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# DNA- In Situ Localization of Human Cytomegalovirus and Immunohistochemical Targeting of Protein Expression of p21 Gene Sequence in Tissues from a Group of Iraqi Patients with Non-Hodgkin's lymphoma

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**Abstract:**

**Background:** Cytomegalovirus (CMV) is highly seroprevalent among all populations and CMV antigenemia and CMV disease are associated with varieties of tumors and / or cancers, among them the hematologic neoplasms, where are known to cause high morbidity and mortality among patients with leukemia, lymphomas and stem cell transplanted patients. The p21 protein (Waf1/Cip1/CDKN1A) is important for regulation transition of G1 to S phase, modulating cell-cycle control, apoptosis and cell growth. Two polymorphisms of p21 gene may effect the function of its expressed protein and thus may participate in the development of cancer.

**Objective:** To analyze the distribution as well as the concordant impact of either p21 expression or HCMV infection on a group of tissues from Iraqi patients with B- & T- cell types of Non-Hodgkin's lymphoma.

**Patients and Methods:** A total number of 60 tissue specimens were examined for the rates of HCMV as well as P21 gene expression. Those samples were belonged to 40 patients diagnosed with Non-Hodgkin's lymphoma (NHL) while the remaining 20 tissues specimens were apparently healthy lymph nodes that have been included as control group for this study. Detection of HCMV was done by ultra- sensitive version of an in situ hybridization kit whereas immunohistochemistry detection system was used to demonstrate the expression of p21 gene.

**Results:** The percentage of CMV-ISH reaction results in the total group of NHL was (60%), where its percentage in NHL of B-cell type was (73.9%) and in NHL of T-cell type was (52.9%). None of the control group showed CMV-ISH reaction. Statistical analysis showed significant difference between these groups ($p<0.05$). ). The high percentage of signal scoring of HCMV-ISH in B-cell NHL was found in high signal score for test was (**41.2**%) while, T-cell NHL cases with moderate score signal was (44.4%). The weak intensity of HCMV-ISH reactions was (47.1%) in B-cell NHL and in T-cell NHL, the high percentage of signal intensity in moderate intensity was found **(55.6%)**. Statistically, they showed significant differences ($p> 0.05$). Expression of p21 gene was observed in 57.5% of total NHL tissues whereas its percentage in NHL of B-cell type was (62.2%) and in NHL of T-cell type was (47.1%).It was found that the significant correlations among the results of HCMV and grade of NHL.

**Conclusions:** The significant detection of HCMV along with P21 gene expression production in NHL patients could support an etiologic role for that virus along with expression of that tumor suppressor gene in the hypothesis of cancer development of patients with Non-Hodgkin's lymphoma.

**Key words:** Non-Hodgkin's lymphoma, HCMV, P21, ISH, IHC.

## INTRODUCTION:

Lymphomas are types of cancer characterized by the malignant increase in specific immune cells (B and T lymphocytes). Non-Hodgkin's lymphoma (NHL) includes all lymphomas except for Hodgkin's lymphoma [1]. Non- Hodgkin's lymphoma (NHL) is a mixed heterogeneous group of malignancies ( more than 30 NHL subtypes). The two most common subtypes (diffuse large B-cell lymphoma and follicular lymphoma) are accounting for about 30% and 20% of all NHL cases, respectively [2].During the past three decades, an increase in the worldwide- incidence of NHL has been consistently reported [1]. In 2013 about 2.96 million people had non-Hodgkin lymphoma and 226,000 died [3].

In United States 2.1% of people are affected at some point in their life. In US, the age-adjusted incidence has doubled during 1975 - 2008 ("SEER Stat Fact Sheets: *2016).* The most common age of diagnosis is between 65 to 75 years old. [4]. In 2005, American cancer society has ranked NHL as fifth most common cancer among U.S. women and as sixth most common cancer among men [5].

During past 50 years, list of classification schemes have been proposed as well as revised [6,7].However, new WHO classification has become an international standard for clinical practice and research. This classification of NHL has incorporating morphology, immunophenotyping, cytogenetic and molecular features, clinical behavior, and some etiological aspects and pathogenesis [8] .

