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A MOLECULAR AND IMMUNOHISTOCHEMICALL STUDY ABOUT THE ROLE OF EPSTEIN –BARR VIRUS IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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ABSTRACT
Epstein Barr virus (EBV) is ubiquitous virus which infects the majority of the human population and is the causative agent of many B-cell tumors this virus expressed many protein and it is associated with a variety of B-cell tumors. Recently many studies have suggested a causal relationship between EBV and Chronic lymphocytic leukemia. In this study we investigated the association between EBV and CLL. Aim of this study the found the correlation between EBV latency proteins in CLL patients. Expression of (EBERs, LMP1, and EBNA2) in bone marrow biopsy was evaluated in 30 CLL patients and 20 as control by ISH and Immunohistochemistry respectively. Increased of EBERs, LMP1 and EBNA2 were significantly higher in CLL patients compared with control LMP1. Our result suggests that these EBV proteins are accusation with CLL.

KEY WORDS: Epstein-Barr virus, Latent membrane protein, EB nuclear Protein, EB encoding –RNAs, Immunohistochemistry, *In situ* hybridization, Chronic lymphocytic leukemia.

INTRODUCTION

Epstein-Barr virus and it's the first virus described to be linked with the human pathogenesis of tumor. In 1968, EBV was recognized to be the etiological factor of infectious mononucleosis (Diehl *et al*., 1968). At the same time, EBV was reported to alter infected B cells to uncontrolled proliferation (Diehl *et al*., 1968; Burkitt, 1969; Hjalgrim and Engels, 2008). EBV is an abundant virus that infects over 95% of early middle age individuals. The virus was exposed in Burkitt lymphoma 30 years ago and since then the cell–virus communication and the host/ virus communication have been widely considered (Anquan *et al*., 2001; Ayln *et al*., 2006). In 1991, researchers first noticed a rare but characteristic type of stomach cancer that had very similar features to nasopharyngeal cancer. When they tested the models and found that, nearly all of them were positive for EBV (Shibata *et al*., 1991). Much newer study reported that EBV association with chronic lymphocytic leukemia. CLL is the commonest type of leukaemia in the western world, accounting for 40% of all leukaemias in individuals over the age of 65 years. The average age of presentation is between 65 and 70 years. The male/female ratio in all populations is about 2:1 (Estella and Claire, 2004). In CLL cases which display EBV infection, EBV markers are however detectable lonely in a subpopulation of tumor cells (Tsimberidou *et al.*, 2006; Tsimberidou *et al*., 2006). Notably, several studies have reported that expression of EBERs noticed by in situ hybridization is associated with progressive or accelerated clinical courses (Tsimberidou *et al*., 2006). CLL cells can become sometimes infected with EBV in vivo provided that mitogenic and activating signals are within the microenvironment (Dolcetti *et al*., 2010). EBV alters B-lymphocyte growth, causing permanent growth transformation by regulated expression of multiple viral genes. These genes comprise three integral membrane proteins, latent membrane proteins 1, 2A, and 2B (LMP), 6 EBV nuclear antigens (EBNA1, 2, 3A, 3B, 3C, and EBNA-LP), then two small, non-coding nuclear RNAs (EBERs). (Tselis and Jenson, 2006). EBV small non-coding (NC) and non-polyadenylated RNAs called EBV-encoded RNA (EBER) (Rickinson and Kieff, 2007). EBERs confer fighting to RNA-dependent protein kinase (PKR)-induced apoptosis upon BL cells (Nanbo *et al*., 2002). Previous researches have also confirmed that EBERs induce the transcription of different cytokines such as (IL-10, IL-9, IL-8) depending on cell type, , insulin-like growth factor-1 (IGF1) in epithelial cells, and these cytokines later act as autocrine growth factors for the EBV-infected cancer cells (Iwakiri *et al*., 2005). EBERs have been shown to enhance the growth and proliferation of epithelial cell lines derived from NPC and GC (Tsang *et al*., 1998). Thus, expression of 1 or more of the EBV late genes may play an important role in inducing proliferation effects and possibly disease progression in CLL patients (Laytragoon- Lewin *et al*., 1995; Dolcetti *et al*., 2010). Latent membrane protein 1 (LMP1), a main viral protein expressed during EBV infection, is a confirmed oncogene in rodent fibroblasts, and along with the nuclear proteins EBNA1, EBNA2, and EBNA3a and c, is considered

essential for EBV transformation of human B cells in vitro (Brennan, 2001) · Through activating viral as well as cellular target genes, EBNA-2 starts the transcription of a cascade of primary and secondary target genes, which finally govern the activation of the resting B-cell, cell cycle entry and propagation of the growth transformed cells. (Maeda *et l.*, 2001).

MATERIALS & METHODS

This prospective study consisted of 50 Bone marrow biopsy were collected from 30 CLL patients and 20 control with average age (40-80 years), the samples were collected directly from patients in Baghdad Teaching Hospital, from the National Center of Hematology and from many private histopathology laboratories that generously helped as and are kindly thanked in the present dedication.

❖ ***In situ hybridization for detection of EBV by EBERs***:

The presence of certain nucleic acid sequences in cells or tissue can be detected with in situ hybridization using labeled RNA Probes. The hybridization results in duplex formation of sequences present in the test object and the specific gene probe.

