Impacts of *bcl2* t(14,18) chromosomal translocation, and EBV *lmp-1* genes on Non-Hodgkin lymphomas

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Abstract : The main objectives of this study are to determine the frequency of *bcl2* t(14;18) chromosomal translocation, and EBV *lmp-1* in NHL Iraqi patients , also to determine the correlation of *bcl2* translocation with EBV *lmp-1* gene. To achieve the intended aims a 46 formalin fixed paraffin embedded (FFPE) blocks were examined included 32 blocks from NHL patients, 10 blocks from reactive follicular hyperplasia, and 4 tonsils as a control group for *bcl2* translocation. Genomic DNA was extracted from these blocks and amplified by polymerase chain reaction (PCR) by using oligonucleotide primers specific for opposing sides of the *IgH* gene rearrangement on chromosome 14 towards the major break point region (MBR) of the *bcl2* gene on chromosome 18 and conserved *JH* sequence on chromosome 14. In addition to specific primers used for EBV *lmp-1*detection. High degree of translocation rate at MBR region (96.9%, and 100%) in NHL and reactive follicular hyperplasia respectively. The present results illustrated positive results of EBV *lmp-1* (43.8%) among 14 (32) NHL, while low percent was shown in reactive follicular hyperplasia 10.0% (1 out of 10). Current results also, revealed that all cases with positive results of EBV have *bcl2* chromosomal translocation with no significant correlation observed between them. This study favored the significant role of *bcl2* t(14;18) and EBV *lmp-1* genes in pathogenesis of lymphoma.. Furthermore, the *bcl2* t(14;18)chromosomal translocation independent of EBV infection.

Key words- Non-Hodgkins lymphoma- *bcl2* t(14;18) gene- EBV *lmp-1* gene.

Introduction

Lymphoma is a cancer of the lymphocytes, a type of white blood cells occurs when cells grow abnormally without of control. Traditionally, two main groups of lymphoma have been distinguished: Hodgkin's Lymphoma (HL) characterized by large polynuclear cells called Reed Sternberg cells, and Non-Hodgkin's lymphoma (NHL)[2].

Non-Hodgkin's lymphomas (NHLs) are the hematologic malignancy with the highest prevalence worldwide. They are broadly classified as B cell or T-cell lymphoma depending on which type of lymphocyte becomes cancerous, B-cell lymphoma is more common than T-cell lymphoma. Also NHL is classified as either slow growing (known as low grade or indolent lymphoma) or fast growing (known as high grade or aggressive lymphoma) [3]. The exact cause of NHL remains unknown. However,research have focused some factors that may contribute to the development of lymphoma including genetic factors, impaired immune system, viruses such as, Epstein Barr Virus (EBV), bacterial causes (e.g. Helicobacter pylori), exposure to chemicals, and heavy smoking [5].

Human *bcl2* gene is a proto-oncogene located on the chromosome 18. Its product is an integral membrane protein called Bcl2, located in the membranes of the endoplasmic reticulum (ER), nuclear envelop,

and in outer membranes of mitochondria [6]. The Bcl2 isexpressed during B-cell maturation and has a central role in the inhibition of apoptosis that modulates the mitochondrial release of cytochrome c, and interaction of apoptosis activating factor. Altered of Bcl2 and p53 protein expression involved in lymphomagenesis[7]. Chromosomal translocations have been observed in up to 90% of NHL cases [8]. These translocations, with or without additional genetic lesions, can precipitate the activation of oncogenes or inactivation of tumor suppressor genes [9]. An important example is thet(14;18)(q32;q21) which leads to constitutive activation of the bcl2 oncogene by the enhancers of the immunoglobulin heavy chain locus(IgH). This chromosomal translocation is typically present in tumor cells of follicular lymphoma but can also be found in other types of NHL [10]. The clustering of the breakpoints on chromosome 18 with or near the *bcl2* oncogene facilitates the application of polymerase chain reaction (PCR) to amplify this *bcl2/JH* breakpoint fragment [11]. Among the most extensively characterized human tumor viruses is EBV, which has been postulated as a risk factor for NHL in the general population [12]. The EBV implicated as a causative agent in certain B-cell lymphoid diseases including endemic Burkitt's lymphoma, gastric lymphoma, aggressive lymphoproliferative disease seen in people who have severe immunodeficiency [13-15], Human Immunodeficiency Virus(HIV)-related lymphomas [16], and lymphomas in the elderly [17]. It has mutagenic characteristics for B lymphocytes cause lysis and destruction on these cells, and cause of the ploy-colonal stimulation of cells, eventually transformation to lymphomas [18].

