Original Article

`In Situ Hybridization for Detection of Latent Epstein-Barr Virus Early Repeats(EBERS) and Mutant-P53- Tumor Suppressor Gene in Patients with Non- Hodgkins Lymphoma

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Summary:

Back ground: Epstein- Barr virus (EBV) is a ubiquitous in that infecting more than 90% of adult population worldwide. Recently, EBV has been linked to the development of variety of human malignancies. P53 gene is mutated in more than 50% of human cancers. Cell cycle dysregulation, measured by p53 protein expression, and latent EBV infection are important in the pathogenesis of Non-Hodgkin's lymphomas.

Objective: To analyze the distribution and impact of concordant p53 expression and latent EBV infection on a group of B & T cell types of NHL.

Materials and Methods: Forty (40) formalin-fixed, paraffin embedded tissue blocks were obtained from lymph nodes biopsies related to patients with NHL.

In addition, biopsies of twenty (20) lymph nodes autopsies were included as apparently normal control group. The clinico- pathological criteria of NHL cases were assessed and In Situ Hybridization(ISH) techniques for mutant p53 and Epstein-Barr Early Repeats(EBERs) detection were performed.

Results: The percentage of EBV-ISH reaction results in the total group of NHL was (60%), where its percentage in NHL, B-cell type was (58.6%)and in NHL, T-cell type (63.7%). None of the control group showed EBV-ISH reaction. Statistical analysis showed significant difference between these groups ($p<0.05$). The percentage of B-cell lymphoma with moderate signal score for EBV-ISH test was (41.4%) while with strong scoring was (17.2%). In the T-cell lymphoma, moderate signal scoring was (18.2%) while strong scoring was(45.5%). Statistically, they showed no significant differences ($p> 0.05$). Mutant p53 gene was observed in 60% of NHLs and dual positivity of EBV and p53-ISH was found in 42.5 % of cases. Regarding the grades, it was found that the higher level of p53 expression was observed in low grade tumors (39.3% ; 11out of 28) NHL.

Conclusion: The present findings indicate that cell cycle dysregulation and EBV-related transformation are important in the pathogenesis of NHL.

Key words: Non-Hodgkin's lymphoma , Epstein Barr virus, Epstein-Barr Early Repeats, Mutant P53, In Situ Hybridization.

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Introduction:

Non- Hodgkin's lymphoma (NHL) is a mixed group of diseases that characterized by the malignant increase in specific cells of immune system (B and T) lymphocyte. There are 40 different types of Non-Hodgkin's lymphoma which can occur at all ages, however the average age of diagnosis is in the 60 year and the incidence of this disease generally increased with age. The disease is more common in men than women and affects whites more often than blacks. Over all, between 1973 and 1997, the incidence of NHL in U.S. grew 81%, (1).The American cancer society has ranked NHL in 2005, as the fifth most common cancer in U.S. among women and the sixth most common cancer among men, excluding non- melanoma skin cancer(2). The high incidence of NHL has been noted worldwide. Particularly in elderly persons >55 years. Concerning gender sub groups, a male predominate throughout all age groups is apparent. Although the NHL incidence has historically been higher in whites and blacks, the increase in high grade NHL and extra nodal disease are predominant. Differences in geographic distribution are striking for follicular lymphoma, which is more common in Western countries than those where Asian who have higher rates of aggressive NHL, T-cell lymphomas , and extra-nodal disease . In the middle East, high rates of intestinal extra nodal disease are observed (3). . The cause of malignant lymphoma remains unclear, but it is related

