

## Association Between Epstein-Barr Virus and Burkitt's lymphoma in Western Iraq. (A molecular case-control study)

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### Abstract

**Background:** Association of EBV with BL is differ according to the geographical distribution and type of BL with a consistent C-MYC chromosomal translocation.

**Objectives:** The purpose of this study is to find out the association between Epstein Barr virus & the characteristic C-MYC as well as the age and gender distribution of Burkitt's lymphoma in Western Iraq.

**Patients and Methods:** In this case-control study we have analyzed paraffin sections from 60 patients with Burkitt's lymphoma (19 endemic BL, 41sporadic BL). Diagnosis of these cases was depend on clinical and histological as well as immunohistochemical bases. Detection of Epstein-Barr virus (EBV) DNA and C-MYC chromosomal translocation by PCRwere performed, using sets of primers flanking the EBNA-1 and the c-MYC chromosomal translocation.

**Results:** Endemic BL affected mostly children aged 3–10 years (mean age: 5.6 years), whereas sporadic BL affected mostly adults aged 22–37 years(mean age:29 years). The sex ratio of the BL was 2:1 (41M: 19F).Study showed that 18/19 of endemic BL and 8/41 of sporadic BL were positive for EBV(positive EBNA1 by PCR).C-MYC chromosomal translocation was detected in 18/19 endemic BL and in 40/41sporadic BL.

**Conclusions:** There was significant association between EBV and endemic BL but no such a relation with sporadic BL. Also there was a strong association between C-MYC translocation in both endemic and sporadic BL. However there was no significant correlation between C-MYC translocation of endemic & sporadic BL and the gender.

**Keywords:** PCR, EBV, Burkitt's lymphoma, C-MYC translocation, Western Iraq.

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### Introduction:

**B**urkitt's Lymphoma(also called small non cleaved cell lymphoma) is a highly aggressive non-Hodgkin lymphoma with an incidence of 2-3 cases per million per year in USA <sup>(1,2)</sup>. While in the African equatorial belt the incidence is 5-10/100000 for endemic BLand the children accounts for up to 74% of childhood malignancies <sup>(3)</sup>.

However, Indian studies ranked BL only third in frequency among children

following precursor T-lymphoblastic lymphoma and DLBCL <sup>(4)</sup>.

The disease is characterized by tumors of the jaw bones and abdomen <sup>(5)</sup>. There are three subtypes of Burkitts lymphoma: endemic Burkitt's lymphoma (eBL), sporadic Burkitt's lymphoma (sBL), and immunodeficiency-associated Burkitt's lymphoma, these show marked variation across different geographical regions with respect to age, primary tumor site and

association with Epstein-Barr virus (EBV) infection <sup>(3,6)</sup>.

Epstein bar virus (EBV) or human herpes virus 4 (HHV4) belongs to DS DNA virus from herpesviridae family, infects more than 90% of the world population and persists for the life of the host, Occasionally, it may switch to lytic infection which cause infectious mononucleosis. Following primary infection, Epstein-Barr virus persists in an asymptomatic immortalizes host cells as part of its latent mode of infection that has been associated with a number of human malignancies <sup>(7,8)</sup>. Most EBV diseases are associated with EBV latency, during which the viral gene expression is limited to the six members of the EBNA family of nuclear proteins, two membrane proteins, and two small polymerase III transcripts. During latency I, Epstein-Barr nuclear antigen 1 (EBNA-1) and the two small non coding Epstein-Barr RNAs (EBERs) are expressed. Latency I is generally associated with the EBV-related Burkitt's lymphoma <sup>(9,10,11)</sup>.

The hallmark of all BL (some reports approximately 75%-90% of these tumors) regardless of their geographical origin exhibit one of three c-MYC/Ig chromosomal translocations leading to the activation and over expression of the c-MYC gene that leads to strong proliferation signals, allowing the cells to grow rapidly as a crucial event in the development of this disease <sup>(12,13)</sup>.