Aims of the study

This study was designed to investigate a group of NHL Iraqi patients to study the impacts of *bcl2*, and EBV *lmp-1* genes as genetic factors that contribute to development of lymphoma

Subjects and methods

The subjects included in this study were represented as formalin fixed paraffin embedded (FFPE) biopsy tissue blocks from nodal and extranodal sites that were obtained from patients who had undergone surgical operation done from them. The collection samples of this study were carried out during the period from July 2012 to May 2013.

These blocks were distributed on the following groups.

1. Thirty two from non-Hodgkin's lymphoma.
2. Ten blocks from reactive follicular hyperplasia.
3. Four tonsils used as a control group for detecting *bcl2* translocation.

The ages of NHL patients ranged between 3-81 years with median age 55 years, and mean ± SD equal 45.718± 23.51 years. The NHL patients enrolled in this study consist from 11 males and 21 female. All biopsies tissue of NHL was taken before treatment.Genomic DNA was extracted from sections of FFPE blocks by using PureLink Genomic DNA Mini kit (Invitrogen).

The *bcl2* t(14;18) chromosomal translocation and EBV *lmp-1* gene were detected by PCR-amplification performed in a programmable thermal cycler gradient PCR system.

The primers sequence of *bcl2/JH* and EBV *lmp-1* used in this study are illustrated below (tables 1, and 2).

Table (1): Sequence of primers used for PCR amplification of bcl2/JH genes.

Primers	Sequence	Size Product	References
MBR JH	5'-TTAGA GAGTT GCTTT ACGTG-3' 5'-ACCTGAGGAG ACGGT GACCA GGGT-3'	220 bp	19-20

MBR- major breakpoint region

Table (2): Sequence of primers used for PCR amplification of EBV *lmp-1 gene*.

Primers	Sequence	Size Product	References
Forward	5'- ATTTATTTTTGCTTGCCATT -3'	*190 bp*	*21-22*
Reverse	5'- GTCTGTCTGTCTGTCCGTCA -3'		

PCR conditions

Genomic DNA was amplified in a final volume of 20 µl (5 µl Genomic DNA+ 3 µl MBR or *EBV lmp-1* forward primer + 3 µl revers primer +5 µlBioneer's master mix with Green Taq DNA polymerase + 4 µl DDW) using the **following conditions of bcl2**: Denaturation at 94 $^{\circ}$C for 4 min. followed by 45 cycles of (denaturation at 94 $^{\circ}$C for 45 seconds, annealing at 55 $^{\circ}$C for 45 seconds. and extension at 72 $^{\circ}$C for 1 min. and a final extension was at 72 $^{\circ}$C for 5 min. then hold at 4 $^{\circ}$C for indefinite time. Then the amplification products were separated by electrophoresis through 1.5% agarose gel stained with ethidium bromide.

While conditions required for EBV are Denaturation at 95 $^{\circ}$C for 5 min. followed by 35 cycles of (denaturation at 95 $^{\circ}$C for 30 seconds, annealing at 58 $^{\circ}$C for 30 seconds. and extension at 72 $^{\circ}$C for 1 min. and a final extension was at 72 $^{\circ}$C for 5 min. then hold at 4 $^{\circ}$C for indefinite time. Then the amplification products were separated by electrophoresis through 1.5% agarose gel (2%) stained with ethidium bromide (0.5 µg/ml).

Statistical analysis

The data were analyzed using SPSS statistical software (SPSS version 16). $P < 0.05$ was considered statistically significant. The distribution and comparison of each was made using the Chi-square test.