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to immune deficiency, ionization radiation, viral infection include HIV, EBV, HCV and HTLV1and others(4).EBV is a typical virus consisting of a core containing a linear, double stranded DNA: an icosahedral capsid. Approximately10-100 nm in diameter, containing 162 capsomeres, an amorphous material that surrounded the capsid, (tegument) and an envelope containing viral glycoprotein spikes on its surface(5). 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, 2, 3A, 3B and 3C in latently infected cell(6) EBV has been classified as a group 1 carcinogen associated with a variety of lymphoid and epithelial malignancies by the international agency for research of cancer {IARC}(7). Evidence of EBV being a monogenic virus is drives from its ability to infect and transform normal human B cells in vitro, resulting in immortalization of these cells and leading to continuous growth of lymphoblastoid cell lines. Moreover, EBV can transform human squamous epithelial cells in vitro. The virus is involved in the development of several human cancers such as nasopharyngeal carcinoma and various lymphoma(8) The small un translated 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 kinas R (PKR0, and inhibits its activation by double-stranded RNAs, protecting infected cells from INF-induced apoptosis(9).EBV encoded small RNAs have however a more prominent role in EBV-mediated growth transformation, as viruses lacking the coding sequence for this RNA were significantly less efficient in generating lymphoplastoid cell lines(LCLs) in vitro, and the cell lines generated proliferation at much lower rates, due to reduced autocrine IL-6 production (10). These observations have been extended to epithelial cells lines, where EBERs induced the expression of growth factors that promote cell survival (11). The wild type p53 gene is a tumor suppressor gene. P53 is located on short arm of chromosome 17 at position p1.4.1), and encodes nuclear phosphoprotein that regulates cell cycle checkpoint and the induction of programmed cell death (apoptosis) in response to DNA damage , cell stress or the aberrant expression of some oncogenes (12). The longer half life of the mutated form product increases the concentration of this product in the nucleus of the affected cells and being accessable for detection using commercially available antibody (13). The percentage of p53 mutation among NHL patients ranged from 16.2% (14) to 62.2% (15).This study is aiming to access the impact of expression of EBV as well as p53 tumor suppressor gene on the histopathological finding of NHL.

Materials and Methods

Forty (40) formalin-fixed, paraffin embedded tissue blocks were obtained from lymph nodes biopsies which included IHC marker study regarded CD3 for T-lymphocyte type of NHLs and CD20 for B-lymphocyte type of NHLs . The age of the patients ranged between 3 to 75 years, the patients samples were collected from the archives of histopathology laboratories of Gazi Al-Hariery hospital, Baghdad Teaching Hospital in Baghdad Medical City and Al-Kadhimiya Teaching Hospital, as well as many private histopathology laboratories that generously helped us. Twenty (20) lymph nodes biopsies were included as apparently normal control group for this study were obtained from the Baghdad Forensic Medical Institute archives. The mean age of these individuals was 43.59 years.We performed ISH techniques in the laboratories in College of Medicine/ Baghdad University and College of Dentistry/ Baghdad University.The diagnosis of these tissue blocks were based on their accompanied records. A consultant pathologist reexamined all these cases to further confirm the diagnosis following trimming process of these tissue blocks. In one hand, 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) EBER RNA. One section was mounted on ordinary glass slide and stained with hematoxyline and eosin, while another slide was mounted on charged slide to be used for ISH for detection of EBV. For the in situ hybridization procedure, the slides were placed in 60c hot-air oven over night then the tissue sections were de-paraffinized and then treated by graded alcohols according to the standard methods and the details of processes for performing ISH reaction with this probe were applied according the instructions of the manufacturing company(Zyto Vision GmbH. Fischkai, Bremerhaven. Germany). The main steps for ISH procedure are:

