Molecular Co-Localization of Human Epstein Barr Virus Infection Associated with the Expressed Protein of Cyclin D1 \Cyclin Dependent Kinase 4 in Hodgkin's Lymphoma Tissues

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Abstract

This retrospective case-control study to assess the impact of Cyclin D1 and CDK4 expression along with EBV infection with Hodgkin's lymphoma (HL).80 lymph node included in this study; (40) formalin-fixed, paraffin- embedded biopsies from Hodgkin's lymphoma (HL), and (40) apparently healthy lymph nodes as control group. EBV detection was done by *CISH* whereas IHC detection system was used to evaluate both Cyclin D1 & CDK4 gene expression.

The positive –*EBERs* CISH reactions detected in 16 / 40 (40 %) of Hodgkin lymphoma tissues while positive - *EBERs* reactions detected in 3 out of 40 tissues (7.5%) in control lymph nodes tissues. Statistically was found highly significant between study groups (P value = < 0.05). Cyclin D1 & CDK4 -IHC were detected in 40% and 52.5% of HL, respectively. Statistically, significant differences noticed among negative, low and moderate scoring of tissues (P<0.05).

Significant EBV gene expressions along with higher percentage of tissues in which Cyclin D1 & CDK4 genes being expressed may be indicator their possible role in the pathogenesis and / or the carcinogenesis of a subset of our Hodgkin's lymphoma cases.

Key word: *EBV; Hodgkin's lymphoma (HL), Cyclin D1;CDK4, CISH, IHC.*

Introduction

As an unusual malignancy, HL is characterized of Hodgkin/ Reed Sternberg cells by the inflamation infiltrates. Two types of HL are recognized on morphologic, immunophenotypic, and clinical bases; classical (cHL) as well as and nodular lymphocyte-predominant Hodgkin lymphomas [(1).]

As a ubiquitous human herpetic virus, EBV has a global seroprevalence of 95%. Usually, EBV infection is asymptomatic in childhood, while in adolescents, infection frequently results in infectious mononucleosis[(2)].

The three forms of cHL, pediatric as well as older adults HL are EBV-positive types, while HL of young adults is EBV-negative type. Moreover, EBV is also associated with the development of other lymphomas, including natural killer / T-cell lymphoma and diffuse large B-cell lymphoma [(3).]

Reed-Sternberg cells are EBV positive in 40% of the times and demonstrate latency II pattern.It was showed that consistent presence of 1 of the 3 chromosomal translocations in BL deregulate the c-myc expression [(4).] Although still controversial, it has revealed a presence of EBV in small proportion of nodular lymphocyte-predominant HL cases · However, the link of classical Hodgkin lymphoma with EBV is most clearly

established, and was first implicated in the pathogenesis of such lymphomas in patients who had raised antibody titers to EBV preceded development of these lymphomas by several years [5].

EBV-positive BL cells have gene expression profile similar to the latency form resembling memory B cells[6] with switching from one to other EBV – latency forms in B-cells might regulate the viral life cycle and redirecting the EBV-positive B cells towards long-term persistency in their memory pools. LMP1 replaces the survival as well as differentiation signals provided by an activated CD40 receptor [7].

The CCND1 gene in hematological malignancy , due to either translocation, amplification, deletions, point mutations of 3′-UTR of t(11;14)(q13;q32) gene, or genetic alteration. Cyclin D1, with cyclin-dependent kinases (CDKs), play a key role in regulation of cell cycle during the G1/S transition [8].

The cyclin D1 gene expression is associated classically with lymphoma while abnormalities of cyclin D1 & CCND1 gene expression were not well studied in nodular Hodgkin lymphomas [9] .

The oncogenic potential of *CDK4* gene was experimentally shown necessary for the transforming and immortalizing effect of *cyclin D1* [10].

Here, we aimed in the present research work was explore both the possible impact of EBERs-EBV as well as to assess the effects of expression of cyclin D1 and CDK4 proteins on the cellular proliferation of lymph node tissues in order to gain further insight into the proliferation profile of a group Iraqi patients with Hodgkin's lymphomas.

Materials and Methods

Few other 4µm - tissue sections were mounted on charged slides for application of CISH & IHC techniques.

For the detection of EBERs by CISH kit (purchased from Zyto Vision GmbH. Fischkai, Bremerhaven. Germany), the Chromogenic In Situ Hybridization procedures were performed according to the manufacturing company instructions.

