Original paper

Co-localization of EBV and Expression of P16- Cyclin-Dependent Kinase inhibitor Protein in Tissues from Patients with Non-Hodgkin's Lymphoma

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Abstract

Background: EBV has been classified as a group 1 carcinogen associated with a variety of lymphoid and epithelial cancers. EBV was evidenced as a monogenic virus from its ability to transform normal human B cells, resulting in immortalization of the infected cells. A tumor suppressor protein, P16, is a cyclin-dependent kinase inhibitor plays a critical molecular role in cell senescence, regulation of the apoptosis pathway and G1 cell cycle arrest**.**

Aim of the study: To analyze the concordant frequency and impact of P16 protein expression and EBV infection on tissues from a group of patients with Non- Hodgkin's Lymphoma (NHL).

Patients and methods: Forty-five (45) formalin-fixed, paraffin- embedded tissues from lymph nodes biopsies were enrolled in this study; (30) lymph nodes biopsies were related to patients with NHL and (15) lymph nodes autopsies have included as apparently normal control group. Detection of EBV was done by ultra-sensitive version of in situ hybridization (ISH) method whereas immunohistochemical (IHC) system was used to demonstrate the protein expression of P16 tumor suppressor gene.

Results: Detection of EBV -ISH reactions in tissues with NHL was observed in 17 out of 30 (56.7%), while in the tissues from lymph nodes autopsies was detected in 6.7% (1 out of 15).Positive P16- IHC reactions were observed in 14 out of 30 NHL cases (47.1%). No P16 positive – IHC reaction was detected in healthy lymph node tissues in the control group. The differences between the percentages of EBV and P16 detection in NHL tissues and control tissues group were statistically highly significant (P value = < 0.0001).

Conclusions: The significant protein expression of P16 tumor suppressor gene as well as EBV infection in NHL in our results could indicate that cell cycle dysregulation and EBV-related transformation are important events in the pathogenesis of subset of NHL.

Keyword: EBV; P16; Non- Hodgkin's Lymphoma (NHL); In Situ hybridization; Immunohistochemistry.

Introduction

Lymphoma is a heterogeneous disease with multifactorial etiologies. It can be classified into two main categories: Hodgkin's Lymphoma (HL) and Non-Hodgkin's Lymphoma (NHL) [(1-2)] Over the recent decades, the incidence of lymphoma has been increased among the population and was steadily raised over the past few years, too [(3)].

Non- Hodgkin's lymphoma represents about 85% of lymphoma cases whereas 15% are Hodgkin's disease (HD) [(4)]. Non-Hodgkin's Lymphoma can occur in lymph node and other organs that contain lymph

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tissues. This cancer may be located in one place in the body but often located in multiple areas throughout the body. This is because cancerous (malignant), lymphocyte often circulate through the body just like normal lymphocyte. Non-Hodgkin's lymphoma can be either B-lymphoma or T-lymphoma depending on which type of lymphocyte become cancerous).

The risk factor of lymphoma has not been determined or clear yet. However, several risk factors have been implicated in the initiation and development of lymphoma, including Epstein - Barr virus (EBV) and P16 protein derrangments [5, 6].

Epstein–Barr virus (EBV) is a gamma herpes virus that has been demonstrated to be necessary for driving infected B cells toward proliferation, immortalization and malignant transformation in vitro [4,7]. This malignant transformation was suggested to be due to the expressions of number of latent EBV viral oncogene such as Latent Membrane Protein-1 (LMP-1) and some nuclear antigens [4, 7-8, 9]. Sequence analysis has defined two strains of EBV: type I and type II (alternatively named EBV A and B) which differ at the domains that encode EBV latent proteins, namely EBERs, and the nuclear antigens EBNA-LP,1,2,3A,3B and 3C in latently infected cells [10].

The small untranslated RNAs (EBER-1 and-2) are accumulated at high levels during all forms of latency and regulate apoptosis through different mechanisms. EBER-1 interacts with the interferon-inducible protein kinase R (PKRO, and inhibits its activation by double-stranded RNAs, protecting infected cells from INF-induced apoptosis [11].