It is indirectly detected using an enzyme-conjugated antibody targeting the tags: the enzymatic reaction of chromogenic substrates leads to the formation of a colour precipitate that is visualized by light microscopy.

Procedure

A: Slides preparation: Serial thin sectioning of (4 m) thickness was done for each paraffin-embedded tissue block and sticking the sedtions on charged slides.Paraffin sections were deparaffinized (Dewax) in oven at 60Co overnight .

B: Deparafin and rehydration was done by serial steps in glass staining jars containing the following:

- Xylene (100%) for 15 minutes (two times).
- Ethanol (100%) for 5 minutes (two times).
- Ethanol (95%) for 5 minutes (one time).
- Ethanol (70%) for 5 minutes (one time).
- Distilled water for 5 minutes. (one time)
- Dry the slides for 5 min at 37℃.
- Wish the slides 3 time by 1xPBS 5 min.

C: Add pepsin solution was applied in tissue and incubatesd in (30-40) in humidity chamber at 37℃ and wishing the slid for 5 min and dry section.

D: Denaturation and Hybridization:

- Vortex the probe and pipette (5-8) µl Probe to the samples.
- Cover slip and denature the slid in humidity chamber in 75c for 5 min.
- Incubate slid overnight in 55c or 60 min.

E: Post-hybridization and Detection:

- Removing the rubber cement Carefully.
- Preparing two wash buffer in jar (in room temperature and in 55℃) for 5 minutes.
- Rinsing the slides in wash buffer at room temperature for 5 minutes and then washing the slides in wash buffer at 55c for 5 minutes.
- Washing the slides in wash buffer at RT for 5 minutes.
- NBT/BCTP was applied and incubated for 90-120 minutes at 37◦C in a humidity chamber.
- Washing 3 times by Deionized water or distilled water for 5minutes.
- Adding nuclear fast Red for 2 minutes.

G: Dehydration:-Dehydrating the sections serial concentration of alchohol:

- Ethanol: 70%,95%,100%(one time) ,100% (2time)and 2 min. for each concentration and finally incubate in xylen 100% for two minutes .

E: Mounting: Add mounting medium (DPX) and Read the slide under light microscopy.

❖ ***Immunohistochemistry :***

✓ Latent membrane protein -1 and EBNA-2

I- principle of the test

The samples were rehydrated and treated with protein blocking agent to reduce non-specific binding of antibodies. The tissues were incubated with Primary AB to binds to specific Ag, Biotinylated secondary Ab to binds to the primary Abs, Streptavidin peroxidase reagent to binds to secondary Ab. The streptavidin binds to biotin on the secondary Abs: then peroxidase serves as the indicator enzyme. The last step addition of peroxidase substrate (hydrogen peroxide) and colored chromogen resulted in the formation of colored in the tissue Ag.

II- Procedure: All steps were done at room temperature

A- Dewaxing: The same method of dewaxing used with ISH was also applied for immunohistochemistry .

B- REHYDRATION :

- Add Xylene: 100% for 15 minutes 2 times
- Add Ethanol: 100 % for 5minutes 2 times
- Add Ethanol: 95% for 5 minutes .
- Add Ethanol: 70% for 5min .
- Wash in D.W for 5min
- Ari Dry section for 5 minuter at 37℃ .

C: Add enough drops of hydrogen peroxide Block to cover the section incubate 10 min .Wash 2 times in buffer and Air dry section.

D: - Retrieval

Unraveling antigenic epitopes by retrieval methods is important for successful immunohistochemically staining and detection of protein .Slides were placed in bath containing retrieval solution 1ml of citric acid +100ml D.W was added and boiled in 95℃/PH=6 for 20min.

- Washed with Buffer for 5min

E: Power block

Enough drops of protein Block were added to cover the sections for10min in 25C˚and slides were put in humidity chamber. Then slides were drained for 5 minutes .

- Slides were air –dried

F-Primary antibody

The slides were covered with enough drops of ready to use primary (anti LMP-1 and Anti EBNA2) after that incubated for overnight in a humidity chamber at RT .After that all the slides were rinsed with PBPs for 5 minutes

G- Apply secondary antibody

Biotinylated coat Anti-mouse was added for 30-45min and buffer was washed for 5 min and was dried.

H-Add streptavidin peroxidase reagent : The slides were covered with enough drops of streptavidin peroxidase and incubated for 15 minutes at RT and a slide was washed for 5 minutes.

I-Chromogen : Enough drops of DAB chromogen were applied in dark room (30µlDAB chromogen +1.5mlDAB substrates) for 10 min and buffer for 5min and slides were dried.

J: Hematoxylin counter stain : Enough drops of haematoxylin were added for 2minutes and Washed by tap water.

L: Dehydration:-dehydrated the sections by using series concentration of alcohol: (100%,95%,70%) %one time) and 100% (two times), two minutes for each solution ; finally incubation in xylen 100% for two minutes .

M: Mounting: Enough drops of mounting media were placed to cover the section and let dry over night at RT .

•DPX +cover slip.

•Light microscopy.

