 37- 13% FR1, which is lower than the previous 24% of studies [30][32].

DLBCL

For the DLBCL type, 10 samples were obtained with positive results out of 15 samples (66%), less than 95% [32] and 80% for the study [23] and 67% for the study [34] and higher than the study of 50% [31] and study of 61% [33] and the same proportion of 66% for study [30] in addition to one sample was obtained out of 15 (6%) for the region FR1, which is lower than the previous studies [32][31][35] and higher than 0% of the study (31), while for the FR2 region was Obtain 5 samples with a positive results out of 15% by 33%, which is less than 40% for study [32] and 50% for the study of [31]. In the FR3 region was obtained 5 samples out of 15 samples by 33%, which is lower than the previous studies 35% [32] and 50% for study [31] and higher than 16% for study [30].

FL

For FL type, 3 samples were obtained with a positive results out of 3 samples by 100%, which is the same as the percentage of study [32][23] and higher than 60% for [30] and 72% for study [33]. The FR1 region did not have a positive result, which is less than the study of 50% [32] and 30% for the study of [31] and the same percentage of study [30]. In the FR2 region was obtained 2 samples with a positive result of 3 (66%), which is higher than the previous studies [32][30] and [31]. The FR3 region was obtained with 2 samples with a positive result out of 3 by 66% higher than 50% for study (32), 60% for study (30) and 13% for study by [31].

SLL

In the SLL type, 3 samples were obtained with positive results of 4 samples by 75%, less than 81% for [33] and 100% by [31] and higher than 50% of study [30]. In the FR1 region didn't show positive results it was the same as the proportion of study [30] and which was lower than the previous study [33], [31]. In the FR2 region, one sample out of 4 by 25% was obtained as a positive result less than 75% for study [36] and 100% for study [31]. In the FR3 region 2 out of 4 was obtained as positive results by 50% it were the same as a study (Stanek et.al 2014) and less than 87% of study [37] and study 100% [31].

NMZL

For the NMZL type, 2 samples were obtained with a positive results out of 2% by 100%, higher than 28% for study [23] and 83% for study [33]. In FR1 region one case showed a positive result out 2 by 50%, more than 28% for the study of [23] and in FR2 region one case out 2 showed a positive result by 50%, which is less than 70% for the study [37] and no positive results was showed in the FR3 region that were less than the study of [37]

MALT

For the MALT type, one sample was obtained with a positive result of 2% by 50% less than 82% of study [23] , 100% [32] and 60% for [33]. In the FR1 region one sample, out 2 was obtained as monoclonal by 50%, higher than the previous study by 33% of study [32] and higher than 0% of study [30]. The FR2 region did not have any positive results same of study [32] and less than 50% [30] and 18% [23]

The FR3 region did not show a positive result, less than 66% of the study of (32) and the results were consistent with [30], which did not show positive results in this region.

BL

For BL type, 2 samples were obtained with positive results from 3 samples (66%) in the just FR3 region higher than 37% for the study of [33].