Immunohistochemistry / Detection system (purchased from Abcam. England) was used to estimation the protein genes products of Cyclin D1 & CDK4 by using specific monoclonal antibody for that antigen determination on the targeted Cyclin D1 & CDK4 protein.

In the current study, and for statistical analysis, Chi –square test was used via Version– 21 SPSS program and P value was significant when p <0.05.

Results

I-Results of EBERs-EBV in Hodgkin Lymphomatous tissues:

Sixteen out of 40 tissues with Hodgkin lymphomas showed positive chromogenic in situ hybridization reactions (constituting 40% of the total Hodgkin lymphoma tissues) (Table 1). The healthy lymph nodes tissues revealed seven and half percent (3 / 40). The differences between the percent of study groups showed statistically highly significant difference (P value = < 0.05).

Table (1): Results of EBERs - Associated HL Using CISH Technique

EBERS-EBV-	HL (no.=40)	%	A.H Control (no.=40)	%	P-value
Negative	24	60%	37	92.5	Z test P=0.003 sign. (P<0.05)
Positive	16	40%	3	7.5	

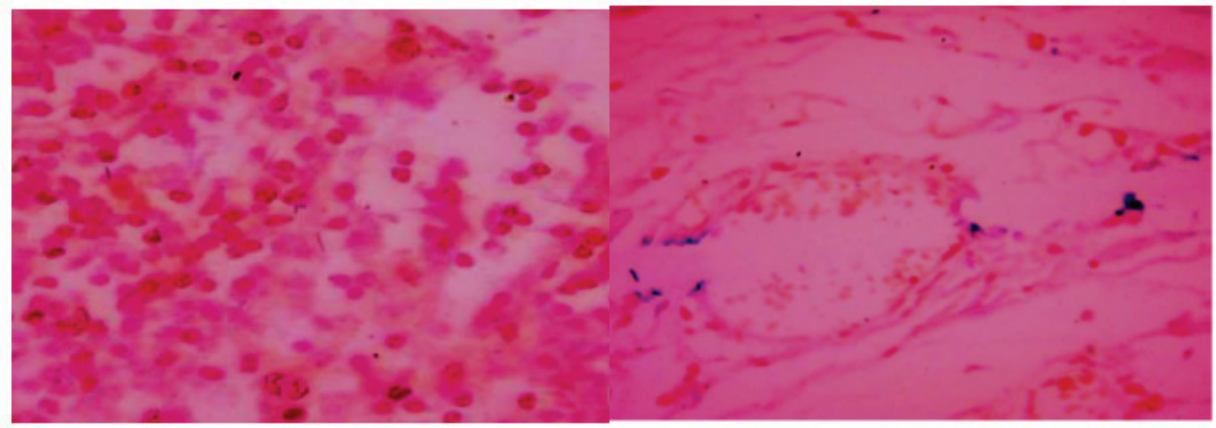

Figure 1: CISH reaction for EBERs- EBV detection in lymph node tissue sections with Hodgkin Lymphoma: (40X). (A) Negative reaction; (B) positive reaction.

II. The Results of Cyclin D1 -IHC Signal Score in tissues with Hodgkin Lymphoma.

Table (2) shows the positive result of Cyclin D1 IHC detection where 52.5 % from HL showed positive signals including 22.5 % in the low score (I) followed by 17.5 % and 12.5% in the moderate score (II) and high score (III), respectively, while the percentage of positive signals in AHC was (7.5%), including 5 % score (I) followed by 2.5 % in score (II) (Fig. 2 A& B). Statistically, significant differences were found among scorings of IHC signals (P<0.05).

Table (2): Cyclin D1 -IHC Score Signaling in Hodgkin Lymphoma

Cyclin D1 -IHC signal scoring		Hodgkin lymphoma(n=40)		A.H. Control (n=40)		P-value
		No.	%	No.	%	
Negative		19	47.5	37	92.5	
Positive		21	52.5	3	7.5	
Scoring	I	9	22.5	2	5	χ2test P=0.03 sign. (P<0.05)
	II	7	17.5	1	2.5	
	III	5	12.5	0	0	

III.CDK4 -IHC expression in patients with Hodgkin lymphoma:

Among the 40 Hodgkin lymphomatous tissues, 45% exhibited positive CDK4-IHC reaction, while in control group 12.5% (Table 3) .

