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Association of t (14; 18) Chromosomal Translocation to Hodgkin's Lymphoma in Iraqi Patients Using Multiplex PCR Technique

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Abstract: Translocation t (14; 18) (q32;q21) is a common cytogenetic abnormality in B- cell lymphoma, especially in non-Hodgkin's lymphoma, but it is rarely seen in Hodgkin's lymphoma (HL). In the current study, t(14; 18) chromosomal translocation was detected in Hodgkin's lymphoma of Iraqi patients by multiplex PCR with multiple primers and direct sequence by Sanger method with Agencourt® CleanSEQ® dye-terminator Removal Kit. DNA was extracted from 50 samples of paraffin- embedded tissue, 25 samples of blood by using QIAamp DNA Mini Kit (Qiagen /Germany) and 10 samples from reactive hyperplasia as control. The t (14, 18) was detected in 26(35%) cases that showed IGH/BCL2 gene rearrangement with a significant difference compared to control ($P<0.01$) or hyperplasia. The detected breakpoints represent as 5(19.2%) for major breakpoint MBR1, 4(15.38%) for MBR2 and 3(11.5%); 2(8%); 7(27%) for 3′MBR1, 3′MBR2 and 3′MBR4 respectively. Break points of MCR1, MCR2, 5′MCR were detected as 4(15.38%); 1(4%) and 2(8%)) respectively. On the other hand, no breakpoint was detected for 3′MBR3. Thus, this study reported an increased frequency of breakpoints in MBR regions in HL patients than mcr cluster and an increase in t (14; 18) translocation in adult's age and in male gander.

In conclusion t (14;18)(q32;q21) was detected in B- cell lymphoma by multiple primers for all three breakpoint regions, the MBR, 3′ MBR and mcr with multiplex PCR amplification

Key words: Hodgkin's lymphoma , t(14;18), IGH/BCL2, MBR.

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Introduction

Lymphoma refers to a general name for cancers that develop in the lymphatic system specifically of T or B lymphocyte cells (1). There are two main types of lymphoma: non-Hodgkin's lymphoma and Hodgkin's lymphoma (2). Hodgkin disease represents approximately 11% of all lymphomas in Western countries and has a unique bimodal age distribution that differs both geographically and ethnically. In developing countries, the early peak occurs before adolescence (3,4).

Hodgkin lymphoma is distinguished by strange- looking cells which are giant

multinucleated looking a bit like "owl's eyes" and known as the Reed – Sternberg cell (5). Pathologists have found another large mononucleotide cells unlike reed Sternberg cells with "no owl eyes" under microscope called Hodgkin cells. Collectively they referred as Hodgkin and Reed-Sternberg cells (HRS) (6,7). There are different types of Hodgkin lymphoma depend on the cells which seen under a microscope, certain abnormal proteins and different clinical presentations. These types are classical Hodgkin lymphoma (Nodular sclerosis Hodgkin lymphoma; Lymphocyte - rich classical Hodgkin lymphoma; Mixed cellularity Hodgkin's lymphoma; Lymphocyte-depleted Hodgkin lymphoma) and Nodular lymphocyte predominant Hodgkin lymphoma (8).

In t (14;18) translocations, the BCL-2 gene from chromosome 18 becomes fused with the immunoglobulin heavy-chain locus on chromosome 14, bringing juxtaposes of the BCL2 gene on 18q21 with the enhancer region of the IGH gene from chromosome 14q32 producing abnormal expression of the BCL2 protein in mature B lymphoid cells (9). Many types of lymphoma exhibit translocations in the genome and this has given much information about the genes involved in lymphoma genesis. The two genes located at the breakpoints are rearranged to form different fused proteins which indicate that different types of this rearrangement were exist in this translocation (10,11). The rearrangements can cause increase Bcl-2 expression which caused resistance to chemotherapeutic drugs, radiation therapy and accumulation of Go cells contribute to chemo resistance (12) or decrease Bcl-2 expression which may promote apoptotic responses to anticancer drugs. The Bcl-2 expression in Hodgkin's lymphoma is frequently observed and almost 54% from classical Hodgkin's lymphoma cases revealed a variable proportion of HRS cells Bcl-2 positive (12,13).

