Prevalence of Epstein - Barr virus types in subtypes of Hodgkin lymphoma in Kerbela city, Iraq.

Hussein Abd Ali Mohamed Sadeq Al Her[1], Prof.Dr.Mohanad Mohsen Ahmed[2] and Consultive . Dr. Haider Jebur Kehiosh[3]

1. B. Sc., microbiology, College of Science, 1991 Al-Mustansiriya University

2. University of kerbala, College of Medicine, Department of Medical Microbiology

3. Al-Hussein Teaching Hospital, Laboratory of Histopathology

Abstract

Background: Epstein-Barr virus (EBV) is a ubiquitous virus associated with lymphomas. However, in Karbala as other parts of Iraq, EBV detection is not a part of the workup for lymphoma diagnosis.

The aim of this study: to study the prevalence of EBV types in the subtype of Hodgkin lymphoma among patients from Kerbala province, Iraq.

Methods: Archival formalin-fixed paraffin embedded blocks from 31 patients with mean age 32.56±15.685 (range from 5 to 70years) diagnosed Hodgkin lymphoma during the period from 2010 to 2018 in Karbala city included in this study. Histological diagnosis was reviewed. Around 25mg of archival tissue were used to extract DNA. DNA extracts were used Polymerase Chain Reaction (PCR) targeting the EBNA3C region of the viral genome. This PCR system offered detection and typing of the EBV. T-test and chi-square were used to evaluate the association between age with the subtype of Hodgkin lymphoma, gender and EBV state.

Results: The majority of the Hodgkin lymphomas belonged Mixed Cellularity HL (MCHL) [n=18, 58%] where Nodular Sclerosis HL (NSHL) was seen in 42 % (n=13). The other subtypes of HL were not reported in this study. Male more affected with HL than female (16 vs.15) and the peak age range of HL 31to 40 years. EBV was detected in 18(58%) out of all cases. EBV type 1 was more common in MCHL were types 2 was the most common among NSHL cases. To our knowledge, this is the first time such data has been generated in Iraq.

Conclusion: most of the HL cases in Kerbela are associated with EBV. there is a difference in the distribution of the EBV types according to the subtypes of lymphoma.

Keywords: Epstein Barr virus type1 and type 2, Hodgkin lymphoma subtype, molecular detection.

انتشار انواع فيروس الابسن بار في الانواع الفرعية للاورام اللمفاوية الهودجكن في مدينة كربلاء ، العراق .

الخلاصة

الخلفية: فيروس الابسن بار هو فيروس شائع الانتشار ويكون عادة مرتبط بالأورام اللمفاوية. ومع ذلك ، في كربلاء وفي مدن العراق الاخرى ، لا يعتبر كشف الفايروس جزءًا من عملية تشخيص الإصابة بالورم اللمفاوي.

الهدف من هذه الدراسة: دراسة مدى انتشار أنواع الفايروس في الانوع الفرعية من الاورام اللمفاوية الهودجكين بين المرضى من محافظة كربلاء ، العراق.

االطريقة: من الشرائح النسيجية المثبتة بالفومالين والمغمورة بالشمع لـ 31 مريض بمتوسط العمر 32.56 ± 15.685 (من 5 إلى 70سنة) المشخصين بورم الغدد اللمفاوية هودجكين خلال الفترة من 2010 إلى 2018 في مدينة كربلاء تم دراستها. تم مراجعة التشخيص النسيجي . استخدم حوالي 25 ملغم من المقاطع النسيجية المخزونه لاستخلاص الحمض النووي. تم استخدام مستخلصات الحمض النووي في تفاعلات الكوثرة باستهداف منطقة الجين EBNA3Cفي الجينوم الفيروسي. استخدم التفاعل لتشخيص الفايروس و تميز انواع فايروس الابسن بار .تم استخدام اختبار تي و مربع كاي لتقييم الارتباط بين العمر بالانواع الفرعية من سرطان الغدد اللمفاوية هودجكين والجنس و مقارنتها مع نتائج تشخيص الفايروس .