Results

♦ **Frequency of *bcl2* t(14,18) chromosomal translocation at MBR region.**

The result of *bcl2* t(14, 18) chromosomal translocation frequency in studied groups were illustrated in table (3). We observed there were a surprising high degree of translocation rate at MBR region (96.9%, and 100%) in NHL and reactive follicular hyperplasia respectively. Our results also did not recorded any positive result at MBR in tonsils (control group). The current results showed highly significant differences (p<0.00) between patients and control group, The result of PCR for detection of   t(14, 18) *bcl2* translocation in studied groups are reveal in figures (1, and 2). The positive results detected in 220 bp for MBR.

Table (3): Frequency of *bcl2* t(14,18) chromosomal translocation at MBR region .

			MBR *bcl2* translocation		
			N	P	Total
Groups	NHL	Count	1	31	32
		% within groups	3.1%	96.9%	100.0%
		% within MBR			
	RFH	Count	0	10	10
		% within groups	.0%	100.0%	100.0%
		% within MBR	.0%	24.4%	21.3%
	Tonsils	Count	4	0	4
		% within groups	100.0%	.0%	100.0%
		% within MBR	80.0%	.0%	8.5%
Total			5	41	46
		% within groups	10.6%	87.2%	100.0%
		% within MBR	100.0%	100.0%	100.0%

- MBR: Major breakpoint region
- NHL: Non-Hodgkin's lymphoma
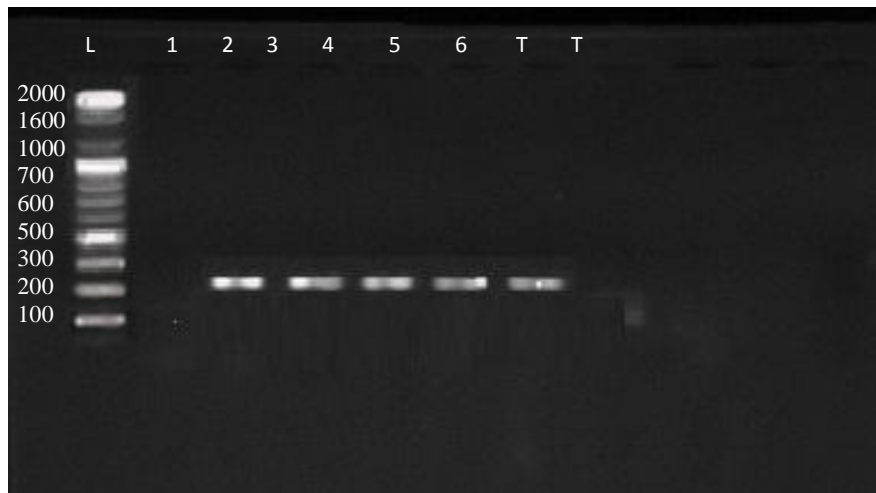- RFH: Reactive follicular hyperplasia
- P value <0.000

Figure (1): The PCR amplified products of *bcl2* translocation on agarose gel (1.5%), 70 volt for 60 minutes. Bands in the size region 220 bp are indicative of the *bcl2* t(14;18) chromosomal translocation of NHL samples.

Lane L: 100 bp ladder, T: Tosile

Lane (2-6) shown positive result of NHL while negative result show in T lanes at major break point region.
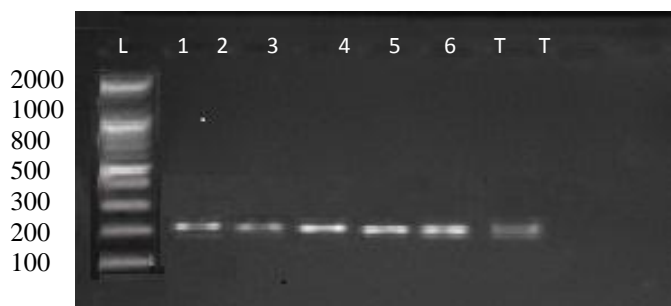

Figure (2): PCR followed by gel electrophoresis for detection of *bcl2* t(14;18) in reactive follicular hyperplasia patients using a 100 pb DNA ladder in lane (L).The positive results at major break point region indicated in lanes 1– 6, the negative results shown in lane (7-8) for (T) tonsils.