The detection of t(14;18) translocation involves the hot spots regions of the Bcl2 gene in chromosome 18 which includ the major breakpoints (MBR) located in the 3′ untranslated region of Bcl2 exon 3 and the minor cluster region (mcr) located in the 3′ region of Bcl2 exon 3 and the IGH gene located at chromosome 14q32.33 which included the breakpoints that are predominantly within the joining region (JH) of the IGH gene (14,15,16).

Material and Methods

The study included 75 Iraqi patients with Hodgkin's lymphoma (44 males and 31 females). The samples include 25 blood samples and 50 samples as formalin fixed paraffin embedded (FFPE) lymph nodes biopsy tissue blocks as well as 10 samples as reactive hyperplasia blocks for control (5 males and 5 females). The blood samples were collected from hematology unit in Baghdad hospital in medical city but paraffin blocks tissue were collected from histopathological laboratories in different hospitals during the period from September 2013 till March 2014. The DNA was extracted from samples using QIAamp DNA Mini Kit (Qiagen /Germany) as described by manufacturer's protocol.

To detect the involvement of BCL2 breakpoint regions in the t (14; 18), nine forward primers were used for bcl2 regions and one reverse primer for JH gene and mixed in three pair of primers. All primers were designed using NCBI and blast system. All DNA extracted

amplified by PCR which performed by using three pairs of primers to detect the MBR, 3′ MBR, mcr region as shown in (Table 1) (17,18) as well as positive controls for each breakpoints. DNA extraction and PCR assays were carried in the Molecular Oncology Unit lab of the Guy's and St. Thomas' NHS Foundation Trust Hospital/London/ United Kingdom.

Table (1): Primer sequence for t (14; 18) assay

Primer name	Sequence (5′-3′)	tube
MBR1(Forward)	CCATAGATTTGAATCTGCTGGTC	t(14;18) tube A (MBR)
MBR2(Forward)	ATATAATGCAATAATGCCACAGAGT	
JH (Reverse)	GGTCACCGTCTCCTCAGGTAAG	
3′ MBR1 (Forward)	CAGTGTTGTATCCAGCAGGTGC	t (14;18) tube B (3′ MBR)
3′ MBR2 (Forward)	GCCACCACACCCTGCTAGTTT	
3′ MBR3 (Forward)	AAGATTTGCTCCCCAGTCATTAC	
3′ MBR4 (Forward)	TCTCTAAACCACGCCAACCAGT	
JH (Reverse)	GGTCACCGTCTCCTCAGGTAAG	
5mcr (Forward)	TGCTTTCGTTTCTTTCAGAAGG	t(14;18) tube C (mcr)
mcr1 (Forward)	TATTTATTGGGCGCTTGCTCA	
mcr2 (Forward)	TTGGATTTGAGATGGCATTCA	
JH (Reverse)	GGTCACCGTCTCCTCAGGTAAG	

MBR: Major breakpoint(Bcl2), JH: Joining region(IGH), mcr: minor cluster region(Bcl2)

All DNA samples were examined for t (14, 18) the amplification was carried out by using HotStarTag®Master Mix Kit (Qiagen / Germany). The amplification condition was given in table (2).

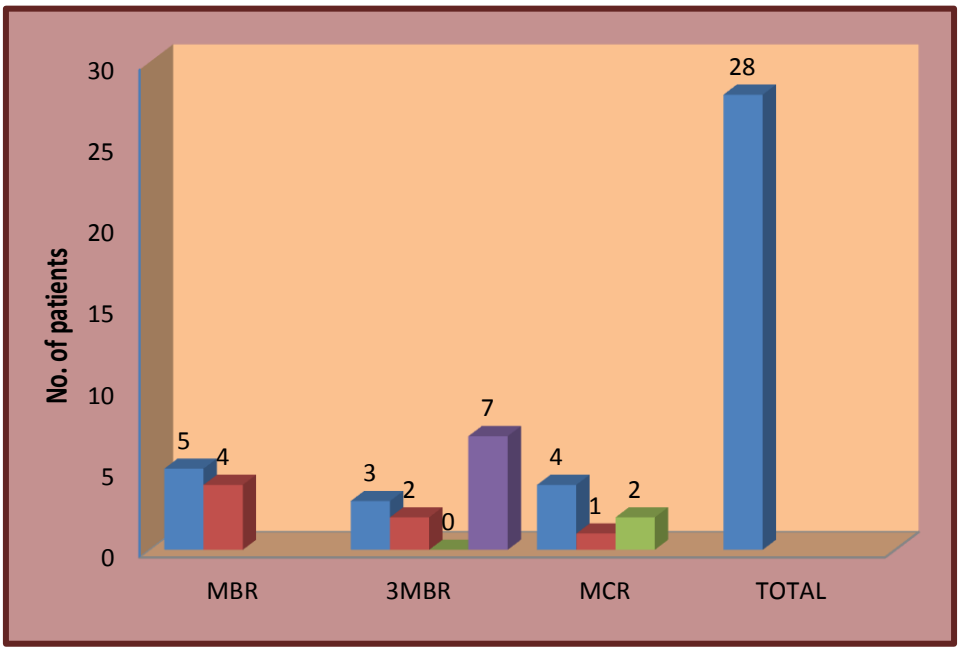
Table (2): Amplification condition used for t (14, 18) assay