النتائج: كانت غالبية الأورام اللمفاوية هودجكين تنتمي إلى النوع الفرعي MCHL حيث كانت 18 (58%)حالة من 31 ثم النوع الفرعيNSHL في 13(42%) حالة. و لم تسجل حالات من الأنواع الفرعية الأخرى من الاورام الهوجكن في هذه الدراسة. الذكوراكثر اصابة من الإناث بالاورام اللمفاوية الهوجكن (16 مقابل 15) . كانت الفئة العمرية من 31 إلى 40 عاما الاكثر اصابة بالاورام اللمفاوية الهوجكن . تم الكشف عن الفايروس في 18 حالة من جميع الحالات (58 ٪). كان EBV1 أكثر شيوعًا في النوع الفرعي MCHL . كان EBV2 الأكثر شيوعًا بين حالات NSHL . على حد علمنا هذه هي المرة الأولى التي يتم فيها تسجيل مثل هذه البيانات في العراق.

الاستنتاج: ارتبطت معظم حالات الاصابات بالاورام اللمفاوية الهوجكن مع فايرس الابسن بار .هناك اختلاف في توزيع أنواع الفيروس وفقا لأنواع سرطان الغدد اللمفاوية.

INTRODUCTION

Epstein–Barr virus (EBV) belongs to the group of gamma-herpes viruses and was the first recognized human oncovirus (Vranic *et al*., 2018). It infects at least 90% of the population worldwide (Dunmire et al., 2018). EBV associated with several diseases whose incidence varies dramatically in different parts of the world (Ahmed *et al*., 2017). EBV is associated with different malignancies in different geographic regions remains puzzling and may be related to EBV genotypic variability through specific disease and geographic associations(Chang *et al*., 2009). For instance: gastric and nasopharyngeal carcinoma in the east Asia (china, democratic people's republic of Korea and Taiwan) (Khan and Hashim, 2014), Burkitt's lymphoma is a common cancer of children in equatorial Africa and Papua new guinea (Hsu and Glaser, 2000) and infectious mononucleosis (IM) adolescents/young adults from western societies (Macsween and Crawford, 2003). EBV-associated lymphomas include NHL and classic Hodgkin Lymphoma (cHL), lymphomas arising in aids patients, immunocompromised individuals, peripheral T-cell lymphomas, angioimmunoblastic T-cell lymphoma, extranodal nasal-type natural killer/T-cell lymphoma, and other rare histotypes (Gala *et al*., 2017). There are two type of EBV, EBV type 1 (EBV1) and EBV type 2 (EBV2). EBV2 varies genotypically from EBV1 in key latency genes [eg, those encoding EBV nuclear antigen 2 (EBNA2), EBNA3a, and EBNA3c](Coleman *et al*., 2017).

The HL accounts for approximately 10% of all lymphomas and 1% of all cancers in industrial countries (Cuceu *et al*., 2018). The epidemiology of HL varies with age at clinical onset. In developing countries, the disorder appears predominantly during childhood and its incidence decreases with age, while in industrialized countries, the incidence rate increase with age (Mozaheb, 2013). The most frequently diagnosed cancers in male and female 15-29 year (Aben *et al*., 2012). In childhood varies by age such that HL is exceedingly rare in infants (Baharvand and Mortazavi, 2014). The HL annual incidence of 2–3 per 100000 in Europe and the United States of America (USA) (Thomas *et al*., 2002). Globally, EBV-positive HLs account for up to 40% of all HL cases, and they have been shown to vary substantially by patient demographic and tumors characteristics. The presence of EBV in HL is strongly associated with specific epidemiological features including male gender, ethnicity, mixed cellularity subtype, children and older adults, lower socio-economic status(Massimiliano Salati, 2014). The World Health Organization (WHO) classifies HL into 5 subtypes – 4 are referred to as Classical Hodgkin Lymphoma (CHL) (Nodular sclerosis classical Hodgkin Lymphoma (NSHL), Mixed cellularity classical Hodgkin Lymphoma (MCHL), Lymphocyte-Rich classical Hodgkin Lymphoma (LRHL) and Lymphocyte-Depleted classical Hodgkin Lymphoma (LDHL) and 1 is classified separately as Nodular Lymphocyte-Predominant Hodgkin Lymphoma (NLPHL). These classifications are based on what the lymphoma cells look like under a microscope (Modkharkar *et al*., 2018). The most common subtype is the NSHL which accounts for 70% of the cases of classical HL, this followed by the MCHL subtype which account for 20 to 25% of the cases (Swerdlow Steven H et al., 2016). In Iraq, the Incidence Rate of HL 2.78% in 2010 (Iraqi Ministry of Health 2010) and 2.36% in 2011 in top ten cancer (Iraqi Ministry Of Health, 2014). The HL not record in top ten cancer in 2009 (Iraqi Ministry of Health, 2009). In Karbala city, HL did not record in top ten cancer in 2009 (Iraqi Ministry of Health, 2009), 2010 (Iraqi Ministry of Health 2010) and 2011 (Iraqi Ministry Of Health, 2014).