Incubation of slides for min at 70°C(e.g. on hot plate), then Incubation of slides for 5 min in xylene. Afther that incubation for 5 min in 100% ethanol (alternatively, dewaxing protocols routinely used in immunohistochemistry procedures, e.g. 2-5 min xylene, 2-5 min 100% ethanol, 2-5 min 96% ethanol, 1-5 min 70% ethanol, can be used. Air drying of sections. then application (dropwise) Pepsin Solution(ES1) to the tissue/cell section and incubate for 20-30 min at 37°C in a humidity chamber. After that we immersed slides in distilled water and drain off the water, air dried sections, then add the probe to the center of a cover slip and place cover slip upside down on target area). Denaturation of the slides at 75°C for 5 min, e.g. on hot plate, then transfered the slides to a humidity chamber and hybridize for 60 min

at 37°C for DNA-targetting probes or at 55°C for RNA-targeting probes) and the post-hybridization and detection process that included removing the cover slip by submerging in 1x wash buffer TBS, then washed for 5 min in 1x wash B\ buffer TBS (prepared by using WB5) at 55°C (should not perform this step on slides hybridized with Zytofast RNA (+) control probe(PF6) as this will reduce signal intensity). Then application of AP-Streptovidin (AB9) drop wise (3-4 drops per slide) to the slides and incubate for 30 min at 37°C in a humidity chamber. Then washed in wash buffer TBS (prepared by usingWB5) and then twice times for 1 min in distilled water and application of NBT/BCIP(SB4) drop wise (4 drops per slide) to the slides and incubated for 40 min at 37°C in humidity chamber. Then _checking the color development in intervals of approx, 5-10 min using microscope. Lastly, Washing three times for min in distilled water. After that covering the sections. Then the sections were embedded in an aqueous embedded medium, then final evaluation by light microscope.On other hand, the detection of p53 by ISH kit using biotinylated Long DNA Probe for Human P53 Gene 8µ g / tube (Maxim biotech Inc, USA) was performed on additional 4µm paraffin embedded tissue section. The details of processes performing ISH reaction with this probe were applied according to the instruction of the manufacturing company (Maxim biotech Inc, USA). The interpretation of results depending on the target being DNA, a positive reactivity in the target cells is indicated by a blue-violet colored reaction product either within the cytoplasm or the nucleus. The main steps of ISH for detection of P53 include:

Rehydration process Rehydration process was done at room temperature which include:Slides were immersed in two changes of absolute ethanol for one minute each, then Immersion in ethanol (95%) for one minute each, after that immersed in ethanol (70%) for one minute each, 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 we done digestion process by add proteinase K to the slides, 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 µ1 of cDNA probe added to each section and slides were covered by cover slips be careful to avoid 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 should be careful not to tear the tissue, 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 were added to tissue sections. Then slides were kept in a humid chamber at 37°C for 20 minutes. Then one to two drops of 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 37°C 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 alchhol, (95%, once for one minute then, 100% twice times for 2 minutes each); cleared by Xylene, then mounted with permanent mounting medium (DPX).

Evaluation of results:

In situ hybridization was given intensity and percentage scores(16), based on intensity of positive signals and number of signals, respectively. A scale of 0-3 was used for relative intensity with 0 corresponding to no detectable ISH reaction, and 1, 2, 3 equivalent to low, moderate, and high intensity of reaction, respectively. Positive cells were counted in ten different fields of 100 cells for each sample and the average of positive cells of the ten fields was determined assigning cases to one of the three following score categories: Score (1) low = 1-25%; Score (2) moderate = 26-50%; Score (3) strong > 50%.