## Patients and Methods

Sixty blocks of formalin-fixed paraffin-embedded lymphatic tissue diagnosed and treated as cases of Burkitt's lymphoma (BL) were collected from different laboratories in Anbar Governorate as well as from Abu Ghraib region during a period between June 2013 and June 2014, these cases were belong a period extended from 2003-2004. Diagnosis of the cases was based on clinical, histological and

immunohistochemical markers and patients referred to oncology centers that confirm the diagnosis. Information and clinical data regarding the tissue blocks including sex, age at diagnosis and anatomic location of the tumor were obtained. Forty one cases were abdomino-pelvic and nineteen cases were oro-facial, 41/60 were male & 21/60 were female (M:F ratio of 2:1), ages ranged from (3-37 years). Sixty cases (2-40 years) represented non neoplastic lymphatic tissue biopsies as a control group were included. This study was performed in college of medicine-Anbar University. Statistical analyses were based on non-parametric methods and a p value <0.05 was considered significant. All biopsies of paraffin-embedded lymphatic sections (BL cases and controls) were subjected for molecular study using conventional PCR method to detect the presence of EBV DNA by amplification EBNA-1 sequence of the virus genome, and C-MYC chromosomal translocation based on the specific primers. DNA using a QIAamp tissue kit (Qiagen-Germany) as described in protocol. DNA purity was assessed through determined the concentration of the DNA by UV spectrophotometry by ratio of the DNA optical density (OD 260) and DNA optical density (OD 280) <sup>(14)</sup>.

## Results:

Current study revealed that endemic BL affected children aged 3-10 years (mean: 5.6 year), whereas sporadic BL was affected adult, aged 22-37 years (mean: 29.2 years), (table 1). There was significant difference between median of age of endemic BL and sporadic BL (T test = 21.62, P value = 0.000). No significant difference in the age between the cases and control group was detected (T test (0.69), (P>0.05). Sex ratio of the BL was 2:1 (41M: 19F), in the control group

35/60(58%) were males and 25/60(42%) were females (Fig.4.5), there was no significant difference in gender between cases and control group ( $X^2 = 0.72$ ,  $P = 0.61$ ).

There were significant gender difference in both types of BL, in endemic BL 14 out of 19 cases were males ( $X^2 = 4.26$ ,  $p$  value= 0.039) and in sporadic BL 27 out of 41 were males ( $X^2 = 15.22$ ,  $p$  value = 0.000)(table 2).

**Table (1): Distribution of Burkitt's lymphomas and control according to age**

	Minimum	Maximum	Mean	Std. Deviation	T test	P value
<b>ENDEMIC</b>	<b>3.00</b>	<b>10.00</b>	<b>5.6842</b>	<b>1.88717</b>	<b>21.62</b>	<b>0.000</b>
<b>SPORADIC</b>	<b>22.00</b>	<b>37.00</b>	<b>29.2439</b>	<b>4.55401</b>		
<b>CONTROL</b>	<b>2.00</b>	<b>40.00</b>	<b>16.2000</b>	<b>11.31640</b>		

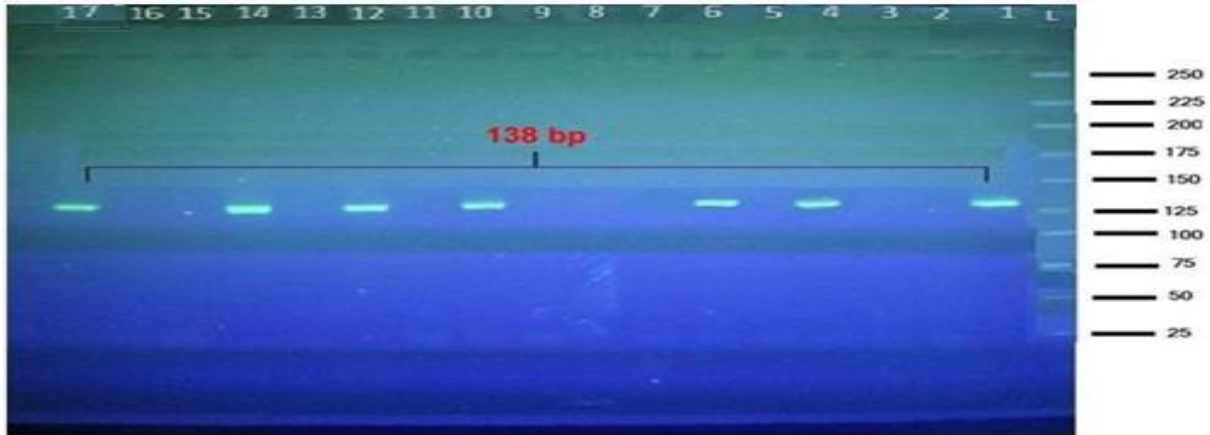
**Table (2): Distribution of Burkitt's lymphomas types and control according to gender.**

