Detection of Epstein Barr Virus lmp-1 Gene in Non Hodgkin Lymphoma Iraqi Patients

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Abstract

Non-Hodgkin's lymphomas (NHLs) are 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.The EBV infects certain epithelial cells and linked to the development of multiple cancers, including NHL, HL, and nasopharyngeal carcinoma.This study designates to determine the frequency EBV *lmp-1* inNHL Iraqipatients, and todetermine the correlation between EBV *lmp-1*and subtypes of NHL.A 42 FFPE blocks were examined included 32 blocks from NHL patients, and 10 blocks from reactive follicular hyperplasia. Genomic DNA was extracted from these blocks using a specific kit and amplified by polymerase chain reaction (PCR) by using oligonucleotide primers specific for EBV *lmp-1* gene .Our result shows EBV *lmp-1* was detected in 43.8 % cases of NHL, and in 10.0% cases of reactive follicular hyperplasia with no significant (P>0.0.5) variation between these groups. In addition to that,incidence ofEBV *lmp-1* was more frequently detected in high grade. From these results we conclude that EBV *lmp-1* plays an important role in NHL development especially in high grade histopathological type.

الخلاصة

يعد اورام الغدد اللمفاوية اللاهودجيكن من الاورام الدموية الخبيثة الواسعة الانتشار في العالم . اعتمادا على نوع الخلية السرطانية يصنف المرض الى اورام الخلايا بي اوتي وتعد اورام خلايا بي اكثر انتشارا من خلايا تي.يصيب فايروس الابشتاين الخلايا الطلائية ويرتبط وجوده مع بعض الامراض منها سرطان الغدد اللمفاوية الهودجكن واللاهودجكن وكذلك سرطان الا نف والرغامي .صممت الدراسة الحالية لتقدير تكرار وجود جين الابشتاين المشفر للبروتين lmp−1في مرضى سرطان الغدد اللمفية الاهودجكن وعلاقته مع الانواع الفرعية للمرض. اظهرت النتائج الحالية ان نسبة وجود جين الابشتاين بار فايروس هي ٤٣.٨% في مرضى سرطان الغدد اللمفية اللاهودجكن مع اعلى نسبة في النوع المتقدم بينما كانت النسبة في الاورام الحميدة ١٠%ممايقودنا الى الاستنتاج بان الابشتاين بار فيروس له دور كبير في امراضية سرطان الغدد اللمفاوية اللاهودجكن وخاصة بالانواع المتقدمة للمرض.

Introduction

Lymphoma is a cancer of the lymphocytes, a type of white blood cells occurs when cells grow abnormally without of control [1].Traditionally, two main groups oflymphoma have been distinguished: Hodgkin Lymphoma (HL) characterized by large polynuclear cells called reed Sternberg cells, and Non-Hodgkin's lymphoma (NHL) [2]. Non-Hodgkin's lymphomas 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.

Researchers have found that NHL is linked with a number of risk factors, but the causes of most lymphomas are unknown. This is complicated by the fact that lymphomas are actually a diverse group of cancers [3].Several viruses have been shown to play a role in the development of NHL like Epstein-Barr virus (EBV), Human T-lymphotropic virus (HTLV), and human immunodeficiency virus (HIV) [4]. Epstein–Barr virus or human herpesvirus 4 (HHV4) belongs to the genus Lymphocryptovirus within the subfamily of gammaherpesviruses. Common features of these viruses are their lymphotropism, their ability to establish latent infection of their host cells and to induce proliferation of the latently infected cells [5].

The EBV infects certain epithelial cells and linked to the development of multiple cancers, including Burkitt's lymphoma, HL, some diffuse large B-cell lymphomas, and nasopharyngeal carcinoma [6]. The association between lymphoma and EBV came from the observation that EBV is the causative agent of infectious mononucleosis [7]. So that patients with a previous history of infectious mononucleosis have an elevated risk of developing lymphoma. The genome of EBV as in figure (1) consists of a linear, double-stranded DNA molecule that is 184 kilo base pairs in length. EBV has a series of 0.5 kb terminal direct repeats (TRs) and internal repeat sequences (IRs) that divide the genome into short and long, largely unique sequence domains [8].