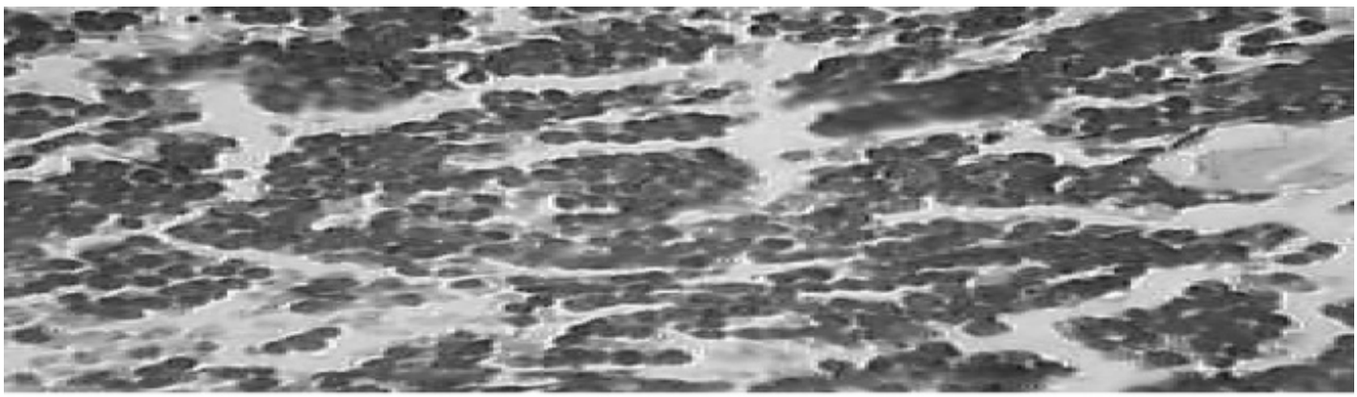
Results:

Table (1) shows 16 case out of 40(40%) were negative for EBV-ISH reactions. The highest percentage of EBV-ISH reaction was found within NHL,T-cell type(7 cases out of 11,63.6%) than that within NHL, B-cell type (17 cases out of 29, 58.6%). None of control group showed EBV-ISH score reaction Statistically, there was no significant difference between the studied groups ($p > 0.05$).

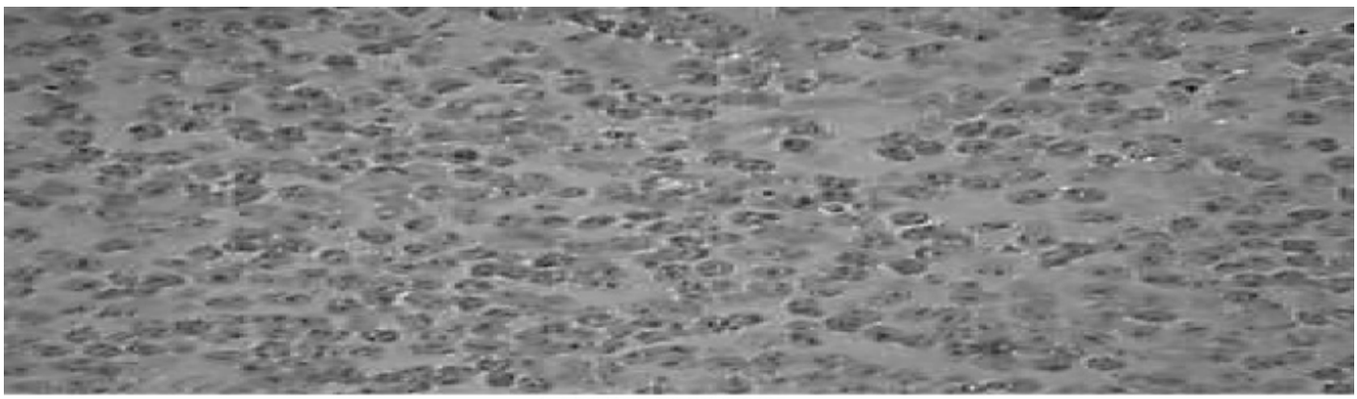
Table (1): Distribution of EBV-ISH results on B and T cell lymphoma groups.