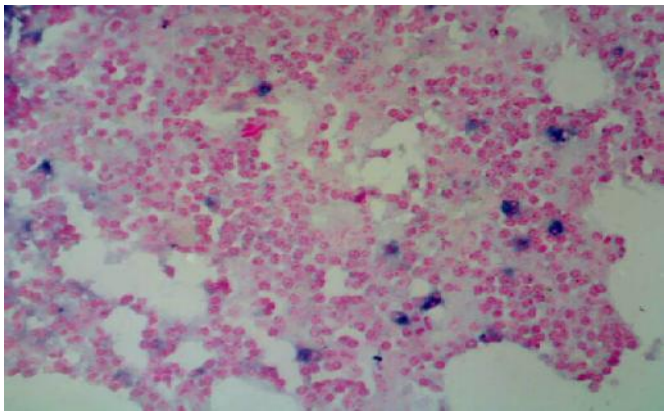
Statistical analysis

Data were translated into a computerized database structure. The database was examined for errors using range and logical data cleaning methods, and inconsistencies were remedied. An expert statistical advice was sought for. Statistical analyses were done using IBMSPSS version 21 computer software (Statistical Package for Social Sciences) in association with Microsoft Excel 2013.

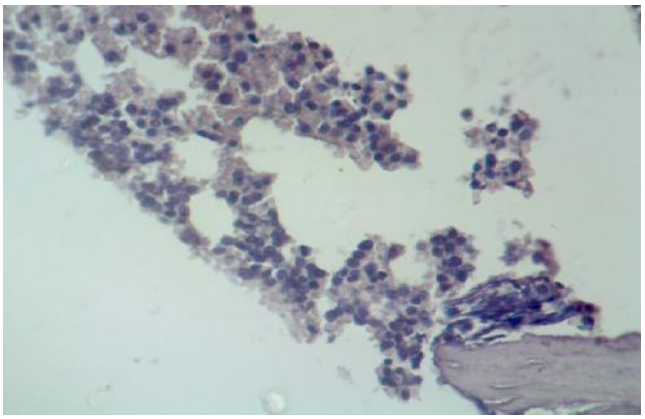
RESULT

Viral markers

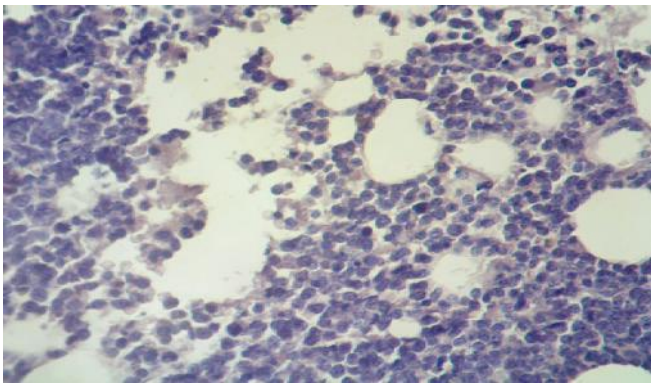
As shown in table (1), all the controls were negative for EBERs. Although the median score and intensity for EBERs was negative for cases group, the mean rank for EBERs score, intensity and composite score (a score resulting from multiplying the score by intensity) was significantly higher among cases compared to controls, figure (1(a), 2, 3, 4). As shown in table (2), all the controls were negative for EBNA2 and 43.3% of CLL patient were positive for EBNA2 . The mean rank for EBNA2 score, intensity and composite score (a score resulting from multiplying the score by intensity) was significantly higher among cases with CLL compared to controls,(patient and healthy control) figures (1(B),5,6,7). As shown in table (3), only 2 cases (10%) of the controls had a positive LMP1 marker. These 2 cases had a low score (+) and a weak to moderate intensity. The median LMP1 score was significantly higher among cases with CLL (+) compared to controls (negative). Similarly the median EBERs intensity was also significantly higher among cases (weak) compared to controls (negative). In addition the median composite score was significantly higher among cases compared to controls, figures (1(C), 8, 9, 10).



A



B



C

FIGURE 1: A) High grade, showing strong nucleic staining of EBERs by ISH (B and C) showed the positive result of (LMP-1 and EBNA-2) by immunohistochemical staining Respectively ,X100)