Step of PCR protocol	Temperature	Time	Number of cycle
Initial Denaturation	95^{O}C	10 min	1 cycle
Denaturation	94 ^{O}C	45 sec	35
Annealing	60 ^{O}C	45 sec	35
extension	72 ^{O}C	1.30sec	35
Final extension	72 ^{O}C	10 min	1 cycle

The PCR products were directly loaded on 2% agarose gels and stained with ethidium bromide together with DNA ladder marker (100 bp). The DNA bands were visualized by UV transillumination. The presence of different bands according to the control and ladder indicated that positive translocation whereas the absence of bands indicated that normal or negative results for t (14;18). The different positive bands which appeared in the gel were cut and purified from gel by QIAquick gel extraction kit (Qiagen/ Germany) then applied for sequence analysis to know where breakpoints occurred and subjected for genotype analysis by using Sanger method with Agencourt® CleanSEQ® dye-terminator Removal Kit for direct sequence analysis to detect the type of breakpoints.

Results and Discussion

The frequency of bcl2 t (14;18) chromosomal translocation was detected successfully in comparison with positive control by using multiple primers defined multiplex PCR and direct sequence. The results showed that there were 26(35%) patients positive for t (14;18) assay with high significant differences compared with control ($P<0.01$). The results also showed a high degree of translocation rate in Hodgkin's lymphoma patients at MBR and low frequency at MCR region as shown in figure (1).



MBR: Major breakpoint(Bcl2), JH: Joining region(IGH), mcr: minor cluster region(Bcl2)

Figure (1): The frequency of breakpoints in the Hodgkin's lymphoma (HL) patients

Out of 26 positive patients to t (14;18) translocation, (25) were detected in tissue samples and one in blood samples. Of 26 positive samples, MBR1 and MBR2 break points were detected in 5(19.2%) and 4(15.38%) samples respectively. 3MBR1, 3MBR2 and 3MBR4 break points were detected in 3(11.5%), 2(8%) and 7(27%) of samples) respectively. The break points MCR1, MCR2 and 5MCR1 were detected in (4(15.38%), 1(4%) and 2(8%) of samples respectively. On the other hand, no 3MBR3 break point was detected in all samples and no break point was detected in reactive hyperplasia as shown in table (2). The results also showed that two patients were with two break points each.

Table 2: The frequency of t (14;18) translocation breakpoints in the HL patients

Patient/ Sample	Mbr1	Mbr2	3mbr1	3mbr2	3mbr3	3mbr4	Mcr1	Mcr2	5mcr1
1 blood	+	+	-	-	-	-	-	-	-
2 tissue	-		-	-	-	-	+	-	-
3tissue	-	-	-	-	-	+	-	-	+
4tissue	-	-	-	-	-	-	+	-	-
5tissue	-	-	-	+	-	-	-	-	-
6tissue	-	+	-	-	-	-	-	-	-
7tissue	-	-	-	-	-	+	-	-	-
8 tissue	-	-	-	-	-	-	-	-	+
9 tissue	+	-	-	-	-	-	-	-	-
10 tissue	+	-	-	-	-	-	-	-	-
11 tissue	-	-	-	+	-	-	-	-	-
12 tissue	-	-	-	-	-	+	-	-	-
13 tissue	-	-	+	-	-	-	-	-	-
14 tissue	+	-	-	-	-	-	-	-	-
15 tissue	-	-	-	-	-	+	-	-	-
16 tissue	-	+	-	-	-	-	-	-	-
17tissue	-	-	-	-	-	+	-	-	-
18 tissue	-	-	+	-	-		-	-	-
19 tissue	-	-	-	-	-	-	+	-	-
20 tissue	-	-	-	-	-	-	+	-	-
21 tissue	-	+	-	-	-	-	-	-	-
22tissue	-	-	-	-	-	-	-	+	-
23 tissue	-	-	+	-	-	-	-	-	-
24 tissue	+	-	-	-	-	-	-	-	-
25 tissue	-	-	-	-	-	+	-	-	-
26 tissue	-	-	-	-	-	+	-	-	-
control	-	-	-	-	-	-	-	-	-
ratio	19.2 %	15.38 %	11.5%	8%	0%	27%	15.38 %	4%	8%
Chi-square value	8.694 **								
**(P<0.01)									
+ : Translocation existence									