			Histopatho logical Diagnosis		Total
			NHL B-cell	NHL T-cell	
EBV Score	-ve	Count	12	4	16
		% within EBV-EBERs	75.0%	25.0%	100.0%
		% within Diagnosis	41.4%	36.4%	40.0%
	Moderate	Count	12	2	14
		% within EBV	85.7%	14.3%	100.0%
		% within Diagnosis	41.4%	18.2%	35.0%
	Strong	Count	5	5	10
		% within EBV	50.0%	50.0%	100.0%
		% within Diagnosis	17.2%	45.5%	25.0%
Total		Count	29	11	40
		% within EBV	72.5%	27.5%	100.0%
		% within Diagnosis	100.0%	100.0%	100.0%

Chi-Square Tests	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3.815	2	.148

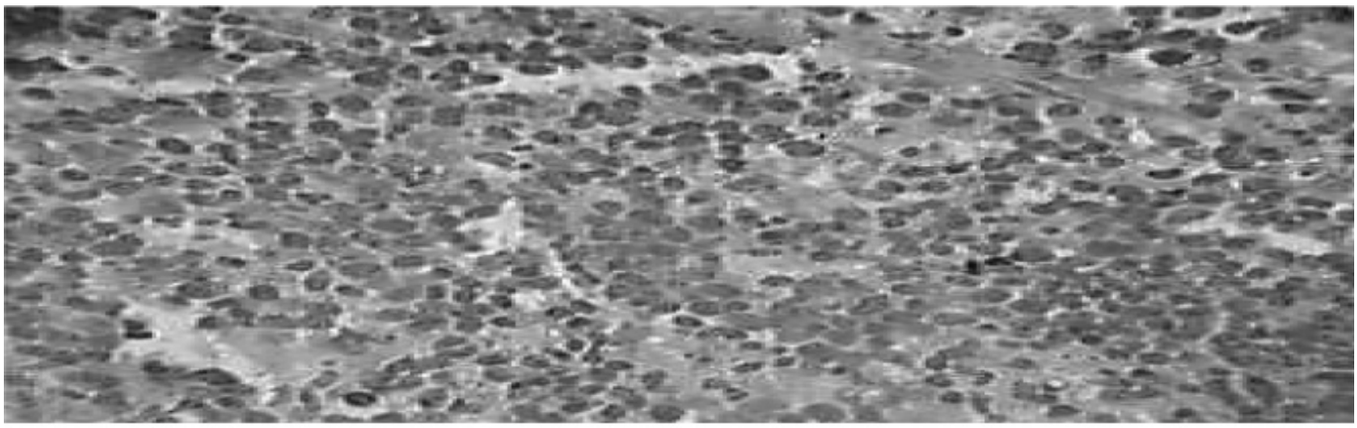


NHL,B-cell Type

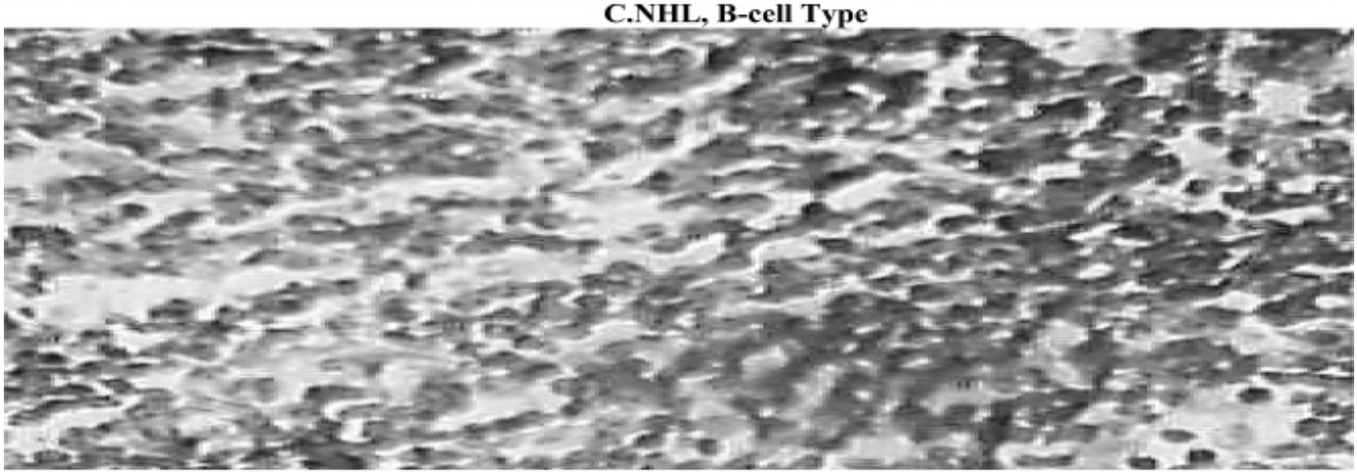


B- NHL,T- cell type