Despite decades of intensive research, the etiology of NHL remains poorly understood, with the only established risk factors were being an infection and immune dysregulation (immunosuppressed populations and individuals with certain auto-immune diseases) [9].Established links have been reported between specific infectious agents and rare NHL subtypes (EBV and Burkitt lymphoma, HTLV1and adult T-cell leukemia/lymphoma, HHV 8 and primary effusion lymphoma, *H. pylori,* and gastric mucosa-associated lymphoid tissue (MALT) lymphoma). However, the aforementioned conditions and factors are relatively uncommon in general populations and can explain only a small portion of NHL cases [10].In addition, Epidemiological studies have suggested that certain environmental and occupational exposures and lifestyle factors may be associated with the risk of NHL. For example, cigarette smoking [11]., alcohol consumption [12]., using hair dyes [13]. ultraviolet radiation [14], BMI and overweight to the risk as well as poor outcomes of NHL [15], [16], organochlorine compounds [17,18], specific polymorphisms in TNF and IL10 genes [19] and toll-like receptor genes [20],immune and inflammation genes [21], Chromosomal translocations, DNA repair genes , alteration of one-carbon metabolism causes alteration of immune function and results in lymphogenesis [22-24].

Cytomegalovirus (CMV) is a common virus, infecting 70%-100% of the global population. Primary infections are generally mild or asymptomatic in the immunocompetent host with latency and persistency in myeloid lineage cells, and are believed to be followed by periodic asymptomatic reactivations of infections [25].CMV infection or reactivation in adult patients are usually occur in immunocompromised hosts, such as patients with AIDS or organ transplantation recipients. In patients with hematological malignancies, CMV infection is infrequent except in those stem cell transplant recipients [26]. In addition to some scattered case reports, only three case series studying patients with CMV infection are available, two on lymphoma and one on leukemia [27], where most patients had CMV before their clinically overt CMV infection occurred, suggesting reactivation of CMV rather than new infection [28]**.** CMV reactivation is a potentially serious threat following treatment of hematologic neoplasms. A uniform definition has classified CMV infection into CMV antigenemia (CMV-A) or CMV disease (CMV-D), depending on the presence of blood CMV antigens or end organ disease, respectively

[29].However, in immune suppressed individuals CMV might cause life-threatening infections [30].

Control of mammalian cell cycle is accomplished via proteins family that binds to and inhibits cyclin-dependent kinases (Cdk) and among the best known of these inhibitors is p21WAF1 (wild-type p53-activated fragment 1; also known as Cipl, SDI1, or Pic-l). P21WAF1 plays a dual cell-cycle inhibitory function at the G1-checkpoint, because it can interact with cyclin/Cdk complexes and also with the DNA polymerase S replicating factor proliferating-cell nuclear antigen (PCNA). Evidence has been provided that the WAF1 gene contains p53-binding sites and the p21WAF1 product is a potent downstream effector of the p53 tumor-suppressor gene function. The expression and function of p21WAF after genotoxic stress and DNA damage seems to be strictly dependent on the presence of functional wild-type. Then a loss of p53 function by gene deletions or point mutations is an extremely common occurrence in human cancers and is relatively frequent in hematologic malignancies [31-33].

The p21- tumor suppressor gene, encodes a 21-kDa protein that belongs to the CIP/KIP family (18), which includes p27 [34] and p57 [35]. The p21 protein mediates interaction between CDK inhibitors and cyclin-CDK complexes [36] and is able to affect the function of most known cyclin /Cdk complexes, blocking DNA replication and cell cycle progression into ***S*** phase. The p21 protein is a CDK inhibitor where its expression leads to either cell-cycle arrest or apoptosis [37]. In addition, p21 expression can also suppress the growth of tumor by inhibiting PCNA-dependent DNA replication [38].

Identifying NHL risk factors is an extremely complex process. However, due to the limited scope of this article, we will address two issues involved in the risk factors of NHL. The main objectives of this article are: First, studying the risk rates of both HCMV and the expression of P21 gene in a group of tissues from NHL- Iraqi patients. Second, identifying their relation to either grade or major types of NHL, namely B- and T-types of NHLs.