		groups			
			ENDEMIC	SPORADIC	CONTROL
<b>Gender</b>	male	Count	14	27	35
		% within factor	74%	66%	58%
	female	Count	5	14	25
		% within factor	26%	34%	42%
<b>Total</b>		Count	19	41	60
		% within factor	100%	100%	100%

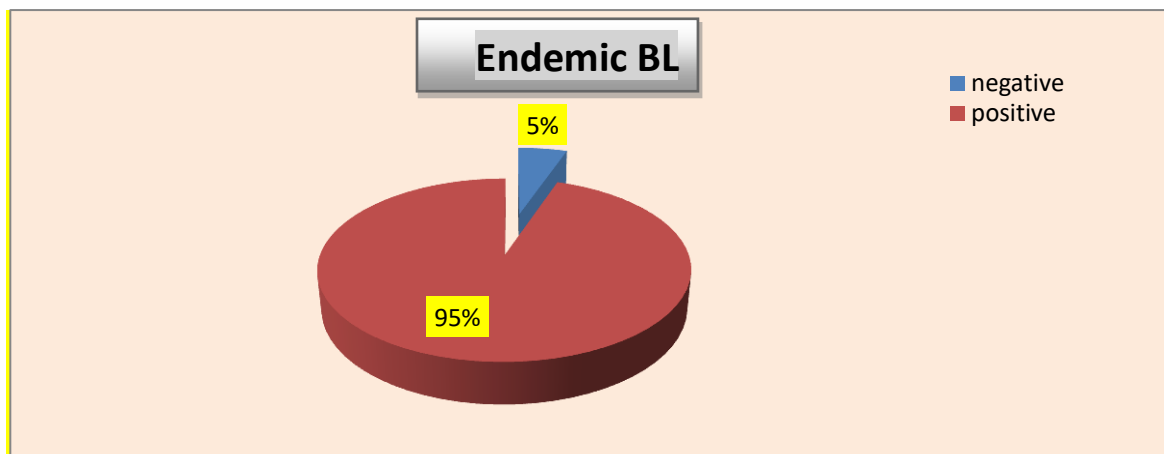
PCR amplification products were electrophoresed on 2% agarose gel stained with red safe as a fluorescent dye to facilitate visualization of EBV DNA under ultraviolet light (300 nm wavelength). The results of descriptive agarose gel electrophoresis revealed visualization small DNA diagnostic bands were detected at 138 base pair fragment (Figure 1). The results obtained by this study showed that 18/19 (95%), (14 males and 5 females) of endemic BL cases and 42 /60 controls were positive for EBV (positive EBNA1 by PCR) ( Figure 2, Figure 7, Figure 8 and

table 3)), while EBV was detected in 8/41(20%), (6 males and 2 females) of sporadic BL cases (positive EBNA1 by PCR) (Figure 3 ,Figure 7, and table 3), There was a significance association between EBV and eBL ( $X^2 = 7.7143$ ,  $P < 0.05$ ) ,where there was a no significant association between EBV and sporadic BL cases ( $X^2 = 0.103$ ,  $p > 0.05$ ) (Figure 8) also there was no significant correlation between EBV-PCR-results of endemic BL and sBL the gender of patient ( $X^2 = 1.99$ ) ( $P > 0.05$ ).