Figure(1:A): NBT/BCIP stained(blue) and counter stained by nuclear fast red(red); A. NHL with negative EBV(EBERs)-ISH reaction(400x); B. Moderate score (Score 2) and moderate intensity of positive (EBERs)ISH reaction(400x).



C.NHL, B-cell Type



D. NHL, T-cell Type

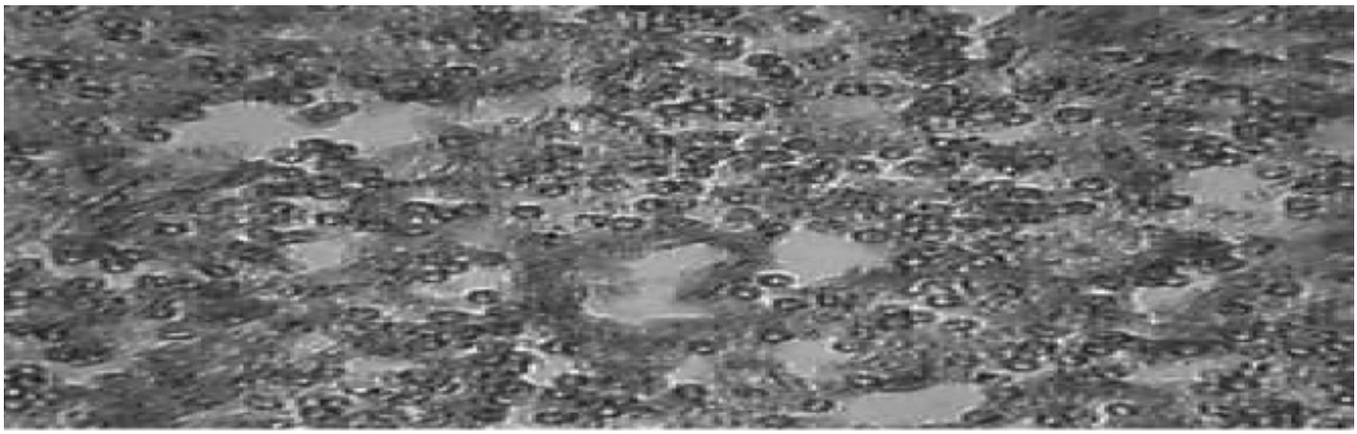
Figure (1:B): NBT/BCIP stained(blue) and counter stained by nuclear fast red(red); C. Strong score (Score3) of positive EBV(EBERs)-ISH and weak intensity reaction(400x); D. Weak score (Score1) and high intensity of EBV(EBERs)-ISH reaction(400x).

Table (2) shows that the highest percentage of p53-ISH reaction was in those with moderate score (12 cases; 30.0%) then followed by strong score(8 cases; 20.0%) and weak score (5cases; 12.5%) P53-negative ISH reactions were noticed in (15 cases; 37.5%). None of control group showed p53 ISH reaction.