The signal scores were as follows: 22.5% (9/40) low score (I) followed by 17.5% and 5% in the moderate score (II) and high score (III), respectively, The intensity signal was shown as intensity I in 25%, and 12.5% as intensity II, and lastly 7.5% as intensity III while in AHC 7.5% as intensity II, and 5% as intensity I (Table 3 & Fig. 2 C& D). Statistically significant difference (P<0.05) found between various intensities (Table 3).

Table 3: The grading of CDK4-IHC score and intensity signals in Hodgkin lymphoma

CDK4-IHC		A.H control		Hodgkin lymphoma (n=40)			P-value (χ2test)
		No.	% out of 40	No.	% out of 40	% out of 17	
Negative		35	87.5	22	55		
Positive		5	12.5	18	45		
Signal Score	I	1	2.5	9	22.5	47.1	0.02
	II	3	7.5	7	17.5	35.3	
	III	1	2.5	2	5	17.6	
Signal Intensity	Weak	2	5	10	25	52.9	0.04
	Moderate	3	7.5	5	12.5	35.3	
	Strong	0	0.00	3	7.5	11.8	

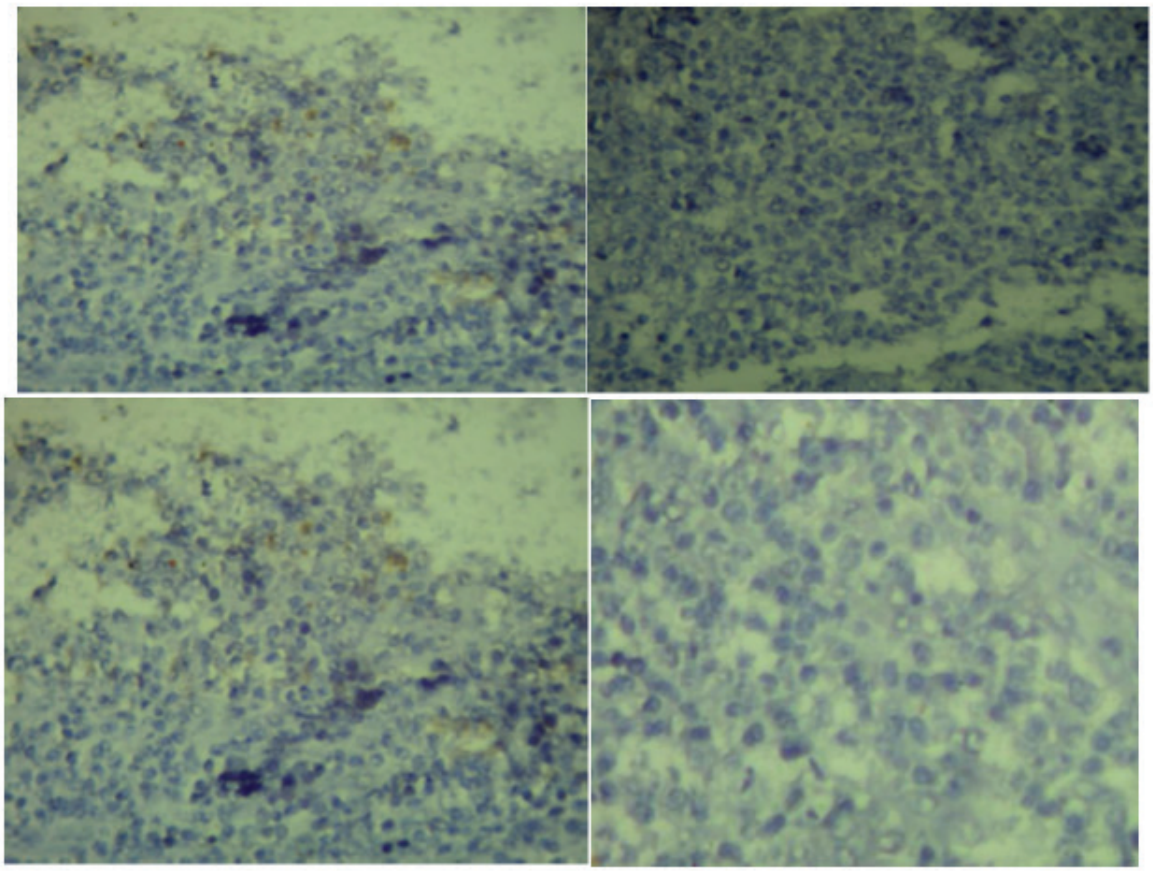

Figure 2: IHC staining of Cyclin D1 and CDK4 protein in lymph node sections with Hodgkin Lymphoma (×40): (A) Negative reaction Cyclin D1; (B) positive reaction Cyclin D1. (C) Negative reaction CDK4; (D) positive reaction CDK4.