Therefore aim of this study: to study the prevalence of EBV types in subtype HL among patients from Karbala province, Iraq.

METHOD

Study design:

This was a laboratory-based retrospective cross-sectional study on archival formalin-fixed paraffin- embedded lymphoma tissue stored from April 2010 to March 2018 in Karbala city. The study was accomplished at the medical research laboratory of medicine collage of Kerbala University. Al-Kafeil super speciality Hospital, Al-Hussein Teaching Hospital and Al-Sajad laboratory are the sites of helping for the collection of samples. All cases diagnosed as lymphoma depending on clinical history, histopathological examination and immunohistochemical finding.

Sampling Frame

From thirty –one patients with Hodgkin lymphomas, archival FFPE tissue stored from April 2010 to March 2018 were collected and studied. The histopathological report of these cases included: all age groups which ranged from 5 years to 70 years old at diagnosis, gender (male16 and female 15), subtype of HL and different anatomical tissue were obtained (cervical 27, axillary 3 and groin 1).

Detection of EBV and its Subtypes in Lymphoma

Section cutting

Sections of tissue block were obtained by the cut at 35- micron thickness by the microtome. Those were transferred to labeled sterile containers. Sterile blades and workplace were kept each sample. After each section, the microtome and workplace were disinfectants with cotton and bleach 10% in distilled water **(Ryan et al., 2004)** to avoid the contamination of samples. Gloves were used in all steps of working.

DNA Extraction

Up to 25 mg tissue sections of FFPE put in 1.5 ml microcentrifuge tube. DNA was extracted by using gSYNC™ DNA Extraction kit (Cat.No.GS100 Geneaid Biotech Ltd. Korea) Purified DNA extraction stored at deep freeze -20 ℃.

Detection of purified DNA

For the purpose of ensuring the purity of the DNA and the absence of any inhibitor for the process of amplification by using PCR, the human β-actin gene (housekeeping gene) was used as internal control. For accomplished amplify the gene fragment, add 1µl DNA primer (conc.10 Picomole) for each (Forward primer: 5‘-GCCATGTACGTTGCTATCC-3‘ and Reverse primer: 5‘-CCGCGCTCGGTGAGGATC-3‘) primers were obtained From(Bioneer, Korea), 2µl genomic DNA as template and 16 µl free nuclease water with 5 µl maxime PCR PreMix. The final volume was 25µl. The PCR mixture was done by Thermal Cycler started with initial denaturation at 95 ℃ for 2 minute, followed by 35cycles including: denaturation at 94 ℃ for 30 second, annealing at 55 ℃ for 1 minute and extension at 72 ℃ for 1 minute flowed elongation at 72 ℃ for 7 min yield than hold 4℃ for indefinite time. Final product was 200 bp. From amplified product, 5µl carry in agarose gel [1.5 gm agraose per 100 ml Tris/ Borate/ Ethylene Diamine Tetra Acetic acid (TBE buffer)] with 1µl ethidium bromide (10 mg/ml) on TBE buffer] by Horizontal Electrophoresis at 70 volts for 60 min. A100 bp ladder suitable for use as molecular

weight standards for electrophoresis. Gel visualized by micro DOC Gel Documentation system and documenting the results in photography picture.