Table(2):The results of p53 ISH scoring in NHL groups:

P53 score	No. of cases	Percent
-ve	15	37.5
Weak	5	12.5
Moderate	12	30.0
Strong	8	20.0
Total	40	100.0

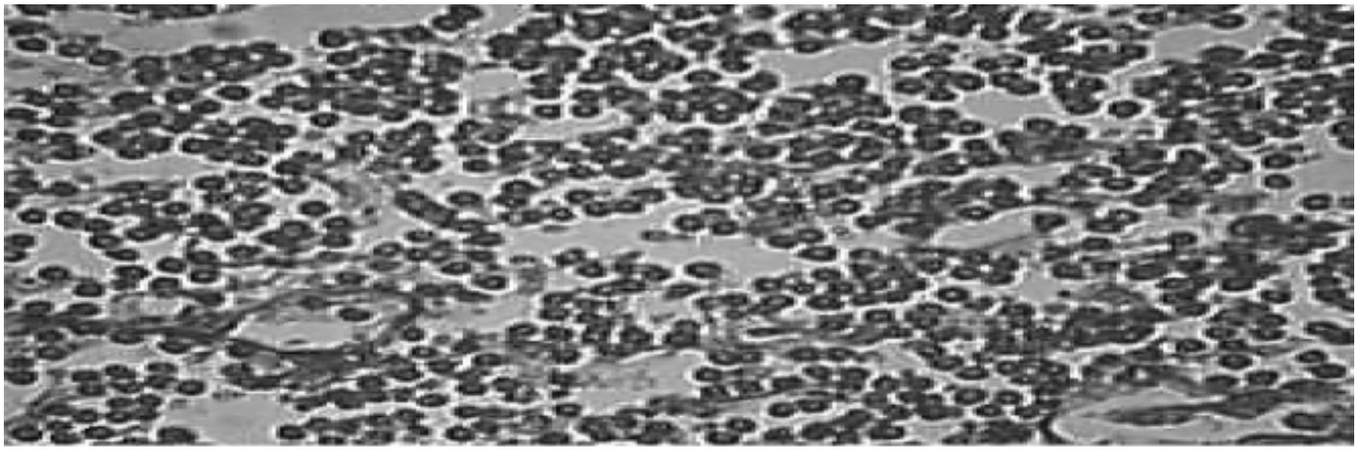
-A – NHL, B-cell type



-B- NHL, T-cell type

Figure(2:A): In situ hybridization for detection of mutated p53 gene in NHL; chromogen stained (blue) and counter stained by nuclear fast red(red); A. Weak score and moderate of p53-ISH reaction(400x); B. Moderate score and weak intensity.

-C- NHL, B-cell type



-D- NHL, T-cell type

Figure(2:B): In situ hybridization for detection of mutated p53 gene in NHL; chromogen stained (blue) and counter stained by nuclear fast red(red); C. Negative ISH- reaction for p53 reaction; D. Strong score and high intensity of p53-ISH-reaction(400x). Regarding table (3), and on assessment the overall results (table 4), 8 out of total number of NHL cases included in this study(constituted 20%, this percentage is not included here because it is an arithmetical assessment in conclusion showed negative p53-ISH &EBV(EBERs)-ISH reactions whereas a percentage of 42.5% (17 out of 40) NHL tissues have positive reactions of both these tests. There was no significant statistical association between both ($p > 0.05$).

Table (3) The results of EBV ISH and p53 ISH signal score among study groups.

			P53 ISH Score				Total
			-ve	Weak	Moderate	strong	
EBV Score	-ve	NO.	❖ 88	1	5	2	16
		% within EBV	50.0%	6.3%	31.3%	12.5%	100.0%
		% within P53-ISH	53.3%	20.0%	41.7%	25.0%	40.0%
	moderate	NO.	3	4	4	3	14
		% within EBV	21.4%	28.6%	28.6%	21.4%	100.0%
		% within P53-ISH	20.0%	80.0%	33.3%	37.5%	35.0%
	Strong	NO.	4	0	3	3	10
		% within EBV	40.0%	.0%	30.0%	30.0%	100.0%
		% within P53-ISH	26.7%	.0%	25.0%	37.5%	25.0%
Total		NO.	15	5	12	8	40
		% within EBV	37.5%	12.5%	30.0%	20.0%	100.0%
		% within P53-ISH	100.0%	100.0%	100.0%	100.0%	100.0%

Chi-square tests	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	7.273	6	.296

Table(4) The results of EBV ISH and P53 ISH in the study groupe in relation to their qualitative assessment as positive or negative ISH reaction.