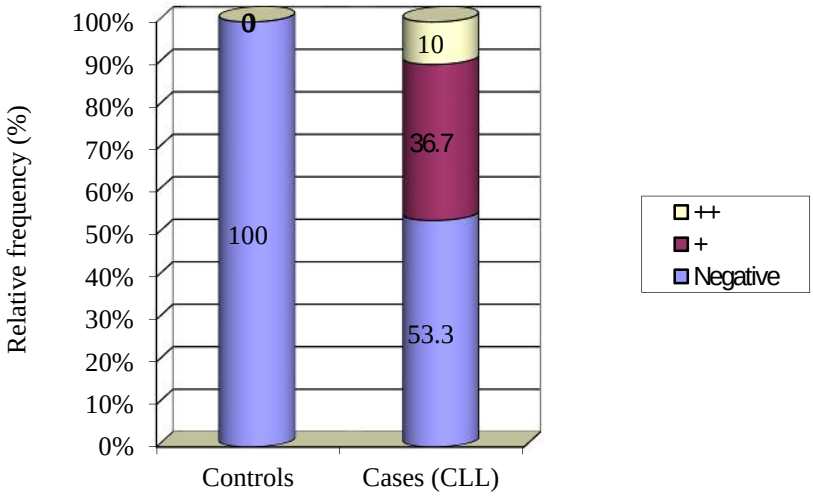


FIGURE 2: Component bar chart showing the case-control difference in EBERs-score

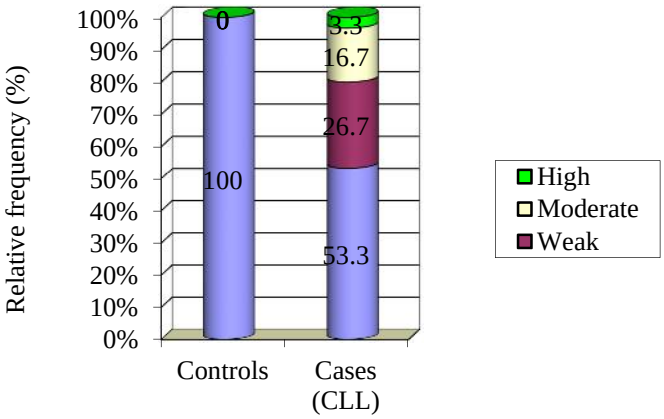


FIGURE 3: Component bar chart showing the case-control difference in EBERs-intensity

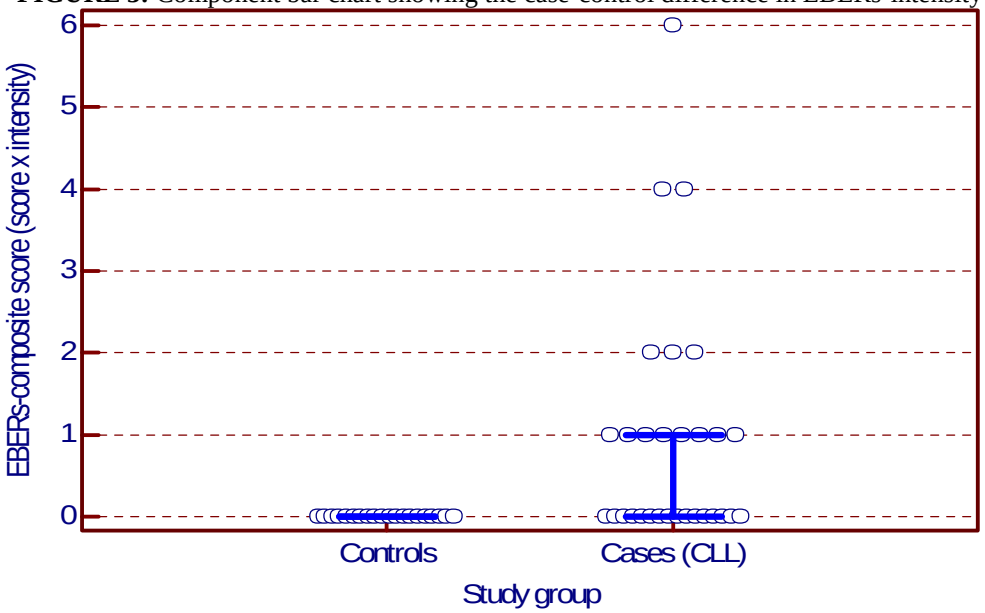


FIGURE 4: Dot diagram with error bars showing the median (with its inter-quartile range) EBERSs in cases with CLL compared to controls

TABLE 1: The case-control difference in median score and intensity and composite score for EBERs viral marker

		Study group				
		Controls		Cases (CLL)		
		N	%	N	%	P
1.	EBERs-score					
	Negative	20	100.0	16	53.3	
	+	0	0.0	11	36.7	
	++	0	0.0	3	10.0	
	Total	20	100.0	30	100.0	
	Range	(Negative to Negative)		(Negative to ++)		<0.001
	Median	Negative		Negative		
	Inter-quartile range	(Negative to Negative)		(Negative to +)		
	Mean rank	18.5		30.2		
2.	EBERs-intensity					<0.001
	Negative	20	100.0	16	53.3	
	Weak	0	0.0	8	26.7	
	Moderate	0	0.0	5	16.7	
	High	0	0.0	1	3.3	
	Total	20	100.0	30	100.0	
	Range	(Negative to Negative)		(Negative to High)		
	Median	Negative		Negative		
	Inter-quartile range	(Negative to Negative)		(Negative to weak)		
	Mean rank	18.5		30.2		
3.	EBERs-composite score (score x intensity)					<0.001
	Range	(Negative to Negative)		(Negative to 6)		
	Median	Negative		Negative		
	Inter-quartile range	(Negative to Negative)		(Negative to 1)		
	Mean rank	18.5		30.2		

TABLE 2: The case-control difference in median score and intensity and composite score for EBNA2 viral marker