Detection of EBV DNA and typing by EBNA3C gene

Used specific primer to detection EBV and determine the types by PCR technique. Specific prime for EBNA3C gene was used for this purpose. The sequence of this primer is 5‘-AGAAGGGGAGCGTGTGTTGT-3‘for Forward and 5‘-GGCTCGTTTTTGACGTCGGC-3‘for Reverse. Final product yield 153bp for EBV type 1 and 246bp for EBV type 2. The PCR amplification accomplished by 1.5 µl each primer (conc.10pM), 6µl genomic DNA as a template and 11µl nuclease-free water with 5µl maxime PCR premix kit. Final volume 25µl. PCR condition started with initially denaturation at 95℃ for 2 minutes followed by 35cycle include denaturation at 94 ℃ for 30 seconds, annealing at 55 ℃ for 45 seconds and extension at 72 ℃ for 1 minute. Finally, elongation at 72℃ for 7 minutes produces than hold 4℃ for an indefinite time. From final amplified product carried 5µl in agarose gel [1.5 gm agrose per 100 ml TBE buffer with 1µl ethidium bromide (10 mg /ml) on TBE buffer] by Horizontal Electrophoresis at 70 volts for 60 minutes. A100 bp ladder was suitable for use as molecular weight standards for electrophoresis. Gel visualized by micro DOC Gel Documentation system and documenting the results in photography picture.

Data Analysis

Data were analyzed with Statistical Package for the Social Science (SPSS) Software Version 22 for Windows. T test and chi-square were used to determine statistically association between EBV state with age, age group and subtype of HL.

Ethics approval

This study is restricted by working at the laboratory and there is not close contact with patients. Ethic’s approval was obtained from Karbala Health Directorate number 3522 on 11 December 2017. All wasted materials and pieces of equipment were collected, packaged and delivered to the Al-Hussein Teaching Hospital for a good and safe disposable by burning.

RESULTS

Detection of subtype of HL dependent on histopathologic report

All the HL blocks which were 31 samples with mean age 31.32±17.421 stored from April 2010 to March 2018 that documented by histopathology report as HL included in this study (Table 1). HL were affected16 out of 31(51.6%) male with mean age 36.37±19.575 and 15 out of 31(48.4%) with mean age 25.39 ±13.398 female (Figure1). HL most affected age group were those aged 31-40 years (Figure2). Of all cases, MCHL was reported 18(58%) out of 31 with mean age 34.33±14.365 while NSHL 13(42%) out of 31 with mean age 27.15±20.832.

Detection of EBV and its types by PCR technique

Beta-actin (a housekeeping gene) was used as internal PCR control to ensure the efficiency of the DNA extraction procedure. Specific primers were used to amplify 200bp fragments. Figure 3 showed 200 bp amplified products were successfully amplified from all DNA extracts. Both type EBV1 and EBV2 were detectable by amplified fragment 153bp for EBV1and 246bp for EBV2 by targeted EBNA3C gene (Figure 4). Of all 31 HL analyzed in this study, DNA of EBV was detected in 18 (58%) out of 31 with mean age 32.65±15.685 while 13(42%) out of 31with mean age 29.62±20.119 were EBV negative

(Figure5). Of all EBV detection in HL, 10(55.56%) out of 18were MCHL while 8(44.44%) out of 13 for NSHL. In HL positive result for EBV, EBV1 was detected in 9 cases and the same number for EBV2.of all cases of EBV1, MCHL was6 versus 3for NSHL. Of all cases EBV2, MCHL was 3 versus 5 for NSHL (Figure 7).

Table 1: characteristic of all HL subtype and statistically comparison

Parameter		MCHL	NSHL	P-value	
№ of cases = 31		18	13	0.806	N.S*
Gender	Male	9	6	0.833	N.S
	female	9	7		
Age Range		12 -70 years	5-70 years	0.168	N.S
Prevalence of EBV		10	8	0.739	N.S
Type of EBV	EBV1	6	3	0.343	N.S
	EBV2	4	5		

***N.S : Non Significant**

Figure 1: Distribution of gender in HL.