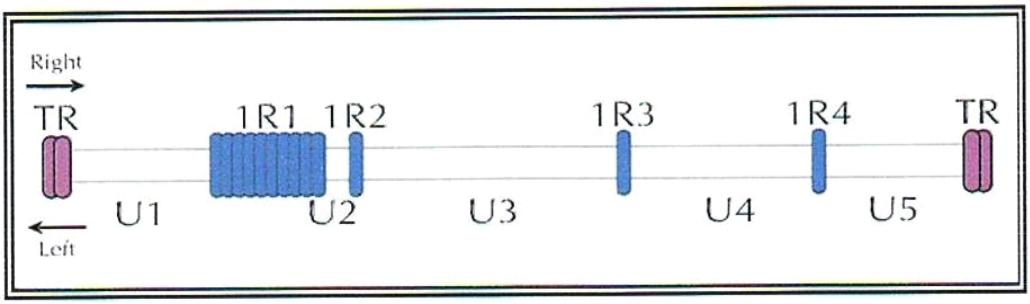

Figure (1): Linear organization of the EBV genome [9].

Multiple EBV proteins can be expressed in infected lymphocytes, among which latent membrane protein-1(LMP1) is thought to be most important for transformation [10]. Many molecular techniques are being used to demonstrate the presence of the EBV, such as the polymerase chain reaction (PCR) and in situ hybridization (IHS). The PCR makes it possible to detect minimal amount of viral DNA in tissues and smears [11].

This study was designates to determine the frequency EBV *lmp-1* in NHL Iraqi patients, and todetermine the correlation between EBV *lmp-1*and subtypes of NHL

Subjects and Methods

The subjects included in this study were represented as formalin fixed paraffin embedded (FFPE) biopsy tissue blocks that were obtained from Iraqi patients and collected from the histopathology laboratories of Iraqi Hospitals and Private

Laboratories.Diagnosis of these tissue blocks were based on the obtained histopathological laboratory records of samples that had accompanied each tissue blocks in each laboratory. Also, a second histopathological reexamination of obtained tissue blocks was done by seniorhistopathologist. The collection samples of this study were carried out during the period from July 2012 until to April 2013. Study groups included 42 FFPE blocks. These samples were distributed as the following, 32 samples from patients of NHL, and 10 blocks from reactive follicular hyperplasia. The ages of NHL patients were ranged between 3-81 years with median age 55 years, and mean ± SD equal 45.718± 23.51 years. All NHL samples were taken before treatment and cases of NHL were classified according to the international working formulation (IWF) of the National Cancer Institute to 3 groups [12] as table (1) elucidate.

Table (1): Non Hodgkin lymphoma subtypes enrolled in this study

NHL subtypes	Number	Age (Mean ±SD) years
Low grade	8	59.750±11.093
Intermediate grade	17	53.235±17.205
High grades	7	9.428±8.462

Formalin embedded blokes enrolled in this study sectioned by microtone, serial tissue sections were cut into 5-15 µm thickness from each tissue block. Genomic DNA was extracted from these sections by using Genomic DNA Minikit (Invitrogen Askutlanda) [13].EBN *lmp-1* gene was detected by using polymerase chain reaction .
Amplification was performed in a programmable MultigeneThermal Cycler PCR (LabnetInternational Inc.USA). Primers sequences were revealed in table (2).