♦ **Frequency of EBV *lmp-1* gene in studied groups**

The EBV *lmp-1* detected by conventional PCR in all tissues of studied groups. The present results illustrated positive results of EBV *lmp-1* (43.8%) among 14 (32) NHL, while low percent was shown in reactive follicular hyperplasia 10.0% (1 out of 10) as shown in table (4). Statistically there were no significant differences (0.052) between studied groups. Otherwise, present results revealed that *lmp-1* clinically consider as a risk factor in NHL development with risk estimate equal to 4 and (95%CI=0.654-29.264). The outcome of amplification of DNA samples of EBV *lmp-1* with selected forward and reverse primer was 190 base pair band which is our target, as in figure (2) illustrate.

Table (4): Frequency of EBV *lmp*-1 in studied groups.

			EBV *lmp*-1		
			N	P	Total
Group	NHL	Count	18	14	32
		% within group	56.2%	43.8%	100.0%
		% within EBV	66.7%	93.3%	76.2%
	RFH	Count	9	1	10
		% within group	90.0%	10.0%	100.0%
		% within EBV	33.3%	6.7%	23.8%
Total		Count	27	15	42
		% within group	64.3%	35.7%	100.0%
		% within EBV	100.0%	100.0%	100.0%

- EBV: Epstein Barr Virus
- *lmp-1*: Latent membrane protein-1
- NHL: Non-Hodgkin's lymphoma
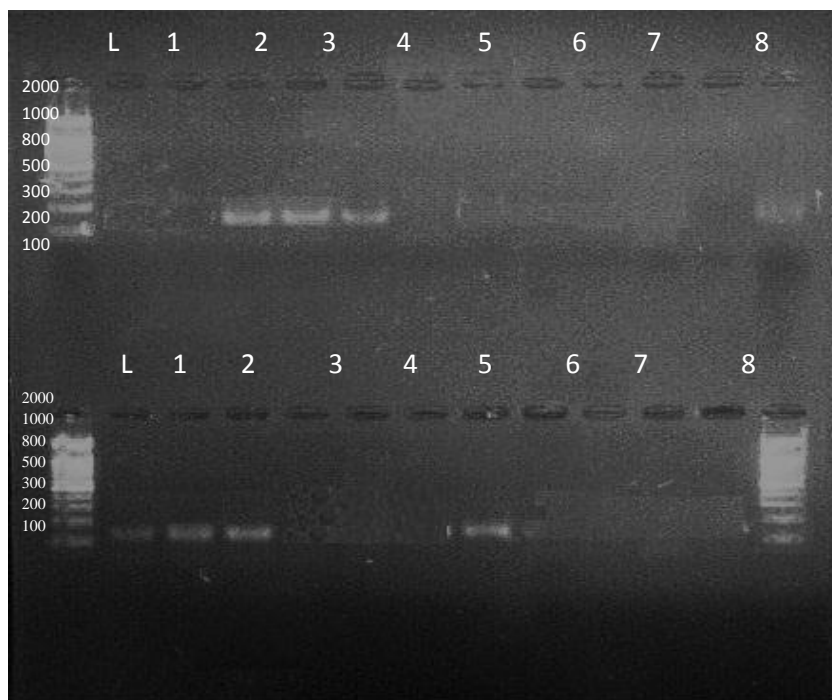- RFH: Reactive follicular hyperplasia
- P value= 0.052

Figure (3): The PCR amplification products of the EBV *lmp-1* on ethidium bromide stained agarose gel (2%), 70 volt for 1 hour. The size region 190 bp are indicative positive result for EBV *lmp-1*.

Lane (L):100 bp DNA ladder
A- Lane (3-5) positive result of NHL.
B- Lane (1-3) positive of NHL, (7) positive of reactive follicular hyperplasia.