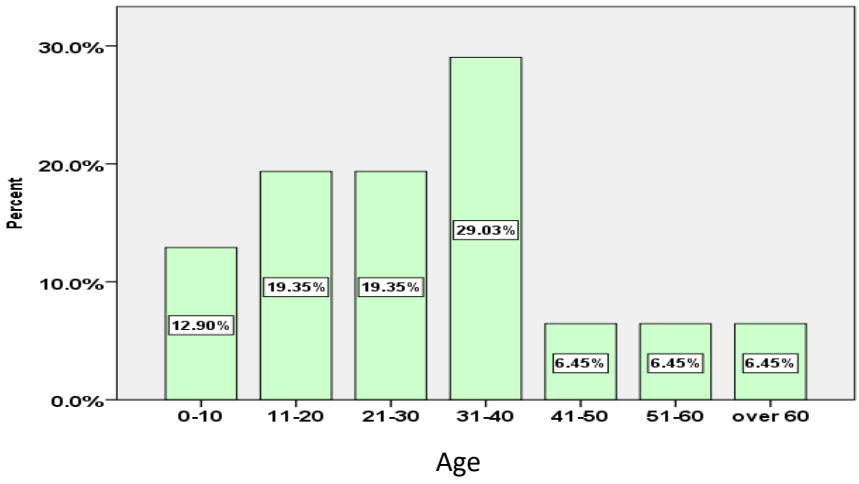

Figure 2: Age range distribution in HL

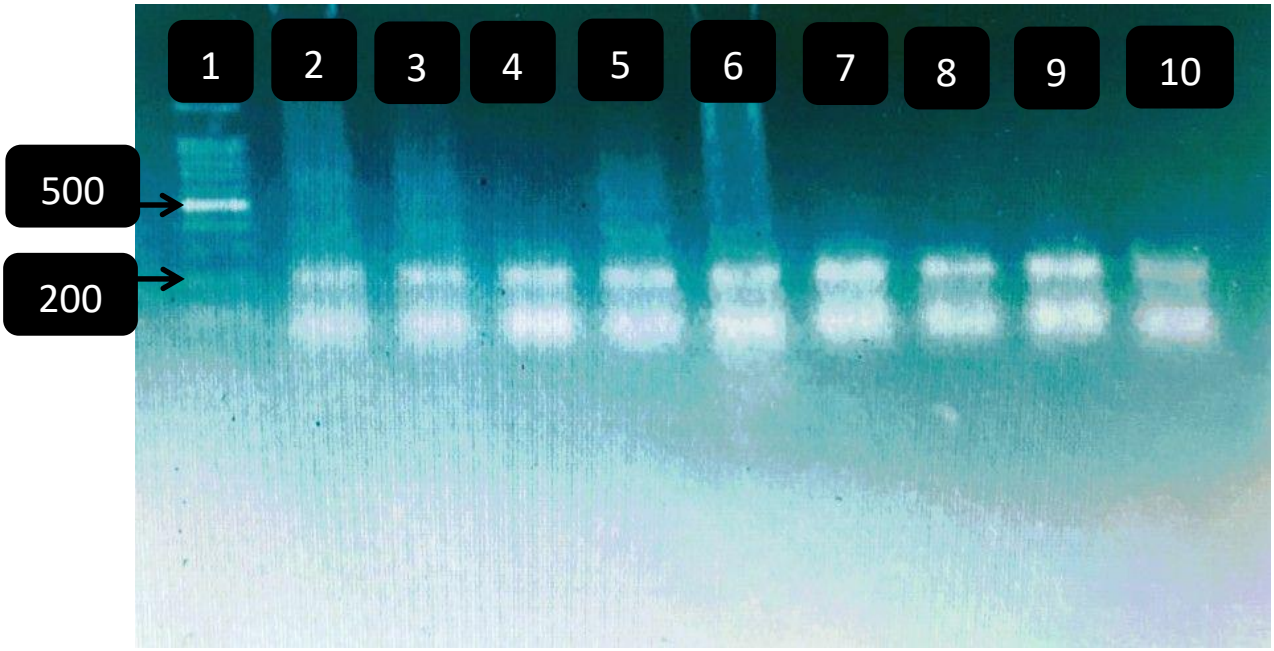

Figure 3: β-actin (control) in selected sample. Lane 1, 100bp DNA ladder, lane2, 3, 4, 5, 6 positive results for EBV1. Lane 7, 8,9,10 positive results for EBV2