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**	**14-15**
Reverse	5'- GTCTGTCTGTCTGTCCGTCA -3'		

PCR conditions

Genomic DNA was amplified in a final volume of 20 µl (5 µl Genomic DNA plus 3 µlforward primer plus 3 µl reverse primer plus 5 µlBioneer's master mix with Green Taq DNA polymerase plus 4 µl DDW) using the following conditions: Denaturation at 95 ^{o}C for 5 min. followed by 35 cycles of (denaturation at 95 ^{o}C for 30 seconds, annealing at 58 ^{o}C for 30 seconds. and extension at 72 ^{o}C for 1 min. and a final extension was at 72 ^{o}C for 5 min. then hold at 4 ^{o}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. Odds ratio (OR) with 95% confidence intervals (CI) were estimated for the effect of high risk translocation.

Results

- **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 illustratedpositive 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 (3). 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 (3): 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: Positive, N: Negative

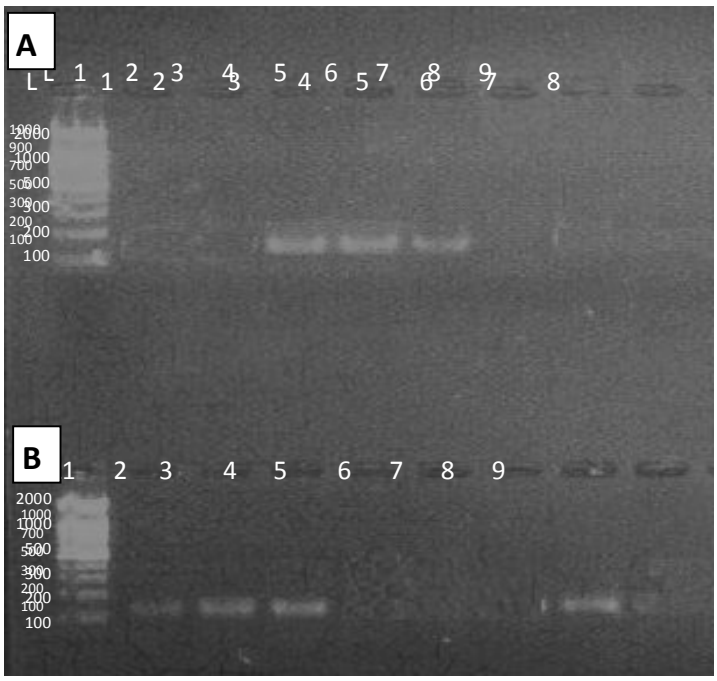

Figure (2): PCR amplification products of the EBV *lmp-1* on ethidium bromide stained agarose gel (2%), 70 volt for 1 hr.
Lane (L):100 bp DNA ladder
PCR products in the size region 190 bp are indicative of positive *lmp-1*.
A- Lane (3-5) positive of NHL.
B- Lane (1-3) positive of NHL, (7) positive of reactive follicular hyperplasia.

- **Frequency of EBV*lmp-1*inNon Hodgkin Lymphomas Subtypes**

This study revealed that the relative incidence of EBV *lmp-1*in high grade of NHL was (71.4%) higher than the intermediate and low grades that showed (41.2%, and 25.0%) respectively. Statistically there was no significant (0.186) variation within grades groups as in table (4). Also, our findings indicated

that EBV*lmp-1* is clinically more effected in high grades when compared with low and intermediate grades (CI 0.53-2.368) with odd ratio <1.

Table (4): Relationships between EBV *lmp-1* and types of Non-Hodgkin's Lymphoma.

			lmp-1		
			N	P	Total
Type	High	Count	2	5	7
		% within type	28.6%	71.4%	100.0%
		% within *lmp-1*	11.1%	35.7%	21.9%
	Inter-medi-ate	Count	10	7	17
		% within type	58.8%	41.2%	100.0%
		% within *lmp-1*	55.6%	50.3%	53.1%
	Low	Count	6	2	8
		% within type	75.0%	25.0%	100.0%
		% within *lmp-1*	33.3%	14.3%	25.0%
Total		Count	18	14	32
		% within type	56.2%	43.8%	100.0%
		% within *lmp-1*	100.0%	100.0%	100.0%

lmp-1: Latent membrane protein-1, P: Positive , N: Negative

Discussion

- **Frequency of EBV*lmp-1* Gene in Studied Groups**

Epstein Barr virus is a very common virus that is already infecting high percent of population worldwide and persisting for the lifetime in the host [16]. It is usually acquired in early childhood in developing countries. Despite the fact that EBV is a common infection, it has been postulated that EBV play a role in the pathogenesis of lymphoma [17].Multiple EBV proteins can be expressed in infected lymphocytes, among which LMP1 is thought to be most important for transformation [10].