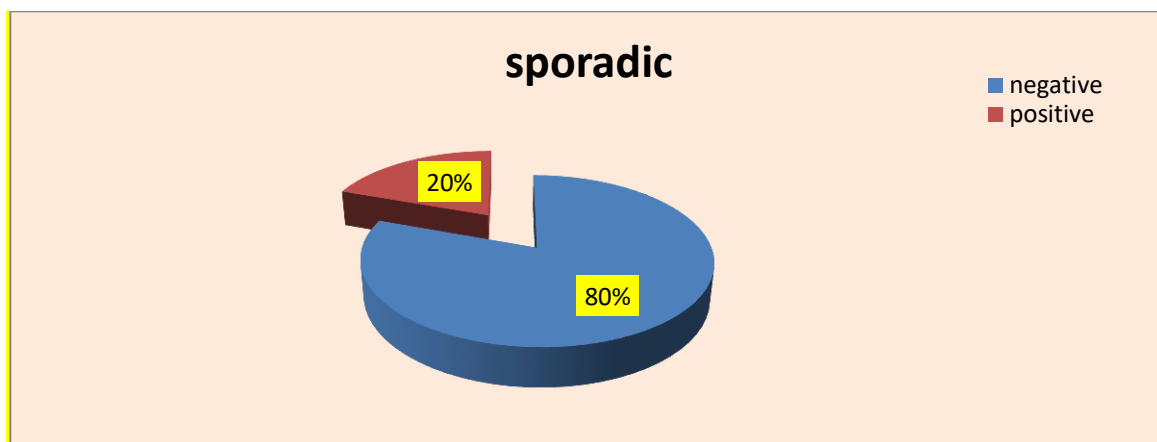
EBNA-1 positive endemic BL were  $\leq 10$  years (table 1). EBNA-1 positive cases of sporadic BL were adult (22 – 37 years)



**Figure (1):** The results of agarose gel electrophoresis (2%) with novel juice stain and voltage 100 volt/cm, bands with amplified gene coding EBNA-1 gene Sequences obtained from BL tumor cells, which showed lane (L) represented DNA ladder, lanes (1-4-6-10-12-14-17) represented positive PCR band (visible band in 138 bp), Lane (2-3-5-7-8-9-11-13-15-16) represented negative PCR band. ladder with (25-250 pb) on the right was used as DNA molecular weight marker.



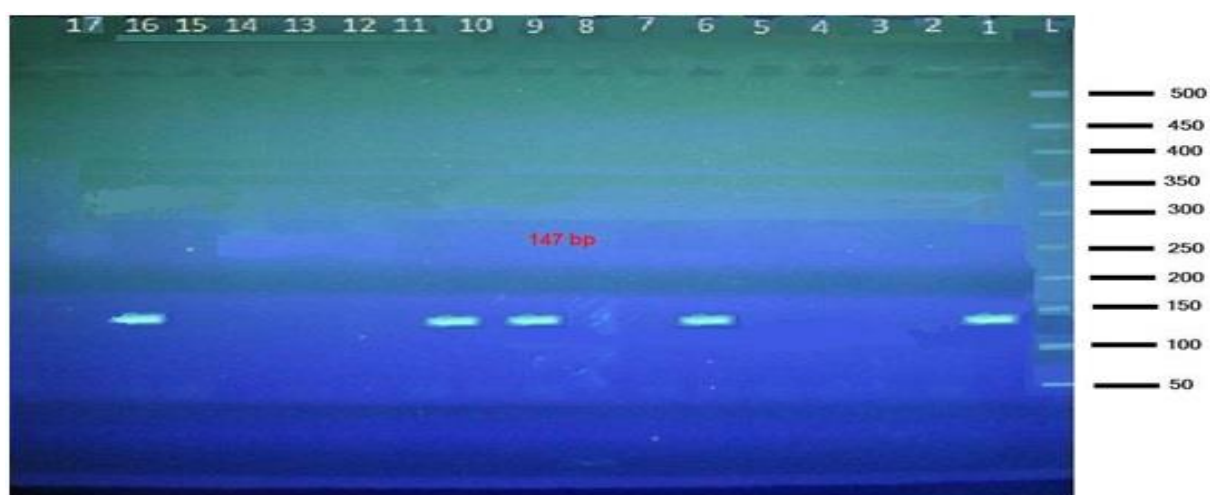
**Figure (2):** Distribution of EBNA 1 in Endemic cases of Burkitt's Lymphoma.