		Study group				
		Controls		Cases (CLL)		
		N	%	N	%	P
1.	EBNA2-score					<0.001
	Negative	20	100.0	17	56.7	
	+	0	0.0	7	23.3	
	++	0	0.0	5	16.7	
	+++	0	0.0	1	3.3	
	Total	20	100.0	30	100.0	
	Range	(Negative to Negative)		(Negative to +++)		
	Median	Negative		Negative		
	Inter-quartile range	(Negative to Negative)		(Negative to +)		
	Mean rank	19		29.8		
2.	EBNA2-intensity					<0.001
	Negative	20	100.0	17	56.7	
	Weak	0	0.0	3	10.0	
	Moderate	0	0.0	8	26.7	
	High	0	0.0	2	6.7	
	Total	20	100.0	30	100.0	
	Range	(Negative to Negative)		(Negative to High)		
	Median	Negative		Negative		
	Inter-quartile range	(Negative to Negative)		(Negative to Moderate)		
	Mean rank	19		29.8		
3.	EBNA2-composite score (score x intensity)					<0.001
	Range	(Negative to Negative)		(Negative to 9)		
	Median	Negative		Negative		
	Inter-quartile range	(Negative to Negative)		(Negative to 2)		
	Mean rank	19		29.8		

TABLE 3: The case-control difference in median score and intensity and composite score for LMP1 viral marker

		Study group				
		Controls		Cases (CLL)		
		N	%	N	%	P
1.	LMP1-score					<0.001
	Negative	18	90.0	13	43.3	
	+	2	10.0	6	20.0	
	++	0	0.0	10	33.3	
	++++	0	0.0	1	3.3	
	Total	20	100.0	30	100.0	
	Range	(Negative to +)		(Negative to ++++)		
	Median	Negative		+		
	Inter-quartile range	(Negative to Negative)		(Negative to ++)		
	Mean rank	18		30.5		
2.	LMP1-intensity					<0.001
	Negative	18	90.0	13	43.3	
	Weak	1	5.0	5	16.7	
	Moderate	1	5.0	10	33.3	
	High	0	0.0	2	6.7	
	Total	20	100.0	30	100.0	
	Range	(Negative to Moderate)		(Negative to High)		
	Median	Negative		Weak		
	Inter-quartile range	(Negative to Negative)		(Negative to Moderate)		
	Mean rank	18.3		30.3		
3.	LMP1-composite score (score x intensity)					<0.001
	Range	(Negative to 2)		(Negative to 8)		
	Median	Negative		1		
	Inter-quartile range	(Negative to Negative)		(Negative to 4)		
	Mean rank	18		30.5		

TABLE 4: The risk of having CLL in the presence of specific viral marker

Positive marker	Controls (n=20)		Cases (CLL) (n=30)					
	N	%	N	%	OR	95% CI of OR	Chi	P
EBNA2	0	0.0	13	43.3	31.6	(3.8 to 264.6)	10.157	0.001
LMP1	2	10.0	17	56.7	11.8	(2.3 to 60)	8.793	0.003
EBERs	0	0.0	14	46.7	36.0	(4.3 to 301)	10.952	<0.001

TABLE 5: Area under ROC curve for score, intensity and composite score of selected viral markers when used as test to diagnose CLL cases differentiating them from healthy controls

	ROC area	P
LMP1-score	0.752	0.003
LMP1-composite score (score x intensity)	0.750	0.003
LMP1-intensity	0.741	0.004
EBERs-score	0.733	0.006
EBERs-intensity	0.733	0.006
EBERs-composite score (score x intensity)	0.733	0.006
EBNA2-score	0.717	0.01
EBNA2-intensity	0.717	0.01
EBNA2-composite score (score x intensity)	0.717	0.01

Risk of having CLL in the presence of specific viral marker

As shown in table (4), the risk of having CLL was significantly increased in the presence of a positive viral marker of any of the 3 tested types. The highest risk was for EBERs, followed by EBNA2, while the lowest risk was for LMP1. The risk of having CLL is increased by 36 times for a subject with positive EBERs, the risk of having CLL is increased by 32 times for a subject with positive EBNA2and the risk of having CLL is increased by 11.8 times for a subject with positive LMP1.

A positive EBNA2 and EBERs markers was significantly higher among CLL cases sta (P-value 0.001 and <0.001 respectively) compared to controls. A positive LMP1 statically was significant among cases with CLL (p0.003) compared to controls figure (11).

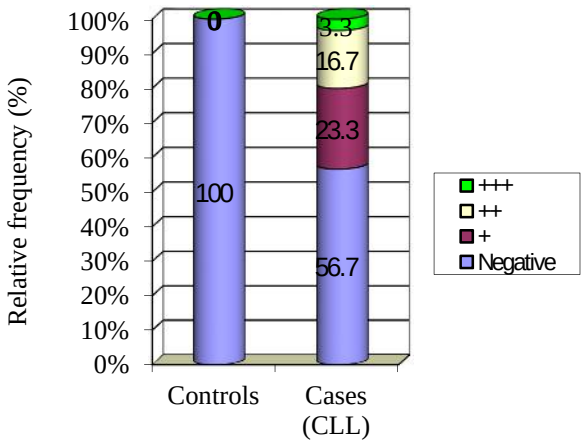


FIGURE 5: Component bar chart showing the case-control difference in EBNA2 score

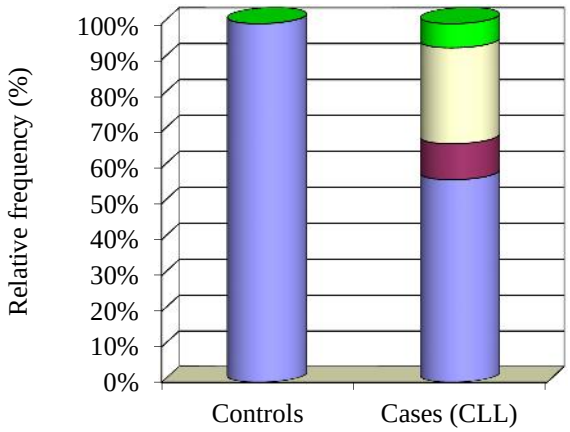


FIGURE 6: Component bar chart showing the case-control difference in EBNA2-intensity