Overall, EBV *lmp-1*detected in our 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 [18-20]. 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 poly-colonal stimulation of cells. Following the contamination of epithelial cells the active replication of the virus leads to the lysis and destruction of the cells [21-22]. 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 [23-24]. 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 [25-26]. The LMP-1 is functionally similar to CD40, acts as a constitutively activated receptor 9 and can activate NF-kB signaling and

downstream genes: the anti-apoptotic *bfl-1* gene [27].

Numerous studies recorded different EBV positivity in NHL case, Gonin*et al.* showed the positivity of EBV in 30% of NHL [28]. 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 [29].Furthermore, Tumwine *et al* showed the frequency of expression of LMP-1 of EBV was detected in NHL patients (34.7%) [30].in contrast with [31] that shows DNA EBV detected in (9/13) (69.2%) of reactive follicular hyperplasia, and low incidence in NHL(4.8%).When compared with previous results mentioned above , we can see many of them in agreement with our results .

Despite the frequency of EBV infection of NHLs is influenced by various factors such as the patient's immune status, the disease histologic subtype, the anatomical site of the tumor, and the sensitivity of the detection method. [32-33]. The current indicated EBV *lmp*1 clinically has a significant role in lymphoma development (95%CI=0.654-29.264) without any history of patients immunity that closely relatedto different studies were reported higher association of the EBV with NHL as high as 80% in other developing countries [34-35].

- **Frequency of EBV*lmp-1*inNon Hodgkin Lymphomas Subtypes**

Epstein Barr virus is an important example of a transforming virus implicated in several NHL subtypes [36].Non Hodgkin Lymphoma in present study was subdivided into three groups, high, intermediate, and low grades. High presence of *lmp-1* genewas detected in high grades (71.4%) of NHL patients.these results were in agreement with some previously studies which also found 30% EBV positive in their NHL cases [37-38]. It is also worth mentioning that Burkitt's lymphoma is a high-grade malignant NHL that is most commonly associated with EBV infection [39-40]. In United States, EBV associated with Burkitt's lymphoma documented in 20 % of patients [16].Previous study observed the carcinogenic role of EBV in Burkitt's lymphoma when find that EBV was a potent transforming virus in culture for the same cell type that develops into Burkitt's lymphoma [41]. Others demonstrated that Burkitt's lymphoma is highly associated with (EBV) in over 95% of cases while in Egypt and Brazil Burkitts lymphoma have also been documented up to 87% of tumors are EBV positive [42 - 43].The roles of EBV contributes to the B-cell cancer pathogenesis by expressing EBV-encoded LMP1, as well as enhancing genetic instability through mutation, translocation, and dysregulated expression of proto-oncogenes [44].Our results also reported low frequency of EBV in low and intermediate grades that indicated the less impact of these proteins in these types that consistence with [40]. Statistically insignificant correlation observed between EBV and subtypes of NHL, but from CI results we can predicate the clinical role of EBV in NHL subtypes especially with Burkitt's lymphoma.

The variable percentages in different studies may be due to the fact that EBV prevalence may be different in different parts of the world.

conclusion

In conclusion the highest frequency of *lmp-1* gene in NHL in our study is 71.1% of high grade and is mostly seen in Burkitts lymphoma. This requires further evaluation to find out whether this positivity is due to co-infection or has a role in pathogenesis.

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