**Figure (3): Distribution of EBNA1 in Sporadic cases of Burkitt's Lymphoma**

PCR amplification products were electrophoresed on 2% agarose gel stained with red safe as a fluorescent dye to facilitate visualization of C- myc gene under ultraviolet light (300 nm wavelength). The results of descriptive agarose gel electrophoresis revealed visualization small DNA diagnostic bands were detected at 147 base pair fragment. (Figure 4). Regarding the association between C-MYC chromosomal translocation and BL, C-MYC chromosomal translocation was detected in 18/19(95%) endemic BL (13 males and 5

females) , 1 male of the 60 controls was positive for C-MYC (Figure 5). C-MYC 40/41((97%) sporadic BL (26 males and 14 females) and 1 /60 males controls was positive for C-MYC (Figure 6, Figure 7, and table 3). There was a strongly significant association of C-MYC translocation in endemic BL (  $X^2=1.083$ ,  $p= 0.000$ ). There was no significant correlation between c-MYC-PCR of endemic BL (  $X^2=1.99$ ,  $P> 0.05$ ) and sporadic BL and the gender (  $X^2=1.99$ ,  $P> 0.05$ ) (Figure 8).



**Figure (4): The results of agarose gel electrophoresis (2%) with novel juice stain and voltage 100 volt/cm, bands with amplified gene coding C- myc gene Sequences obtained from BL tumor cells which showed lane (L) represented DNA ladder, lanes (1-6-9-10-16)**



represented positive PCR band (visible band in 147 bp), Lane (2-3-4-5-7-8-11-12-13-14-15-17) represented negative PCR band (no bands), lader with (50-500 pb) on the right was used as DNA molecular weight marker.

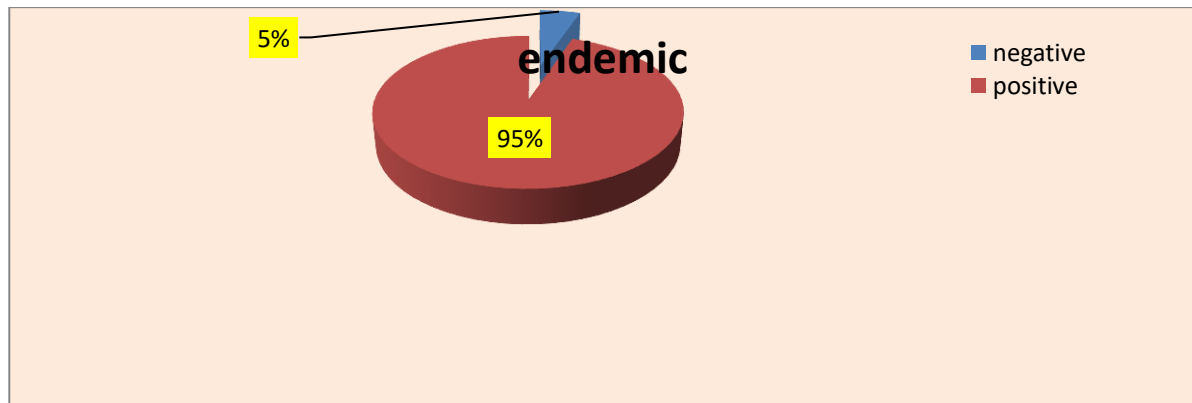


Figure (5): Distribution of C-MYC in Endemic cases of Burkitt's lymphoma.