The total of positive cases were 26 but the total of breakpoints were 28 because some samples were noticed more one breakpoint among three regions and in the same region some samples appeared more one breakpoints therefore in the gel electrophoresis appeared more than one band in the same sample (figure 2).

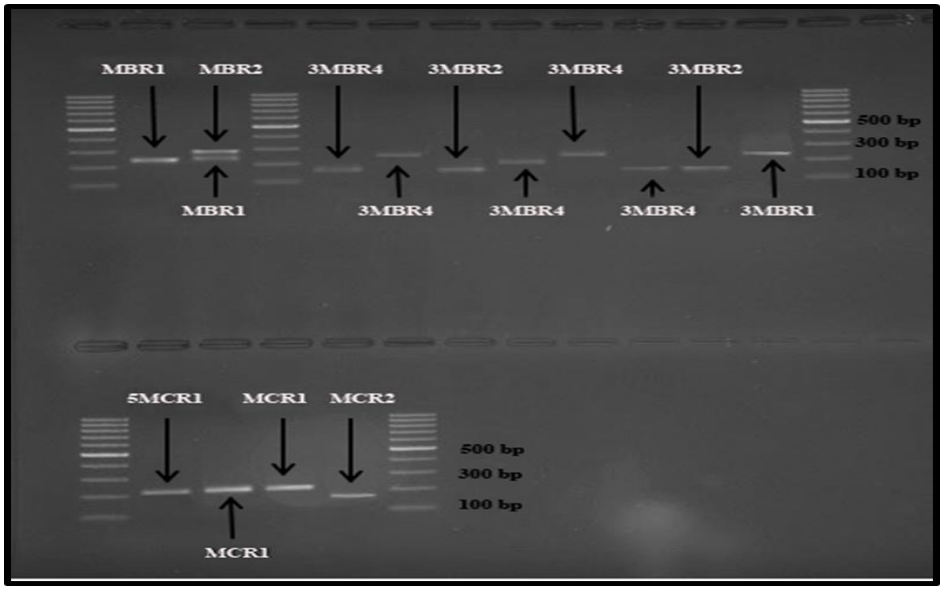


Figure 2: The positive cases of HL samples for t (14, 18) translocation. The PCR samples were electrophoresed on 2% agarose gel for 45 mins, stained with ethidium bromide and visualized by UV

The association of HL patient ages and translocation t (14, 18) was as in table (3). The results showed an increase in frequency 20 (77%) of t (14, 18) in patients with age ranged from (55-76) years. The results also showed an increase in translocation frequency among male HL patients 18(69.2%) than females table (4).

Table3: Distribution of study samples according to age group

Age (years)	patients		control	
	No.	%	No.	%
<19	7	9.33	1	10
20-29	16	21.33	1	10
30-39	5	6.7	1	10
40-49	3	4	3	30
50-59	11	14.70	2	20
60-70	19	25.33	2	20
>70	14	18.66	0	0
Total	75	100	10	100
Chi-square value	---	8.927**	---	9.753**
** (P<0.01)				

Table 4: Distribution of study samples according to gender

Gander	Hodgkin's disease		Control	
	No.	Ratio %	No.	Ratio %
Male	44	58.67	5	50
Female	31	41.33	5	50
Total	75	100	10	100
Chi-square value	---	6.829 **	---	0.00 NS
** (P<0.01), NS: Non-significant.				

The positive breakpoints t (14;18) patient samples were sequenced. The results analyzed by blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The results showed that the patient breakpoints were with different size of breakpoint as compared with the sequence of the positive control (table 5).