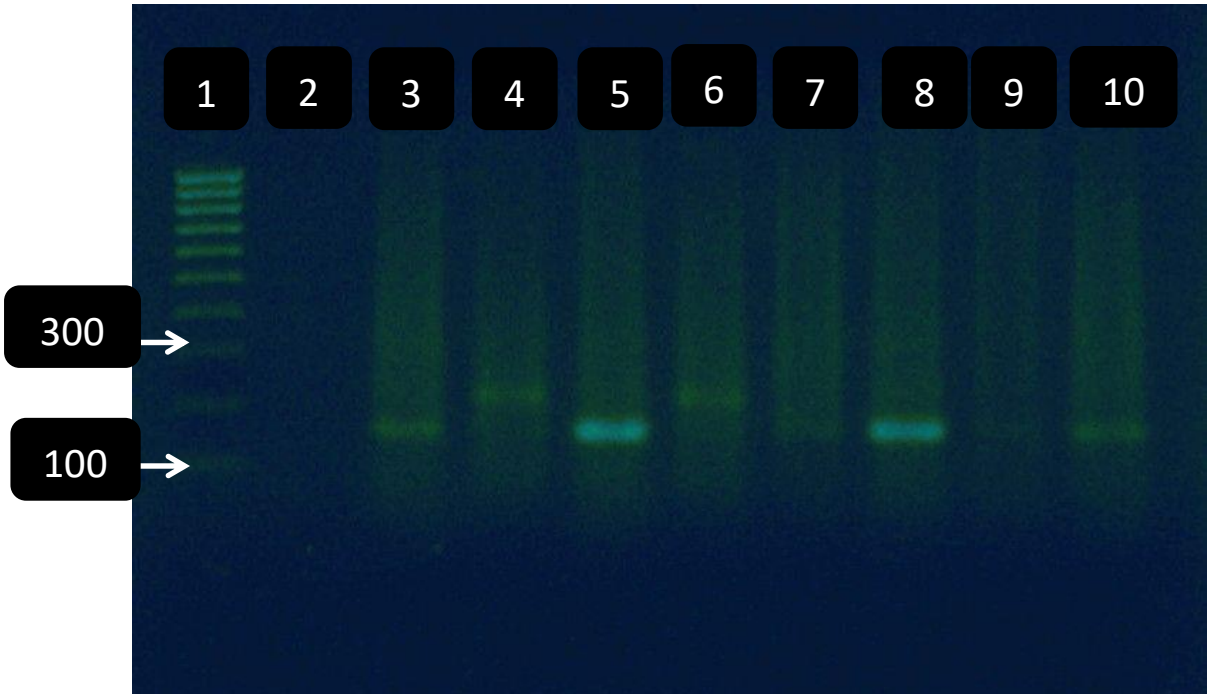

Figure 4: EBV detection and subtyping. Lane 1, 100bp DNA ladder .Lane2 negative control , lane 3positive control for EBV1,Lane 4positive control for EBV2,Lane 5,7,8,9,10 positive result for EBV1. Lane 6 positive e results for EBV2