IV. Correlations among the studied markers (EBERs-EBV; Cyclin D1 and, CDK4) in lymph node tissues from patients with Hodgkin lymphoma:

A significant was highly between EBERs / EBV and Cyclin D1 in Hodgkin lymphoma (r = 0.443, P = 0.003). Similarly, between CDK4 & grade of Hodgkin lymphoma (r = 0. 398; p= 0.044) was noticed. No significant correlations among EBERs / EBV and other parameters (Table 4).

Table 4 Spearman's rho of the studied parameters in Hodgkin lymphoma.

Spearman's rho		Age groups (years)	Grade	EBERs-EBV	Cyclin D1	CDK4
Grade	r	.165				.398*
	P	.104				.044
EBERs-EBV	r	0.040	0.133			
	P	0.765	0.412			
Cyclin D1	r			.443**		
	P			.003		
CDK4	r	0.158	0.432	0.483		
	P	0.347	0.006*	0.003*		

* highly significant

Discussion

The present results revealed that grade I - malignant lymphomas have affected our Iraqi studied patients at an earlier age than that those expected globally, a finding that could be attributed either low mean age & inadequate screening of these patients or to the small enrolled samples in the current study, when compared to those abroad studies. More researches are to be done to elucidate the causes for such frequency of these grade I - lymphomas, as fronted by the majority (54.5%) of low-grade lymphomas results in western countries [11].

In Iraq, Epstein-Barr virus and hCMV were studied in tissues from Non-Hodgkin lymphomas, [12,13] while in Hodgkin's Lymphoma tissues and up to our best knowledge only one study on Human T Cell Lymphtropic Virus Type-1- genes was studied [14] but no research study on EBV was found.

A pathogenic role for EBV in HL has been suggested by the finding of monoclonal EBV genomes in HRS cells indicating role for EBV as an early event, prior to clonal expansion, expressing type II latency genes (i.e. EBNA-1, LMP-1, LMP-2 , EBERs and BARTs RNAs) [15].

The loss of a functional BCR and its signaling components are combined to prevent virus replication during tumor development and maintenance of EBV-associated HL [16].

The epidemiologic studies demonstrated an increased risk to HL in those patients with infectious mononucleosis and further showed that they had an elevated antibody titers against EBV antigens preceding their disease ,and EBV -positive tumor cells in a subset of their tissues [17].

In the current study, we found 16 \ 40 tissues with HL showed EBV positive CISH (40%) .Flavell and Murray,[18] , who found in the HL triggering mechanisms of pathogenic, or as reflection to the presence of depression in the immune regulation, rather than immunosuppression.

Another form of latency of EBV infections that are lacking EBER expression might present and as several previous studies by Bonnet *et al*.,[19] & Sugawara *et al*.,[20] have suggested. Another possibility is that this virus transformed the progenitor cells of Hodgkin's lymphomas by a "hit and run" mechanism .The EBV relation to this disease depends on many factors, including gender, age , ethnicity, resident country, and histological subtype.

Cyclin D1 partners are also present in breast tumors and colorectal cancers [21]. In Cho *et al*.,[22] study of NLPHL cases in correlated with CCND1 gene abnormalities, "20%" of LP cells were positive for "CD20"/"cyclin D1". The cdk4 hyperactivity has been well reported in many types of cancers, particularly melanoma, lung cancer and lymphoma [23].

The current result of cdk4-IHC detection showed that 45% (18/40) exhibited positive CDK4-IHC reaction in Hodgkin lymphoma group, while in control group 12.5% (5\40). Hernandez *et al*., [24] found overexpressed cdk4 in 21% of MCL cases. The results are consistent with Bockstaele *et al*. [25], was studied over two decades ago the biochemical and genetic characterization of D-type cyclins, their cyclin D-dependent kinases (cdk4 and cdk6), and the polypeptide CDK4/6 inhibitor $p16^{INK4}$ and revealed role of mammalian cells in regulation of the entry into regulation of pRb dependent manner in cell division cycle.

EBV along with *Cyclin D1* and *cdk4* over- expression in Hodgkin lymphoma patients of the current study are supporting an etiologic roles for that virus along with these genes in Hodgkin lymphoma development.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: None

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