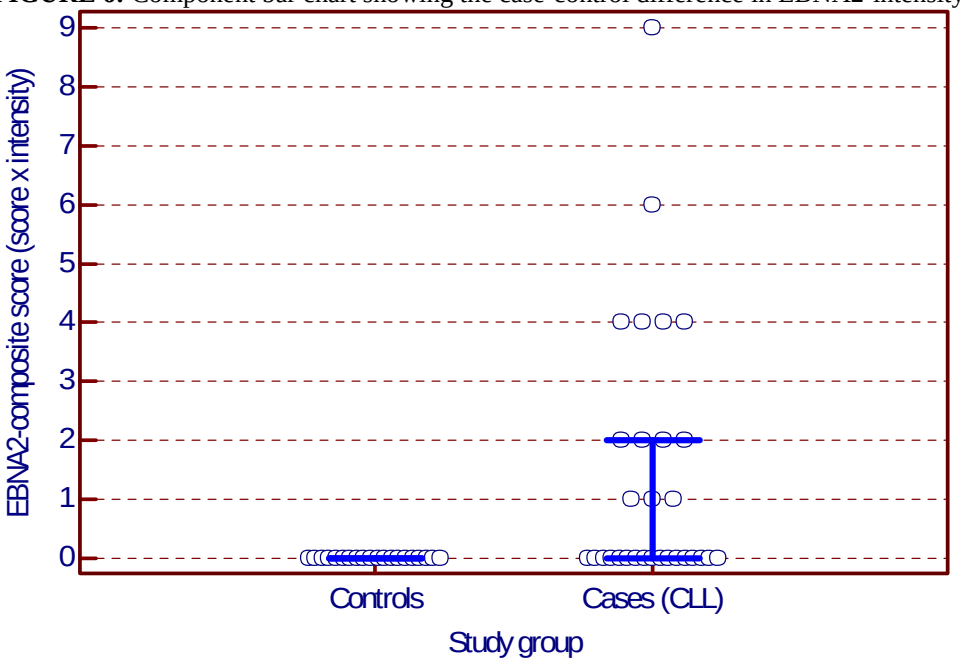


FIGURE 7: Dot diagram with error bars showing the median (with its inter-quartile range) EBNA2 in cases with CLL compared to controls.

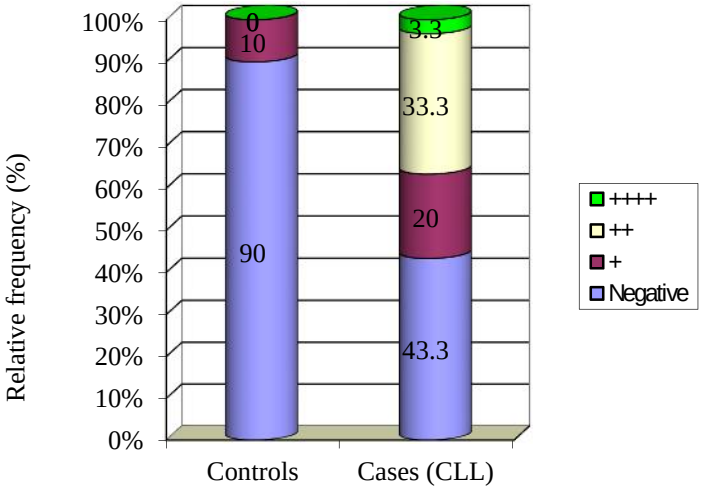


FIGURE 8: Component bar showing the case- control difference in LMP1-score

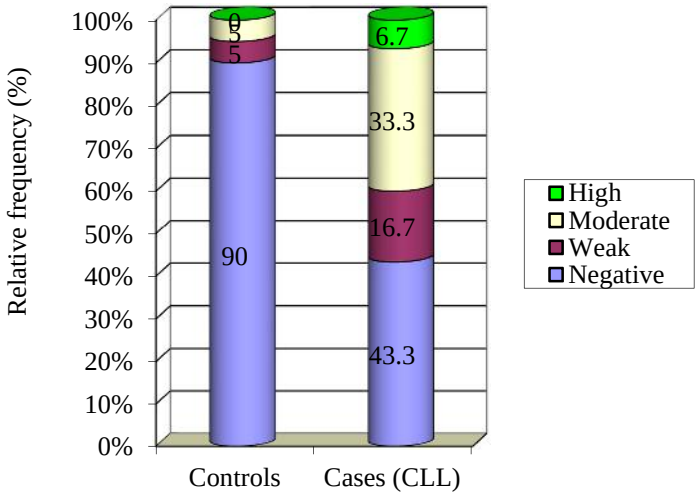


FIGURE 9: Component bar chart showing the case-control difference in LMP1intensity