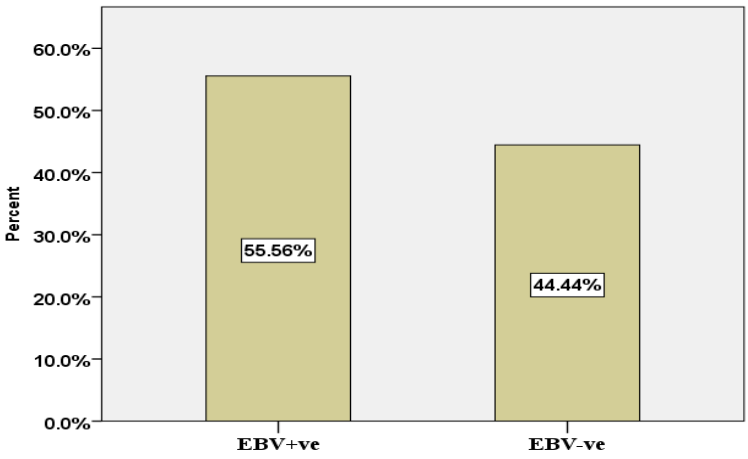

Figure 5: EBV DNA detection in all HL

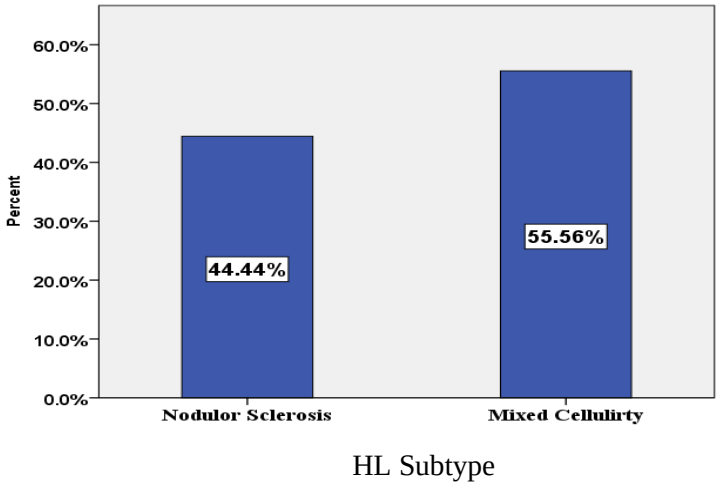

Figure 6: EBV DNA detection in all HL subtype

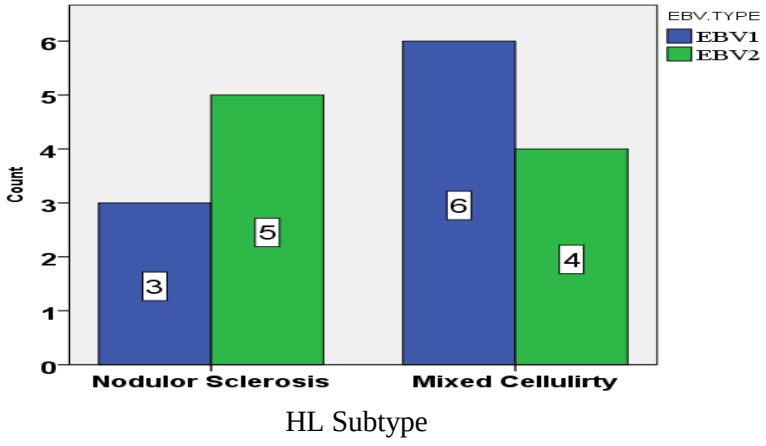

Figure 7: EBV types detection in each subtype of HL

DISCUSSION

Epidemiologic, serologic and molecular studies have all suggested that EBV is involved in the development of a significant proportion of lymphoma (Prabhu and Wilson, 2016). The identification of strain variants is an effective approach to investigating virus transmission and persistence (Sitki-Green **et al**., 2003). Diagnostic information regarding the prognosis and treatment of EBV-associated malignancies was more dependent upon the accurate morphological classification of these tumors than on whether EBV was identified (Michelow *et al*., 2012).

From the review of the results of persons diagnosed with the HL, we find that the age of less than 30 years was high, taking into account that the highest rate was between the age of 31 - 40 and these results correspond to studies in Iraq (Al-Barzinji, 2006) , Lebanon (Sader-Ghorra et al., 2014) , but they differ with studies in the United States (Shenoy et al., 2011). In developing countries, HL appears more during childhood and its incidence decreases with age, while in developed countries, young children are rarely affected by HL in contrast with young adults where incidence increase with ages (Al-Tonbary, 2012). HL is an uncommon neoplasm of B-cell origin with an incidence that varies significantly by age, sex, ethnicity, geographic location and socioeconomic status (Salati et al., 2014).

In this study finding, MCHL was more than half of the HL subtype follow NSHL without any record another subtype of HL without any statistically significant association between HL subtype to age range (chi-square analyses failed because 92.9% of data have expected count less than 5) and HL subtype to gender (chi-square had p-value 0.833 greater than0.05). This results consistent with Iraqi studies (Ridha et al., 2006), (Dina Wael *et al*., 2012), Jordan (Almasri, 2004) Iran (Hashemi Bahremani et al., 2007) but not trend with another Iraqi study (Saeed, 2009), Saudi Arabia study (Al Diab et al., 2003) and another Iranian study (Shamloo *et al*., 2017) that regarding NSHL more common. This finding not consist with common and known about NSHL that approximately 70% of all CHL, and perhaps an even higher proportion in developed countries, NSHL is the most frequent subtype and its incidence has continued to rise over the past decades (Eberle et al., 2009). The result of this study are consistent with results of the study conducted by study finding conducted by (Thomas *et al*., 2002) who found the most common subtype among the young adults is NSHL while the frequency of MCHL increases with age for this reason, this study compatible with type II patterns in rural areas of developed countries dependent Correa and O'Conor35 concept (Flavell and Murray, 2000).