Table 5: The Hodgkin's lymphoma patients t (14;18) breakpoint size

Mbr1	Mbr2	3mbr1	3mbr2	3mbr3	3mbr4	Mcr1	Mcr2	5mcr1
217bp	250bp	176bp	181bp	0	220bp	261bp	134bp	233bp
207bp	171bp	180bp	150bp		181bp	256bp		185bp
149bp	180bp	219bp			171bp	179bp		
156bp	195bp				128bp	180bp		
145bp					141bp			
					244bp			
					132bp			
5	4	3	2	0	7	4	1	2

After the sequence analysis for all 28 breakpoint sequence and compared with NCBI by (http: // nblast. ncbi. nlm. nih. gov/Blast.cgi) and compared with construct fragments sequence (positive control). The breakpoints sequence that its product size equal 200bp or more that were submitted to the **NCBI** (National Center for Biotechnology Information) site for registration in the **Gene Bank database** and the results were registration in the (DNA Data

Bank of Japan) **(DDBJ)** also we registrate the results in the **EMBL** (European Molecular Biology Laboratory) under title (**Homo sapiens DNA, IGH/BCL2 transition region, isolate: hodgkins-bcl2-t(14-18) mbr-p.2**). Gene bank database ID number (accession number) specific for these results were also given which is begin from **LC041134 to LC041142**) and considered as a document reference about Iraq people data that is little in Gene Bank database.

Malignant lymphoma is a cancer that arises from lymphoid cells of either B-cell or T- cell. B lymphoma form approximately 90-95% of all lympho-proliferative disorder(18). According to the Iraqi Cancer Registry in 2010, the most common tumors are the breast cancer followed by cancers of the lymphatic system, lung, brain and bladder. Childhood cancer is 8-10 times more common than in the west (19). The major causes or pathogenesis of Hodgkin's disease is thought to the escape of Hodgkin's and Reed-Sternberg (HRS) cells from apoptosis (programed cell death) in molecular analysis of HRS cells several pathogenic mechanisms related to the anti-apoptosis. The major anti-apoptotic mechanism found in B- cell lymphoma is the deregulated expression of proto-oncogene (BCL2) that occurred by t (14; 18) (20).

The frequency of IgH/BCL-2 gene rearrangements from our HL patients was compared with data from different geographic regions. These results agreed with the results reported by Noriega *et al.*, (21) who noticed that t (14,18) translocation in the MBR regions was higher than another breakpoint regions but disagreed with the results of Al-Qaysi *et al.* (22) who reported that the high frequency of breakpoints in MCR region of BCL2 gene also our results not compatible with the results of Alijani *et al.*, (23) who observed that the high frequency of t (14,18) occurred in the MCR region (38% from 43% positive) while in the MBR region reported low frequency (2% in MBR and 3% in 3MBR from 43% positive cases).

In the present study, correlation of age, gender and the occurrence of t (14,18) translocation was observed, which is similar to the results of Alijani *et al*., (23) which was carried out on Iranian population and similar to the results of studies from western countries (Montoto *et al*., (24); Harris *et al*., (25)) that were carried out on the western population, the studies noticed that t (14,18) increased with age adult and a higher incidence of this translocation was reported in males as compared to females (60%). The results of direct sequence analysis of the product reported the highest possible degree of accuracy and reliability and the results of alignments all perfectly matched with DNA sequence anticipated. The differences in percentages of bcl2 t(14,18) translocation detected in the present study compared with data from other studies might be attributed to variations in histological diagnosis, the absence of standardized primers used, environmental factors, the type of sample is another important source of variation since different studies used different tissue specimens, whether fresh, frozen or paraffin embedded also the quality and sensitivity of the techniques used in these studies may be effect on the differences among studies (26). Despite there were several studies on the molecular cloning of the t (14;18) chromosomal breakpoint, the precise mechanism by which this inter chromosomal recombination occurs

remained unresolved. Little is known about the mechanisms leading to tumor progression and resistance to therapy.
The challenge in the treatment of HL patients today is not only to achieve cure, but also to avoid long- term toxicity (27). The chromosomal translocation is believed to be a specific marker of the malignant clone and a hallmark of the disease. It is therefore, widely used for diagnosis and for monitoring disease progression, minimal residual disease, and response to therapy (26).

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