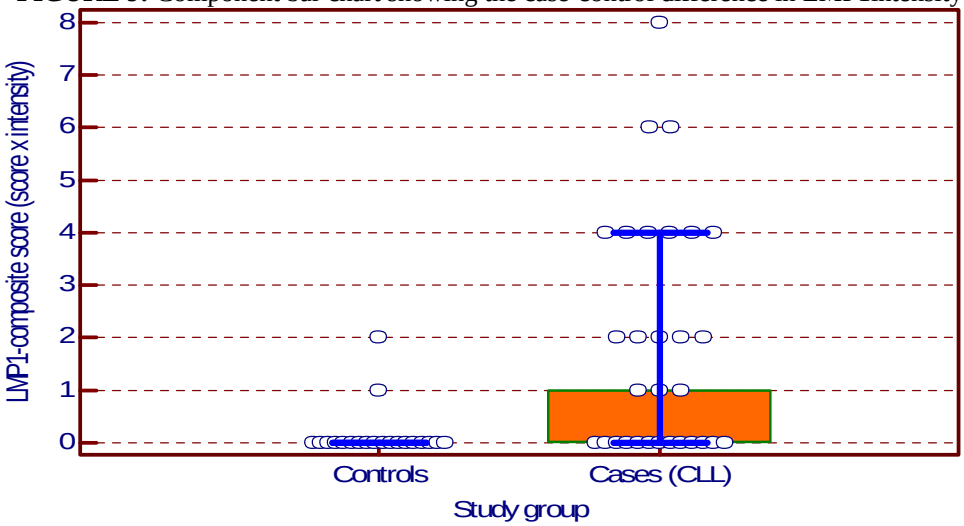


FIGURE 10: Dot diagram with error bars showing the median (with its inter-quartile range) LMP1 in cases with CLL compared to controls.

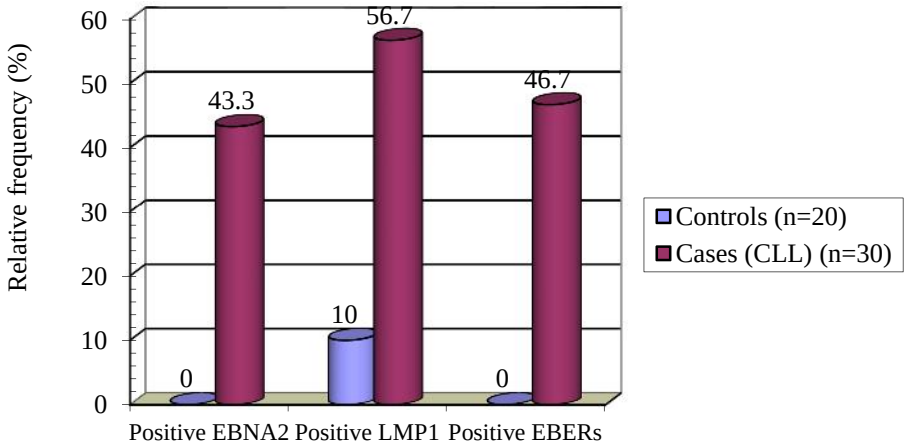


FIGURE 11: Bar chart showing the case-control difference in positivity rate of selected viral markers

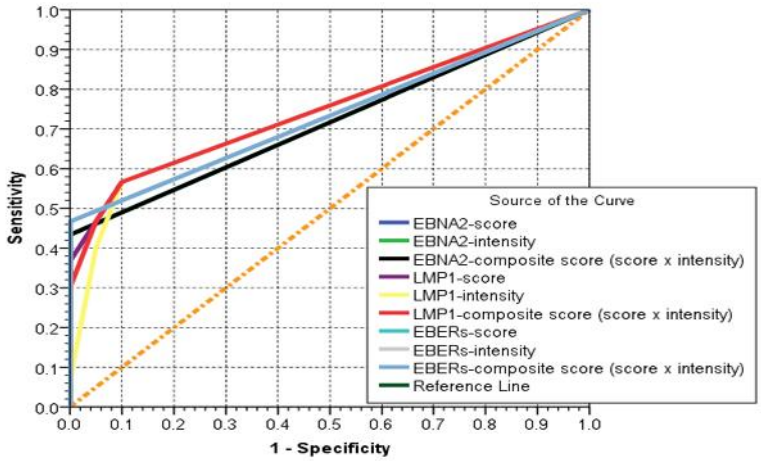


FIGURE 12: ROC curve showing the tradeoff between sensitivity (true positive rate) and 1-specificity (false positive rate) for score, intensity and composite score of selected viral markers when used as test to diagnose CLL cases differentiating them from healthy controls.

Association between study parameters

The association between interleukins and the 3 viral markers was assessed among subjects in the cases group only, since the outcome measurements were mainly negative among controls which made these parameters constant and fails to show any association.

Viral markers

As shown in table (5) and figure (12), the three viral markers were tested in 3 forms: score, intensity and composite score. The 3 forms of each viral marker measurements were associated with a similar propensity for discrepancy between cases with CLL and controls (since none of the controls had a value other than negative, with a minor exception for LMP1).