The percentage of EBV infection in HL was slightly higher than half without any statistically significant association between EBV infections to the subtype of HL (chi-square had p-value 0.739greater than0.05). The result of EBV infection in HL dose not consists with Iraqi study which was reported of EBV infection: 44(86%) out of 51 cases (Di Napoli *et al*., 2013), 15(37.5%) out of 40cases (Mohammed, 2009), 45(90%) out of 50 cases (Subh and Ghasaq, 2012) and negative result in all 3 cases (Al-Hasnawy *et al*., 2016) but relatively close with another which reported 21(66%) out of 30 cases (Dina Wael *et al*., 2012). Comparison of this study with EBV infected with HL to other studies outside of Iraq find that relatively close with the Jordanian studies, which have reported 43% out of 100 cases (Sughayer *et al.*, 2014) Egyptian study 28(63%) out of 45 cases (Audouin *et al*., 2010). With respect to geographic variation, EBV rates in HL from North America, Europe and Japan have been reported to vary between 20-50% (Quintanilla-Martínez *et al*., 1995), while several studies from Central and South America showed an incidence rates varying

from 50 to 95% (Chang *et al*., 1993). In a report from China, evidence of EBV expression in cases of HL reached 65% (Zhou *et al*., 1993), and in a large series from Kenya, EBV was

detected in 92% of HL cases (Leoncini *et al.*, 1996). Many studies suggest a genetic predisposition to develop EBV associated HL (Massini *et al*., 2009). It seems that factors other than socioeconomic profile and age distribution influence the relationship among HL development, its morphologic features, and EBV infection. The HL continues to be a fascinating disease, and more studies are necessary to understand the peculiarities of its geographic and age distributions and the true influence of EBV infection on its pathogenesis(de Oliveira *et al*., 2002)

In this study NSHL subtype more affected with EBV than MCHL. This not consists with Iraqi study, which has reported 79% MCHL, 16% LDHL and 5% for NSHL and LPHL of all 25cases EBV positive result with HL (Ridha et al., 2006). As well as this finding not compatible with Turkey study, this has reported 18(63%) MCHL to 5(16%) NSHL out of 30cases (Bağır *et al*., 2018), Jordan study MCHL 20(80%) of all 26 cases to NSHL20(30%) out of 66 cases (Sughayer *et al*., 2014), Iranian study MCHL10(50%) out of 20 cases follow NSHL8 (28%) out of 28cases (Tanyildiz *et al*., 2015), china study 20(76.9%) out of 26 cases MCHL to 20(44.7%) out of 44cases NSHL (Qin et al., 2018) and Hungary 23(50%) MCHL follow NSHL 16(35%) out of 47 cases (Keresztes *et al*., 2006) but consist with south Pakistan study which has reported5 (83.33%) out of 6 cases NSHL to38(79.16%) out of 48 cases MCHL (Azhar *et al*., 2016).

From the reflection and reviewing the results in this study found that the type of EBV1 equalize EBV2 in patients with HL in Karbala city without any statistically significant association between EBV types to the age range (chi-square analyses failed because 100% of data have expected count less than 5), EBV types to gender (chi-square analyses failed because 100% of data have expected count less than 5) and EBV types to HL subtype (chi-square analyses failed because 100% of data have expected count less than 5). To the best of our knowledge, no study from the kerbala city and Iraq investigated the link between the type of EBV and HL subtype. The tow type of EBV distinguished by the differences in EBNA-2gene, since the divergence in EBNA-2 reveals only 54% homology between the two types (Münz, 2015). Interestingly, it was found that the EBV types noticeably differ in their transformation abilities. For instance, the EBV type 1 transforms the B lymphocytes more willingly than type 2 *in vitro*, and when a recombinant type 2 virus acquired the Type 1 EBNA-2A gene, it gained the transforming ability of type 1 virus (Rickinson et al., 1987). One study suggest that EBV2 is an important human pathogen that present in a wider geographic distribution of HIV associated NHL than originally thought (Boyle *et al*., 1991).

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