The P16, a tumor suppressor protein, is known as a Cyclin-Dependent Kinase (CDK) inhibitor (also known as INK4a) that plays a critical role in the molecular mechanisms of cell senescence, regulation of the apoptosis pathway and G1 cell cycle arrest [12, 13]. In the G1 cell cycle regulation, the binding of P16 to the Cyclin-Dependent Kinase 4 and 6 (CDK4/6) leads to arrest cell in the G1 phase which, in turn, prevents the cell cycle progression from the G1 phase to the S phase [12,13]. Therefore, the loss of P16 can release cell from the G-phase which, in turn, causes cells to progress from the G1 phase to the S phase, while, the increased expression of P16 can cause the human cells to undergone senescence [12, 14].

Epstein–Barr virus (EBV) has the ability to promote cell cycle progression following the initial infection of primary resting B-lymphocytes and to cause cell cycle arrest at the onset of the viral replicative cycle. Various mechanisms have been proposed for the proliferative effects, including the up-regulation of cyclin-D2 by the viral EBNA-2 and EBNA-LP proteins, direct binding of EBNA3C to the retinoblastoma protein (pRb), and down-regulation of the p16-INK4A tumor suppressor by the viral LMP1 product [15]. There is compelling evidence that the viral EBV protein can down-regulate the expression of p16-INK4A in primary human fibroblasts [16]. This occurs in part through effects on the localization of critical transcription factors [17]. However, at present it remains uncertain whether any of these routes are sufficient to explain the ability of EBV to manipulate the host cell cycle in infected cells.

Based on these data, this additional work is to clarify whether there is a correlation between the loss of P16 expression and presence of EBERs-EBV among our patients diagnosed with Non-Hodgkin's lymphoma.

Materials & Methods

The study was designed as a retrospective one. It has recruited 45 selected formalin fixed, paraffin embedded lymph node tissue blocks. Among them, thirty (30) formalin-fixed, paraffin embedded tissue blocks were obtained from lymph nodes

biopsies. The age of the patients from where these tissue blocks were obtained ranged between 33 and 77 years. The patient samples were collected from the archives of histopathology laboratories of Al-_Hussein Medical city (Kerbala). AL-Hilla Teaching Hospital (Babylon) Alsadar Teaching Hospital (Al-Najaf), as well as many private histopathology laboratories. Fifteen (15) lymph nodes autopsies that were included as apparently normal control group for this study were obtained from the archives of Babylon; Al-Najaf and Kerbala Medical Forensic Medicine Institutes. They were age- and -sex-matched with the study group.

Primarily, the diagnosis of these tissue block were based on the obtained histopathological laboratory records of lymph node biopsy samples that had accompanied each tissue blocks in each hospital laboratory. A second confirmatory histopathological reexamination of obtained tissue blocks was done by a consultant histopathologist.

The detection of EBV by ISH kit (Zyto Vision GmbH. Fischkai, Bremerhaven. Germany) was performed on 4µm paraffin embedded tissue sections using digoxigenin-labeled oligo-nucleotides probe which targets Epstein-Bar-Virus (EBV).

For the in situ hybridization procedure, the slides were placed in 60C˚ hot-air oven overnight then the tissue sections were de-paraffinized and then incubation of slides for 15 min (twice times) in xylene then treatment by graded alcohols via incubation for 5 min in 100% ethanol (twice times). The same dewaxing protocols were routinely used for immunohistochemistry procedures were used: 15 min xylene (twice times), 5 min 100% ethanol(twice times), 5 min 96% ethanol (one time), 5 min 70% ethanol(one time, and finally immersion in distilled water for 5 minutes to remove residual alcohol. After that, slides were allowed to dry completely by incubating them at 37°C for 5 minutes. Then digestion process by adding proteinase K to the slides, and then the slides were incubated at 37°C for 15 minutes. Then the slides were dehydrated by immersing them sequentially in the following solution at room temperature for the indicated times, distilled water for 1 minute, 70% ethanol for 1 minute, 95% ethanol for 1 minute and 100% by incubating them at 37% for 5 minutes. Then we add the 20 µl of cDNA probe added to each section and slides were covered by cover slips and avoiding trapping any air bubbles. After that probe and target DNA were denaturated by placing the cover slipped-slides in pre-warmed oven at 95°C for 8-10 minutes, slides were transferred to a pre-warmed humid hybridization chamber and incubated at 37°C for overnight. Then the slides were allowed not dry out at any time during the hybridization and staining. All reagents used during hybridization and detection were warmed to room temperature. At the next day, slides were soaked in pre-warmed protein block at 37°C until the cover slips fell off, and then the slides were allowed to remain in the buffer for 3 minutes, at 37°C after cover slips were removed. After that we add streptavidin-alkaline phosphatase conjugate reagent to tissue sections. Then slides were kept in a humid chamber at 37°C for 20 minutes. Slides were rinsed in detergent wash buffer for 5 minutes and then drained. After that One to two drops of 5-bromo3-chloro3-indoly/ phosphate/ nitro blue tertrazolium substrate-chromogen solution (BCIP/MBT) were placed on tissue section. Slides were incubated at 37C˚for 30 minutes or until color development was developed completed. Color development was monitored by viewing the slides under the microscope. A dark blue colored precipitate form at the complementary site of the probe in positive cells. Then the slides were rinsed in distilled water for 5 minutes, then counter staining process by immersion of the slides in Nuclear Fast