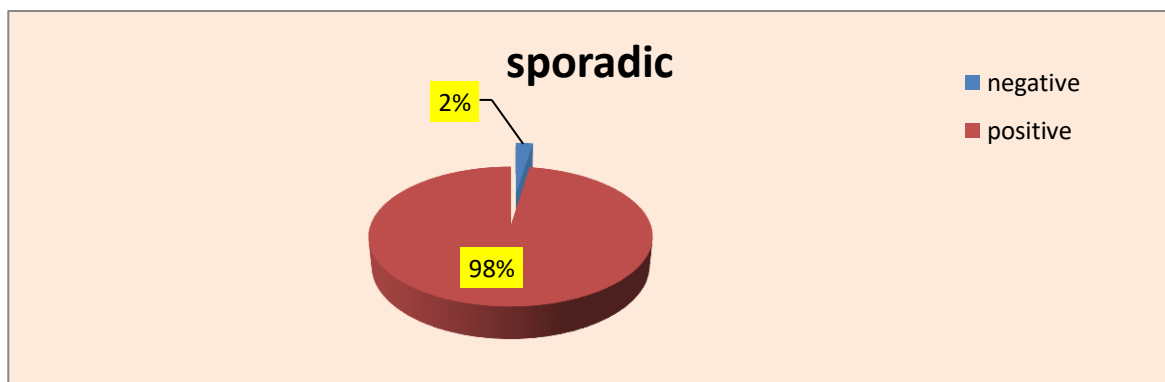
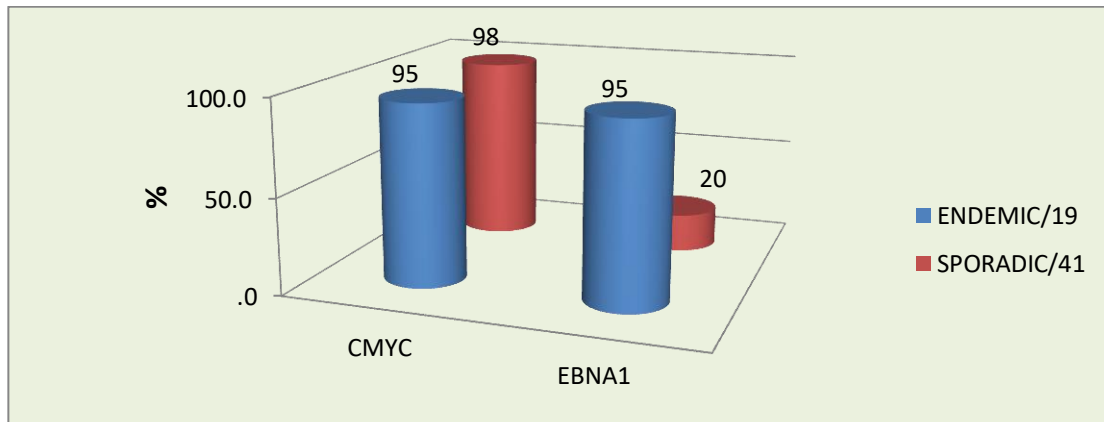


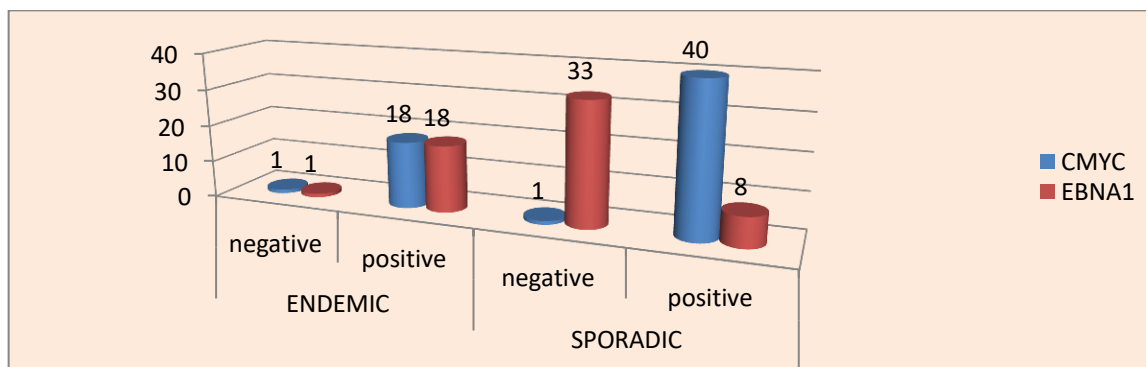
Figure (6): Distribution of C-MYC in Sporadic cases of Burkitt's lymphoma using Conventional PCR method.