DISCUSSION

Viral markers

EBERs

Epstein- Barr virus with variety in B-cell tumors including Burkitt's lymphoma, Hodgkin lymphoma, human immunodeficiency virus, post transplantation lymphoma disorder and Chronic lymphocytic leukemia. Many studies reported that CLL patient had evidence of EBV infection by In situ hybridization for EBERs and detection of EBV-encoded EBER transcripts is considered the gold standard for localizing latent EBV in tissue samples, as EBER transcripts are universally expressed in all EBV associated tumors (Tsimberidou *et al*., 2006; Cohen *et al*., 2014). The presence of EBERs has been shown to correlate with progressive or accelerated clinical course including transformation to Richter's large cell lymphoma (Ansell *et al*., 1999, Tsimberidou AM *et al*., 2006). However EBERs is also found in quiescent EBV latency where no protein is produced and that may be a suboptimal marker for proliferation or transformation capability. In this study all controls showed negative result EBERs but in patients the result show that 14 (46%) out of 30 CLL patients were positive with EBERs. Results obtained are nearly compatible to study which has been done by (Tsimberid *et al* ., 2006) who reported that (38%) of CLL patients had evidence of

EBV infection proved by EBERs positively in tumor cells. Result demonstrated in this study was accordance with (Timberidou *et al*., 2006) referring that 12 out of 32 CLL patient has appositive result. On the other hand (choen *et al*., 2014) found that 8 of 75 (10.7%) cases showed EBERs expression restricted to 5–10% of tumor cells. This contriver in the above results may be related to that ISH process depends on the RNA staining and the concentration of RNA in the cell .this method affected by many factors, including the RNA present in the cell and concentration of RNA. This technique is very sensitive.

EBNA2

Many studies reported that EBNA2 support cell proliferation since strong over expression of one of EBNAs direct target genes C-Myc in a sufficient way to induce proliferation of EBERs positive cell (Polack *et al* ., 1996)

In this study result showed that from a total of 30 CLL patients, EBNA2 was present in 34% of them which is statically significant as compared with the control. This result is highly compatible with a study reported by (Bandobashi *et al*., 2005)who found that 19-30% of CLL cell expressed On the other hand our result incompatible with (Laytragoon-Lewin *et al*., 1995) who didn't found EBNA2 in all cases of CLL and (Aman and Mellsted, 1991) who found EBNA2 is positive in 1% of CLL population. The accurance of EBNA2 in this study was quite low of we compare with many result who found high percent of EBNA2 in B-cell and this result are detected in the early phase of the disease, because as we mention above that EBNA2 play a very important role in the early proliferation of the cell but in the present study all of the sample has been taken in the late stage of the disease which may explain this low occurrence of EBNA2 in this study.

LMP1

Latent membrane protein (LMP1) is a viral mimic of human tumor necrosis factor receptor (TNFR), drives from cell cycling through nuclear factor kappa B, and blocks apoptosis through Bcl-2-associated X protein. LMP1 transfection alone is sufficient to immortalize B cells. Expression of one or more of the EBV late genes may play an important role in inducing proliferation effects and possibly disease progression in CLL patients and the detection of LMP-1 expression in tumor cells of a fraction of CLL cases particularly intriguing and deserves further investigation. (Tarrand *et al*., 2010; Dolcetti and Antonino , 2010).

In this study results obtained revealed a total of 30 patients with CLL, LMP1 was present in 17(56.6%) and in only 2 cases (10%) of the controls had a positive LMP1 marker, Table(4.4) this result is not in agreement with other study who has been done by (Tarrand, *et all;* 2010) who found that EBV LMP1 mRNA transcripts were found in (14%) of the CLL cases, but only 1% of the healthy controls ($P < .0001$), other study done by (Ohshima *et al* ., 1997) showed that the expression of LMP was found in three cases from (14) of B cell lymphoma with integrated EBV .

Bandobashi,k, *et al;2004* found that the infected B-Cll cells exhibit an unusual EBV program, they express the nuclear proteins but not latent membrane protein 1 (LMP-1). EBV infected B-CLL cells and can be regarded as model for this viral program. In B cells the regulation of LMP-1 is executed mainly by EBV encoded nuclear antigen 2 (EBNA-2), interacting with several cellular proteins and these complexes bind to specific sequences in the LMP-1 promoter. The biologic explanation for variation in LMP1 expression levels is not well understood. From the above result we found that 10% of control have got LMP1 (positive) result which could be explained as an induction for starting cell transformation and as I refer before these controls are apparently healthy which means that may be these positive cells with LMP1starting the earliest stage of transformation but not passing to a cancer cell but It can be considered as an important marker which give as a question that may make these cells capable to transform to a cancer cell.

CONCLUSION

This study revealed the following conclusions:

In situ hybridization technique is successful method in the detection of EBV and its association with tumor stage and positive EBERs.

Imminohistochemistry is of valuable value in the diagnosis of LMP1 and EBNA2 in relation to age and gender .

RECOMMENDATION

- Large sample size of chronic lymphocytic leukemia and other type of leukemia like acute lymphocytic leukemia or acute myeloid leukemia to improve the role of EBV.
- Study the role of Bcl-2 which association with EBNA-2 and LMP-1 activation and cell proliferation.
- Detect the integration of LMP-1 in cellular nucleic acid by PCr anf sequencing assay.
- Study the mutation gen in CLL cells and the role of EBv in this type of mutation.

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