♦ **Interrelationship between EBV *lmp- 1* and *bcl2* t(14;18) chromosomal translocation.**

Table (5) shows the correlation of EBV *lmp-1* with *bcl2* t(14;18) chromosomal translocation. Current results revealed that all cases with positive results of EBV have *bcl2* chromosomal translocation with no

significant correlation observed between them. This study also, revealed patients with EBV *lmp-1* clinically would have a risk factor for MBR *bcl2* t(14; 18) chromosomal translocation with (95% CI 1.914 – 4.480).

Table (5):Correlation of EBV lmp-1 with MBR bcl2 t(14;18) chromosomal translocation.

			EBV *lmp-1*		Total
			-ve	+ve	
MBR	-ve	Count	0	1	1
		% within MBR	0.0%	100.0%	100.0%
		% within EBV	0.0%	6.7%	2.4%
	+ve	Count	27	14	41
		% within MBR	65.9%	34.1%	100.0%
		% within EBV	100.0%	93.3%	97.6%
Total		Count	27	15	42
		% within MBR	64.3%	35.7%	100.0%
		% within EBV	100.0%	100.0%	100.0%

- EBV: Epstein Barr Virus
- *lmp-1*: Latent membrane protein-1
- MBR: Major break point region

Discussion

As in many other hematologic neoplasms [23,24]. The NHL cells harbor specific chromosomal translocations, these involve the *IgH* loci including the   t(14;18) (q32;q24) translocation that consider as the most common aberration detected in NHL [25] . This 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].

Current results revealed that the frequency of *bcl2* t(14;18) translocation at MBR (major breakpoint region) was (96.9%, and 100%) in NHL and reactive follicular hyperplasia respectively.. In addition to that, this translocation was not detected in MBR tonsils that used as a control group as shown in table (3). The PCR product illustrated in figures (1, and 2).

The most frequent translocation in human lymphoma is the t(14;18) (q32;q21) which juxtaposes a putative oncogene *bcl2* from 18q21 with one of the six J segments of the immunoglobulin heavy chain locus on chromosome 14 [27]. Xiang Zhi-fu*et al*. have shown the approximately 60% *bcl2* gene breakpoints occur within the 150 bp MBR at the untranslated region in the third exon of *bcl2* gene, in comparison 30% of breakpoints occur at MCR (minor cluster region) [28]. The clustering of breakpoints at MBR and MCR has made it possible to use PCR amplification to identify cells containing *bcl2/JH* gene rearrangement, and this tumor-specific molecular marker can be used to follow the natural history of lymphoma and search for minimal residual disease [29].

The results that conducted from present study appear to concur with the other reports, two studies observed that *bcl2* t(14;18) translocation occurs in 57% to 90% of patients with follicular lymphoma and in 9% to 30% of patients with DLBCL; 55- 60% of translocations occur at the MBR, whereas 10-20% occur at the MCR [30,31]. In addition to that, this translocation detected in 14 of 15 (93%) of the samples that were positive translocation for each MCR and MBR regions these results may be due to the a proximity of these two regions, which leads to overlap among the primer pairs [32]. Furthermore, translocations in *bcl2* studied by Olga *et al*. were demonstrated breakpoints at MBR in 118 patients (50%) and MCR in 11 patients (5%). The remaining 51 PCR-negative cases were evaluated with FISH methods and found to contain the *bcl2/ IgH* translocation in 37 cases (72%) [33] .

On the other hand, results of present study indicated a high percentage (100%) of this translocation in reactive follicular hyperplasia patients. The t(14;18) translocation can be detected in non-malignant circulating lymphocytes of healthy subjects was fueled an extensive debate on the role of the translocation in NHL and at large on the biological steps underlying NHL development. Additional events, possibly involving exposures to carcinogenic agents are required for t(14;18) positive cells to develop a fully malignant phenotype [34], it has been suggested that t(14;18) positive cells in healthy individuals are under control of immunological mechanisms [35,36].