Table (3): Distribution of EBNA1 and C-MYC in Endemic and Sporadic of Burkitt's

	ENDEMIC 19/60		SPORADIC 41/60	
	negative	positive	Negative	positive
CMYC	1	18	1	40
EBNA1	1	18	33	8



**Figure (7): Distribution of EBNA1 and C-MYC in Endemic and Sporadic cases of Burkitt's lymphoma.**



**Figure (8): Distribution of EBNA1 and C-MYC in Endemic and Sporadic BL in Healthy control group using Conventional PCR method.**

We concluded that there was a significant association between EBV and endemic BL but there was no such association in sporadic BL. This study proved a strong association between C-MYC chromosomal translocation with both endemic and sporadic BL. There was no significant correlation between

theresults and gender of endemic BL ( $X^2=1.99$ ) ( $P> 0.05$ ) or gender of sporadicBL ( $X^2=1.99$ ) ( $P> 0.05$ ).

## Discussion

According to the SEER (Surveillance Epidemiology and End Results) database, the yearly incidence of Burkitt's lymphoma/leukemia in the United States across all ages constituted 0.4% of all lymphoid tumors<sup>(15,16)</sup>.

Burkitt's tumor cells demonstrate a characteristic t(8;14) translocation which activates the c-MYC oncogene. The highly consistent detection of EBV DNA and proteins in tumour cells accuses EBV in endemic Burkitt lymphoma. In addition, EBV is itself monoclonal in tumour cells, suggesting that the initial EBV infection occurs in a single precursor B cell as an early tumour-inducing event<sup>(16)</sup>. This study is performed to find out the association between Epstein Barr virus & the characteristic t(8;14) in Burkitt's Lymphoma patients from western Iraq.

In current study the abdomino-pelvic tumor (sporadic BL) was identified in 41 out of 60 (72%), while the oro-facial site (endemic BL) was 19 out of 60 (28%). This figure was similar to studies from Brazil, United States and Europe<sup>(17,18,19,20)</sup>. Al-Attar et al. found a higher frequency of sporadic BL in Iraqi in 1979, they showed that a primary abdominal tumors were documented in 90% of BL patients.<sup>(21)</sup> Also such predominance of sporadic BL in the present study agreement to other studies from Cameroon, Jordan, Saudi Arabia, and the UAE<sup>(22,4,23,24,25)</sup>.

Endemic BL patients were aged 3-10 years (mean age is 6). These ages were similar to earlier studies from Senegal<sup>(26)</sup>, Cameroon<sup>(27)</sup>, Bahia (Northeastern Brazil)<sup>(17)</sup>, equatorial regions of Africa, Papua and New Guinea<sup>(28)</sup>.

Our data indicated that the common age at presentation of sporadic form of the disease (sBL) was ranged 22 - 37 (mean 29 years), similar to the previous studies outside Africa<sup>(16)</sup>.

In contrast to African regions, SEER database the incidence of BL in adults (> 20 y) was roughly 5 times higher than the incidence of the disease in children (< 20 y)<sup>(19)</sup>, this reinforces the need to carry out genetic and environmental studies per age class search for biological factors involved in age-specific risk in BL.

Male/female ratio in this study was 2:1, similar to most of previous reports<sup>(1,29,30)</sup> and in agreement with the other researcher who showed that BL is more frequent in males, mainly in low risk areas like Europe<sup>(31)</sup>, but it was differ from African reports where males and females were almost equally affected, However in the intermediate areas, sex ratio was found to be variable<sup>(32)</sup>.