BCL

In the case of BCL type, the specimens were classified as non hodgkin's only, not diagnosed to the specific type. 5 samples were obtained with positive results out of 8 by 62%, In the FR1 region, there were 2 out of 8 by 25%. In the FR2 region, one positive result was shown out 8 (12%). In FR3 2 samples showed a positive result out 8 cases by 25%. In this study, the Source of DNA was obtained from the FFPE blocks and which obtained 26 samples as a Monoclonal results and these including all species used in this study and 10 samples showed a polyclonal or did not showed any bands or bands that were not specialized that called "false negatives" This was because DNA extracted from FFPE was low purity and DNA extracted from FFPE may affect to PCR process, including several factors such as thickness of the cutting tissue, or the place of cutting tissue which may be not in the location of the tumor cell , the method of embedding process by paraffin , the time of embedding process, the type of paraffin used in the process of embedding and the DNA extraction procedures such xylene or some Materials such as (Marin oil) and the use of high temperatures in wax dissolving that may inhibit PCR [30][38][39]. Also, DNA safety depended on the duration of keeping samples in the hospital before PCR analysis and that the best results were shown when using blocks with a maximum duration of two years [40][30]. The DNA extracted from FFPE was somewhat degraded or broken or short and to solve this problem the primers were designed by BIOMED 2 in Less than 400bp [13][41]. Hence it is necessary to evaluate the quality, integrity, and amplifiability of the DNA extracted from FFPE tissues using a multiple control gene PCR [13][42]. A further obstacle to the successful analysis was the limited number of scattered B-cells in a background of many polyclonal reactive cells masking the monoclonal population [43][44][45]. The other reason for non-appearance of bands or negative result is because of an important factor was the type of somatic hypermutation (SHM), which occurs in most of the B cell lymphoma that was founded in the germinal center, such as DLBCL and FL occurring in germinal center / post-germinal center (GC) (PGC), and these tumors had a somatic mutation in the IGH gene [46][47].The somatic mutation interfered with complementarity-determining (CDR) region for IG gene which allows the process of affinity maturation of the antibodies to occurs that were targeted the antigens by B cell. This process also occurs in the region of framework (FR) found in IGH gene , which most sequences and

primers are designed for this region [48] [49] and contain a large number of mutations in the body of the cell [31]. This leads to a reduction the ability of the primer to binding in the DNA and makes it substandard and due to the loss of target sequences for binding to primers or changing the sequence of the target region by the primer [35]. The other reason is the inappropriate annealing of the primers to the PCR chain relative to the sequence of rearranged nitrogen bases [13], but this problem was solved somewhat by BIOMED-2 to design multiple primers for each of the FR regions with 7 primers of VH for each region of the FR [13]. Another reason why it is impossible to achieve a 100 % success rate in detecting monoclonality of IgH and TCR genes in lymphomas is the heterogeneity of the targeted DNA sequence. To avoid possible non-homology, more sets of primers should be applied [50]. Furthermore, negative results may be caused by unusual IgH gene configurations that may arise from partial or incomplete rearrangements or arise in IGL, IGK [15] and by chromosomal translocations involving the JH germline gene segment on chromosome 14 [51][52]. The other problem is the appearance of pseudo-positive bands and there are several ways to solve this problem, such as analyzing by GeneScan (GS) or by analyzing by polyacrylamide heteroduplex (HD) [52], or by using sequence technique after the PCR process [53]. Several studies have used GS technique [54][26]. In this study, the heteroduplex technique was used. A heteroduplex analysis is a simple, fast and inexpensive method to analyze PCR products in the case of a rearranged Ig gene. In a heteroduplex analysis, PCR products are denatured at a high temperature at 95°C degrees and subsequently recombined by cooling them at 4 to stimulate the homo-hetero duplex formation, which is necessary to separate the monoclonal bands into polyclonal backgrounds detected by nondenaturated polyacrylamide gel 6 %. In this study, the electrophoresis was used in the form of polyacrylamide gel (PAGE) instead of agarose gel in order to provide sufficient accuracy and enhance the detection of the dominant regions within the polyclonal swathes [55]. The use of PAGE Gel instead of agarose Gel for many reasons, the PAGE can absorb larger amounts of DNA without significant loss in quantity. The DNA recovered from the polyacrylamide gel is extremely pure, chemically stable, cross, good for separation of low molecular weight, chemically stable via gel binding [56]. Many studies also found more accurate and better results when using PAGE gel instead of agarose [57] [58]. Also, when using the heteroduplex technique, the single-stranded or mutation sequences are detected by the PAGE gel, which is not readily detected by agarose gel [59]. In conclusion, the used of joined three IGHFR1, FR2 and FR3, rearrangements to assay the clonality of B-cell NHL, has demonstrated a good diagnostic tool for the finding of NHL, especially in patients without a clear morphological status of lymphoma.

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