EBV(EBERs)	P53-ISH		Total
	-ve	+ve	
-ve	8 20%	8 20%	16 405
+ve	7 17.5.5	17 42.5%	24 60%
Total	15 37.5%	25 62.5%	40 100%

Discussion:

The estimated EBER 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, Burkitt's lymphoma and post transplantation lympho-proliferative diseases (18). 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 (19). Since different types of NHL were included in this study and because limited numbers of cases were tested, these factors made is difficult to draw conclusion as to what extent our results reflect the actual association between the virus and this group of diseases collectively.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-Hodgkins lymphomas and EBV occurrence of this infection in our country. The present results, in one hand, are in agreement with other studies in Korea done by Cho et al (2008)(21) 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 (22) 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%). (23) 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(17). 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 (24). 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 (19) A suggesting that a genetically determined susceptibility, possibly based on certain HLA types, could result in an abnormal response to primary EBV infection in our country and as supported by other findings of other researchers (25). 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 suggested morbidity, suggestion 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. Furthermore, 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. Many reasons can be put forth to explain the wide variation in the 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 (26)and the prevalence of the various diseases differs in different geographical regions and this may be ascribed to genetic and environmental etiologic factors (20) In addition, 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. Moreover, some of these studies have investigated a restricted number of diseases and the number of cases in the other cohorts studies certainly influences the significance of these results (27)Lymphomas in patients, which usually express multiple Epstein-Barr virus nuclear antigen latency proteins & EBERs mRNA, frequently demonstrate p53 mutations (28). The importance observation of increased serum p53 protein as well as and serum antibodies along the EBV proteins preceded the diagnosis of NHL and synergistically predicted the risk of subsequent development of such cancer (29).To assess the importance of latent EBV and the expressed p53 protein in lymphogenesis in patients with NHL, we compared the percentage of the latent EBV and mutant p53 expression in lymphomas arising in patients with NHL. In this study, (45%) of EBV-infected NHL have positive signals p53 protein in their tissues whereas (15%) of NHL have showed neither EBV-positive reaction nor p53-over expression. Also the same trend of dual-positivity (i.e. 42.5%) as well as dual- negativity (i.e. 20%) of both -EBV& -P53 ISH reactions were noticed in our series of NHL tissues that were tested for both tissues parameters. Our results are consistent with another results reported by David (2000)(28) who found the percentage of p53 & EBV was 50%.Our series of NHL tissues that showed high EBV latent infection could be related to an immune-suppressive status for different reasons of such phenomenon (29). This possibility is supported by the findings of Salloumn et al(29) who found the simultaneous presence of latent EBV and p53 expression in lymphomas arising in their patients with connective tissue disorders(CTD) who were immune suppressed with methotrexate (MTX). This could also suggest that an important and possibly unique component of the pathogenesis of cell cycle in these lymphomas involves dysregulation through latent EBV infection with concurrent p53 protein expression which was less often identified in (CTD) patients not immune-suppressed with (MTX)(29).

Conclusion:

In conclusion, these findings indicate that cell cycle dysregulation and EBV-related transformation are important in the pathogenesis of our series of NHL.

Author Contributions:

1. Dr. Saad H. Mohommed Ali:(Study conception, practical part of ISH, design & acquisition of data analysis, interpretation of data and critical revision).
2. Dr. Salim R. Hamoodi (Design, interpretation of data & histopathological examination).
3. Ameer H. Abdul Ameer(Collection of samples, practical part of ISH, drafting of manuscript, and critical revision).

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