Red stain for 30 seconds, then washing process was followed by immersion the slides for 1 minute in distilled water. After that sections were dehydrated by ethyl alcohol (95%) once for one minute then, 100% twice times for 2 minutes each); cleared by Xylene, then mounted with permanent mounting medium (DPX).

Immunohistochemistry / Detection system (abcm. England) was used to demonstrate the P16 protein encoded by P16- tumor suppressor gene. This technique is based on the detection of the product of gene expression (protein) in malignant and normal cells using specific primary monoclonal mouse antihuman antibodies for specific epitope on that nuclear targeted protein.

A specific primary Ab will react with its corresponding antigen in the tissue, and then the biotin-labeled secondary antibody will bind to that primary Ab. When the conjugate (Streptavidin bounded enzyme) is added, the biotinylated Ab will form a complex with the enzyme-conjugates streptavidin, and by adding the chromogenic substrate, a colorimetric reaction will happen at the antigen binding site.

The type of the chromogenic substrate depends on the type of the enzyme used. Thus, DAB (3, 3" diaminobenzidine) substrate offers the greatest sensitivity in the horseradish peroxidase enzyme system as a colorimetric chromogen, and a brownish precipitate will from at the antigen binding site [18].

Evaluation of lmmunohistichemical staining

A biotinylated, cross-adsorbed, and affinity purified secondary anti-mouse IgG was used to detect primary antibody-antigen complexes adhered to a glass microscope slide, following reaction with an enhanced detection reagent, proper and accurate application of kit instructions led to appearance of a brown precipitate in positive cells on tissue sections. Quantification of P16 protein expression was evaluated under light microscopy at X100, X400, and X1000. The counting of positive cells was performed at X1000.

IHC was given intensity and percentage scores, based on intensity of positive staining and number of cells staining, respectively. A scale of 0-3 was used to measure relative intensity with 0 corresponding to no detectable IHC reaction and 1, 2 and 3 equivalents to low, moderate and high. Positive cells were counted in ten different fields for each samples and the average of positive cells of these ten fields was determined assigning cases to one of the 3 following categories:

1. Score 1: 1-25%.; 2.Score 2: 26-50%; and 3. Score 3: >50% [19].

Chi –square test was used to detect the significance of variables in our study. All the statistical analysis was done by SPSS program (Version– 17) & P value was considered significant when $p < 0.05$.

Results

Detection of EBV -ISH reactions in tissues with NHL was observed in 17 out of 30 (56.7%). High the frequency of EBV in B-cell type of NHL was (58.6%) and in NHL (T-cell type) was detected in (63.7%). In the tissues from lymph nodes autopsies EBV was detected in 6.7% (1 out of 15).Statistically, there was significant difference between the frequency of EBV in the NHL and control groups (P value = < 0.05) (Table 1 & Figure 1).

The P16-Positive IHC reactions were noticed in 14 (46.7%) NHL cases. The highest score percentage of P16-IHC reactions was observed in those NHL tissues with moderate score (7 cases; 50%) then followed by those NHL tissues with weak score (4 cases; 28.6%) and NHL tissues with strong score (3 cases; 21.4 %).P16-negative IHC reactions were noticed in (16 cases; 53.3%). None of apparently healthy lymph nodes tissues in

the control group showed P16- IHC reaction.