The result of present study is high closely with the results of Dolken*et al*., and Schuler *et al.* that indicated this translocation shows in 30–80% of healthy individuals, also highly-sensitive PCR techniques have allowed for the detection of a low number of t(14;18) copies in peripheral blood lymphocytes and other normal lymphatic tissues [37,38].In addition to that, the high prevalence of the t(14;18) translocation among healthy individuals would indicate that perhaps overexpression of the Bcl2 protein as a result of this transformation may not sufficient for malignant transformation [39-41].Multiple EBV proteins can be expressed in infected lymphocytes, among which LMP1 is thought to be most important for transformation. In healthy infected individuals, outgrowth of EBV-transformed B lymphocytes is prevented by the presence of intact T lymphocyte-mediated immunity [18].The most common methods were used to detection of this virus in the different lymphomas include IHC, RISH and PCR [42].

The EBV *lmp-1* gene was investigated in FFPE samples of NHL, and reactive follicular hyperplasia enrolled in this study. The PCR assays based on *lmp-1* amplification in order to determine the frequency of EBV associated NHL in Iraqi patients. Overall, EBV*lmp-1*detected in extant study among all NHL was 43.8% (14 out of 32 cases), among reactive follicular hyperplasia was seen in10 % (1 out of 10 cases).

Many research revealed that the EBV was associated with lymphoma [43-445]. Naturally EBV has a receptor on B lymphocytes called complement receptor CR21 or CR2 which at the same time has mutagenic characteristics for B lymphocytes. In other words, it is the cause of the polycolonal stimulation of cells. Following the contamination of epithelial cells the active replication of the virus leads to the lysis and destruction of the cells [46,47]. Moreover, EBV causes the infected B cells to replicate and this leads to a genetic mutation in new B cells and eventually transformation to lymphomas [48,49]. Also, LMP-1 is expressed in many EBV-associated cancers and is responsible for most of the altered cellular growth properties that are induced by EBV infection [50,51]. The LMP-1 is functionally similar to CD40, acts as a constitutively activated receptor 9 and can activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signaling then expression of genes that keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die via apoptosis, and downstream the anti-apoptotic *bfl-1* gene [52]. Numerous studies recorded different EBV positivity in NHL case, Gonin*et al.* showed the positivity of EBV in 30% of NHL [53]. Others illustrated the EBV positivity was seen in 12.7% of (9 / 71) cases while the high EBV genomes were detected in 68% of all NHL [54]. Furthermore, Tumwine *et al.* showed the frequency of expression of LMP-1 of EBV was detected (34.7%) in NHL patients [55]. in contrast with Hiraiwa*et al.* that shows DNA EBV detected in (9/13) (69.2%) of reactive follicular hyperplasia, and low incidence in NHL (4.8%) [56].When compared with previous results mentioned above, we can see many of them in agreement with our results.

In this study it was attempted to investigate the correlation between *bcl2* translocation and EBV. Our result revealed that *bcl2* translocation independent of EBV infection but clinically EBV plays an important role in *bcl2* translocation occurrence then lymphoma development (95%CI 1.914–4.480) with risk estimate 2.929. The Current result was similar with the several studies have reported the presence of clonal chromosome translocations in EBV associated malignancies [57,58]. Gruhne*et al.* showed the specific roles of three EBV latency proteins. They demonstrated that the EBV nuclear antigens EBNA-1 and EBNA-3C, and LMP-1, independently promote genomic instability, as detected by nonclonal chromosomal aberrations, DNA breaks and phosphorylation of histone H2AX (the H2AX contributes to the nucleosome-formation and therefore the structure of DNA). Whereas DNA repair is inhibited in LMP-1 expressing cells through downregulation of the DNA damage-sensing kinase such as ataxia telangiectasia mutated (ATM), reduction of phosphorylation of its downstream targets Chk2 and, inactivation of the G2 checkpoint [59].

Conclusions

The high frequencies of major break point chromosomal translocation among NHL suggest that *bcl2* t(14;18) has an essential role in pathogenesis of lymphoma. Also, the detection of EBV *lmp-1* gene among NHL proposes that EBV plays an important role in lymphoma development too. Furthermore, the *bcl2* t(14;18)chromosomal translocation independent of EBV infection.

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