In the current study, EBV was detected in 18 out of 19 (95%) in eBL and in 42 out of 60 (70%) in control cases (significant association,  $p < 0.05$ ) (Fig. 2) and in 8 out of 41 (20%) of the sBL (no significant association,  $p > 0.05$ ), (Fig. 3). These findings were consistent with previous studies from United States<sup>(1,2)</sup> that found EBV was present in the majority of endemic cases of BL (up to 100%) and in only 15% to 30% of sporadic cases and also similar to other studies from Europe<sup>(29)</sup>, northeast of Brazil<sup>(17,33)</sup>, Africa<sup>(34)</sup>, Argentina & Chile<sup>(35,36)</sup>, and Iran<sup>(8)</sup>. In contrary to our results, studies from Southeast and Central West regions of Brazil<sup>(7,35,37,38)</sup> that obtained different frequencies of association between BL and EBV, varying from 50% to 72%.

In the present study all of EBV+ endemic BL were 10 years and under, with a median age of 7 years. This finding is in close to the previous study reported in Brazil where they found that a significant association between EBV infection and lower ages.



A high frequency of EBV+ BL in younger children was also observed in Northeastern Brazil<sup>(39)</sup>.

The different rates of EBV association between endemic BL and sporadic BL in the present study may be associated with the risk of exposure to infections as EBV infection occurs very early in life, and >90% of children are infected by 6 year and these may increase the frequency of EBV in endemic BL, whereas the low frequency of EBV in sporadic BL may be related to the infection that occurs during the first 2 or 3 decades of life and this explanation was consistent with the recent studies from Brazil that suggests in underdeveloped areas socioeconomic status could be a very important factor in determining the rate of EBV association as measured at the individual and regional levels, possibly compromising the host immunologic status as the Brazilian socioeconomic diversity is well known. The population living in the southeast enjoys a higher standard of living compared to that in the northeast<sup>(29,39,40)</sup>.

Our explanation regarding the different rates of EBV association between endemic BL and sporadic BL was supported by recent studies from southeast of Brazil that showed in industrialized countries, seroconversion occurs during the first 2 or 3 decades of life a low association of EBV<sup>(34,41)</sup> and to previous studies from Uganda<sup>(42)</sup> that showed 100% of children are EBV-seropositive before the age of 3, and a correlation between age at seroconversion and socioeconomic status was documented many years ago. We Concluded that there were a statistically significant relationship between endemic BL and detection of EBV DNA by the PCR method supported the an important role of EBV in the pathogenesis of pediatric classical endemic BL in Iraq. There was a low background incidence of sporadic which is rarely

associated with EBV but because of the high prevalence of EBV infection in the control group and low number of sporadic BL, a similar study with greater number of patients can be helpful for the final decision.

In the current study, C-myc chromosomal translocation was detected in 18 /19 cases (95% ) of eBL and 1/60 (1.8%) of the control (strongly significant ,  $p= 0.000$ ) (Fig.5:Table 3), and in 40/41 (98%) of the sBL (strongly significant ,  $p= 0.000$ ) (Fig. 6: Table 3). Our finding regarding C-myc chromosomal translocation was consistent with previous studies that found C-myc translocation is the hallmark of BL and EBV is a cofactor in the pathogenesis of endemic BL and irrespective of its geographical location and its relationship with EBV infection<sup>(43)</sup>.

EBV association and C-myc with Burkitt's lymphoma were similar to previous studies that showed EBV is a transforming virus that is present in every BL cell and strongly supported that EBVs role as a tumor virus in the development of BL<sup>(44)</sup>.

Two Burkitt's lymphoma cases were negative for C-myc translocation in this study ,in agreement recent evidence which indicated that infrequent cases may lack an identifiable MYC translocation, the explanation for which is still uncertain, though suggesting the existence of pathogenetic mechanisms alternative to genetic alterations and other chromosomal abnormalities are also frequently observed in BL<sup>(44,45,46)</sup>.

## Conclusions:

We concluded that in western Iraq there was a significant association between EBV and endemic BL but no such relation with sporadic BL.

The rates of eBL and sBL were similar to that registered in most "non-African" regions (Jordan, Saudi Arabia, UAE, Iran, Brazil...). However, eBL comprised only 28% of BL cases in this region, this may limit the efforts that

aimed to decrease BL via eradication of EBV. Also there was a strong association between C-MYC chromosomal translocation in both endemic and sporadic BL cases.

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