The percentage of positive P16-tumor suppressor gene expression that associated with positive EBV- ISH reaction was constituted (47.1%: 8 out of 17 cases) in NHL group, while the percentage of positive P16 expression was (52.9%:9 out of 17 cases) in NHL tissues that showed EBV-negative reaction by ISH technique (Table 3 and Figure 2). The statistical analysis, according to the results of scoring, show significant association of P16 with EBV ($p < 0.05$) when group of NHL was compared to control group**.**

Discussion

Initially, NHLs in most instances arise within lymph nodes. However, up to 40% of the time they are present at extra nodal sites. Among these extra nodal cases, 2-3% may arise primarily in the oral cavity and jaws. [20] The most frequent non-epithelial neoplasms in the oral cavity and maxillofacial region are NHLs, representing the second most common group of malignancies there followed by squamous cell carcinoma. [21]

Additionally, these lesions are difficult to recognize because there are no pathognomonic oral clinical symptoms, though chronic tumefaction is the most frequent clinical feature. [22]

Viral infections, especially by Epstein-Barr virus (EBV), have been implicated in lymphomagenesis. More than 90% of adults worldwide are infected with EBV [23]. EBV preferentially infects B-lymphocytes through the binding of the receptor on the surface of B-cells. After primary infection, the virus remains in an asymptomatic latent state. [24]

Table 1. Distribution of EBV-ISH results on B and T cell lymphoma groups.

			Histopathological Diagnosis		Total
			NHL B-cell type	NHL T-cell type	
EBV-ISH reaction Score	Negative	Count	8	5	13
		% within EBV	61.5%	38.5%	100.0%
		% within Diagnosis	26.7%	16.7%	43.3%
	Low	Count	2	1	3
		% within EBV	11.8	5.9	17.6
		% within Diagnosis	6.7	3.3	10
	Moderate	Count	7	3	10
		% within EBV	41.2%	17.6%	58.8%
		% within Diagnosis	23.3%	10%	33.3%
	Strong	Count	3	1	4
		% within EBV	17.6%	5.9%	23.5%
		% within Diagnosis	10%	3.3%	13.3%
Total		Count	20	10	30
		% within EBV	70.6%	29.4%	100.0%
		% within Diagnosis	66.7%	33.3%	100.0%

Pearson Chi-Square	Value	Df	Asymp. Sig. (2-sided)
	2.658	2	0.152

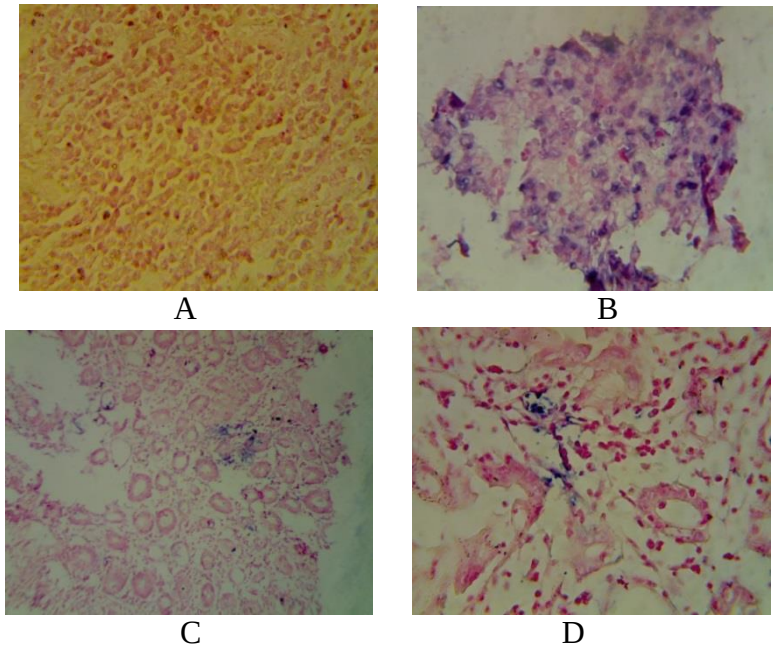

Figure (1): In situ hybridization for detection of Epstein Barr virus in NHL; NBT/BCIP stained (blue) and counter stained (red) by nuclear fast red; A. NHL-B cell type with negative EBV-ISH reaction (40 xs). B. NHL-T cell type with positive signal with strong score and high intensity of EBV-ISH reaction (40 xs); C. NHL-B cell type with moderate score and moderate intensity of positive EBV-ISH reaction (40 xs); D. NHL-T cell type with weak score and moderate intensity of EBV-ISH reaction (40 xs).

Table 2. Frequency distribution of immunohistochemical results for P16 protein according to their signal Scoring.

Signal P16 Scoring		Apparently Healthy Lymph Nodes (n=15)		NHL (n= 30)	
		N	%	N	%
Negative		15/15	100.0	16/30	53.3
Positive		0	0.00	14/30	46.7
Scoring	Weak	0	0.0	4/14	28.6
	Moderate	0	0.0	7/14	50
	Strong	0	0.0	3/14	21.4
P value		<0.001 significant			

Co-existence expression of EBV- EBERS and P16 –protein in NHL tissues:

Table 3. Frequency distribution of Co-localized EBV- EBERS and P16- protein in tissues with NHL.

Studied groups				EBV- EBERS-ISH		Total
				Positive	Negative	
NHL	P16 IHC Reaction	Positive	N	8	9	17
			%	47.1	52.9	100
		Negative	N	5	8	13
			%	38.5	61.5	100
		Total	N	13	17	30
			%	43.5	56.5	100
Control Group	P16 IHC Reaction	Positive	N	0	0	0
			%	0	0	0
		Negative	N	0	15	15
			%	0	100	100
		Total	N	0	15	15
			%	0	100	100
Pearson Chi-Square				Value	Df	Asymp. Sig. (2-sided)
				7.273	6	0.296

A **B**

Figure 2. Immunohistochemical detection of p16 in NHL; DAB chromogen stained (brown) and counter stained by Mayers hematoxyline (blue).A. Negative IHC-reaction for p16 reaction(40x). B. Positive p16 IHC- reaction with moderate score and weak intensity signals (40 xs)

Under circumstances in which the host cellular immunity fails to control EBV-induced B-cell proliferation, infected carrier B-cells can transform from their latent state into malignant cells. The finding of clonal EBV-infected cells supports the concept that EBV was present in the cells before neoplastic transformation, and therefore EBV may play a role in the pathogenesis of some kinds of lymphoma. [25]

In this study, detection of EBV -ISH reactions in tissues with NHL was observed in 17 out of 30 (56.7%), where the frequency of EBV in B-cell type of NHL was (58.6%) and in NHL (T-cell type) was detected in (63.7%).Our results are compatible with another study in China done by Zhang et al (2010) (20)who found the percentage of EBV in NHL, B-CELL type was(51.6%)while in NHL.T-cell type was (65.5%). These results indicate that the difference of positivity rates between (T&B) NHL types are not significant ($p > 0.05$)and in turn suggesting an intimate correlation between these two immunophenotypes of non-Hodgkin's lymphomas and EBV occurrence of this infection in our country. The estimated EBV positive rate was to be 7% for NHL, B-cell type cases. A high percentage of positivity is usually found in diffuse large B-cell lymphomas, Burkett's lymphoma and post transplantation lympho-proliferative diseases [26]. In previous studies NHL, T-cell type, especially angioblastic T-cell lymphomas from Eastern and Western countries, demonstrated frequent association (55% to 97%) with EBV infection [27]. The present results, in one hand, are in agreement with other studies in Korea done by [28] who found the percentage of EBER positivity among NHL, T-cell type was (47%)while our study regarding NHL, B-cell type was in disagreement with these results since they found 6.9% of their NHL, B-cell types were positive for EBV EBERS. Also our study is in disagreement with other study done in Malaysia by [29] who found the results of EBV ISH techniques are (10.5%) among NHL, B-cell type which was lower than its counterpart in the NHL, T-cell type(77.8%) [30].

It was found that the target cell of EBV infection is the human B-lymphocyte, yet, in recent years more evidences have shown by many studies that the human T-lymphomas with EBV infection are also found [31]. The EBV-specific receptor CD21CR2) which is expressed on B-cell and developing T-cell but not on mature peripheral T-cell is well known [32]. In individuals with normal immune system a sustained T-cell infection by EBV occurs only rarely, raising the possibility that the infection of the T lymphocytes &their subsequent unregulated growth could be caused, at least in part, by a defect in the

immune surveillance [27]. Many researchers have focused on T-NHL etiologic and pathologic studies. In this respect an in vitro experiments, it was discovered that immortalized cell lines will be difficult to be transformed from normal T lymphocyte infected with EBV. However, the clinical data showed that EBV detection rate in some types of human T-NHL is higher than that in B-NHL that have morbidity, suggested that T-NHL is more relevant with EBV infection. All these contradictory phenomena indicate that there may be different mechanisms between (T&B) lymphomas infected with EBV.

Many reasons can be put forth to explain the wide variation in the current and previous results. The most important of which are the great diversity of diseases included within the entity of NHL, where each exhibiting different rate of association with EBV [33]. The types of NHL most commonly associated with EBV infection are: the Burkitt's lymphoma (BL), large B-cell lymphoma and NHL arising in the setting of AIDS-associated immune suppression. A part from the BL in which EBV association is consistently high, the association with other types of NHL is variable. In addition, the prevalence of the various diseases differs in different geographical regions and this may be ascribed to genetic and environmental etiologic factors [34]. Moreover, the extent to which different types of NHL impair the immune response, in particular those that lead to defective T-cell regulation was another effecter factor. Furthermore, some of these studies have investigated a restricted number of diseases and the number of cases in the other cohort's studies certainly influences the significance of these results [35].

It is important to consider the geographic distribution when discussing the association between EBV and NHL. However, the number of normal samples is relatively small and further studies are required to corroborate these findings. Histopathologically, B-cell lymphomas are the most common phenotype in head and neck [36, 29]. Although most are DLBCL, other types such as Burkitt's lymphoma, T-cell and natural killer cell lymphomas are seen in different sites of oral cavity and maxillofacial region [37]. They found a frequency of 26.5% for EBV infection associated with NHLs in our cases. EBV was found to be positive in 50% of DLBCL cases and this ratio was 11.8% in low grade B-cells. In contrast to our results, Leong et al. showed a higher association of EBV with T-cell lymphomas than with B-cell lymphomas, especially in immunocompromised patients. [38] In this study, the association between P16 expression and EBV expression in malignant cells of NHL was studied. With respect to all clinical factors, our data revealed that expression of EBV and P16 expression association was found between the all NHL cases. These observations suggest that the P16 expression may be an independent event and not necessarily associated or linked with EBV expression in NHL malignant lymphoma. Our finding is not in agreement with previous studies which demonstrated that the loss of P16 expression was more frequently observed in EBV LMP-1 positive carcinomas than EBV-LMP-1 negative carcinomas [39, 40-41, 42-44]. Taken together, our findings combined with other findings suggest that EBV has different molecular biological effects upon infection and transformation of different cell types. Furthermore, based on previous investigations, EBV infection can cause the loss of P16 expression and/or inactivation of *p16* gene through hypermethylation of its promoter region and these changes are attributable to the gene product of the LMP-1 of EBV [43, 45]. concerning the EBV /P16 relationship, the loss of P16 expression in HL apparently correlates very well with EBV LMP-1 expression, suggesting that EVB infection might be responsible for the loss

of P16 expression or silencing the *p16* gene in in NHL. They finding also suggests that lymphoma represents a heterogeneous group of diseases in terms of the LMP-1 expression and loss of P16 .Also, they finding apparently lends further support to notion that, the role of EBV infection in the loss of P16 expression is sometimes contradictory or controversial [45,46].

Conclusion

Our results demonstrated that there was a positive correlation between expression of the EBV oncoproteins and the decrease P16 tumor suppressor protein in NHL lesions. This finding suggests that EBV oncoproteins expression might play a role in the loss of P16 expression in NHL malignancy.

The present study offers further evidence for the notion that lymphoma is a heterogeneous malignancy, and the pathogenesis and progression of lymphoma subtypes might be linked to several molecular alterations and environmental factors. Therefore, we believe that further understanding of the molecular and environmental mechanisms are necessary for both factors in the pathogenesis and development of lymphoma.

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