## MATERIALS AND METHODS

The study was designed as a retrospective one. It has recruited 60 selected formalin fixed, paraffin embedded tissue blocks; among them, 40 patients diagnosed with Non-Hodgkin lymphoma (NHL) while the remaining 20 tissues specimens have been included as apparently normal control group . They were obtained during **histopathological** diagnosis for different pathological cases; some lymph nodes appear of normal histological architecture used as control cases. Tissue sections of lymph node stained with ordinary H & E stain showed preserved lymph node architecture with well-defined cortex & medulla surrounded by thin fibrous connective tissue capsule and a large number of follicels were seen in the subcapsular cortical area. Specimens were selected for analysis based on two criteria: (i) the presence of sufficient material for analysis and (ii) histological evaluation that demonstrated that the samples contained at least 30%tumor cells (in the case of the cancer samples). Representative sections from each case were paraffin-embedded for staining with hematoxyline and eosin to assess the histopathology diagnosis. The diagnosis of these tissue blocks were primarily based on their accompanied records. Following trimming process of these tissue blocks, a consultant pathologist reexamined all these cases for further confirming the diagnosis.

In one hand, 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 HCMV. In other hand, the detection of HCMV 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 Human Cytomegalovirus DNA.

The main steps for ISH procedure were: incubation of slides for 18 hr at 70°C on hot plate, then rehydration process was done at room temperature which include : slides immersion 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 a 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 a routine dewaxing protocols were used; 2-5 min xylene, 2-5 min 100% ethanol, 2-5 min 96% ethanol, 1-5 min 70% ethanol. Then air drying of sections and pepsin solution application to the tissue sections and incubated for 20-30 min at 37°C in a humidity chamber. After that immersion slides in distilled water and drain off the water, air dried sections. Then addition of the probe and denaturation of the slides at 75°C for 5 min on hot plate, then transferred the slides to a humidity chamber and hybridize for overnight at 37°C and then post-hybridization and detection processes that included removing the cover slip by submerging in 1x wash buffer TBS, then washed for 5 min in 1x wash buffer TBS at 55°C. Then application of Rabbit-anti-DIG - antibodies to the slides and incubate for 90 min at 37°C in a humidity chamber. Then slides were rinsed in detergent wash buffer for 5 minutes (twice times) and then drained. Then application of Anti-Rabbit-AP-Polymer drop- wisely to the slides and incubate for 90 min at 37°C in a humidity chamber. Then slides were rinsed in detergent wash buffer for 5 minutes (twice times) and then drained. After that one to two drops of 5-bromo3-chloro3-indoly/phosphate/nitro blue tertrazolium substrate- chromogen solution (BCIP/NBT) were placed on tissue sections. Slides were incubated at 37°C for 2 hours or until color was developed completely. Color development was monitored by viewing the slides under the microscope. A dark blue colored precipitate forms 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 90-120 seconds, then washing process was followed by immersion the slides for 1 minute in tap water. 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 (Abcam. England) was used to demonstrate the expressed protein of P21 tumor suppressor gene. This technique is based on the detection of the product of gene expression in malignant and normal cells using a specific monoclonal antibodies, i.e. primary antibody for specific epitope (usually mouse anti-human monoclonal antibody) , which binds to nuclear targeted protein 37°C for overnight. The bound primary antibody is then detected by secondary antibody ( usually rabbit or goat anti mouse ) , which contains specific label ( in this context we used peroxidase labeled polymer conjugated to goat anti- mouse immunoglobulin ) . The substrate is DAB in chromogen solution, positive reaction will result in a browning color precipitate at the antigen site in tested tissues.

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. After that streptavidin-alkaline phosphatase conjugate reagent was added to tissue sections. Then slides were kept in a humid chamber at 37°C for 20 minutes.

Chi –square test was used to detect the significance between variables of our study. All the statistical analyses was done by SPSS program (Version– 19) & P value was considered significant when $p < 0.05$.

## RESULTS:

**I. Distribution of patients with Non-Hodgkin´s lymphoma and healthy control group according to their age:**

The archival specimens collected in this study were related to NHL patients whom ages were ranged from sixteen to eighty three years. The mean of age of total patients group was(42.74±19.45)years where the mean of age of the patients with NHL B-cell type was higher(40.16±18.36) years than patients with NHL T- cell type (28.34±12.03) years and healthy controls (29.87± 18.21) years (Table 1 ).

**II. Distribution of the patients with Non-Hodgkin´s lymphoma according to their gender:**

In this study, the number of total males with NHL was higher (27cases; 67.5%) than total females with NHL(13 cases;32.5%) (Table 2).

The percentage of males with NHL B-cell type(39.5%) was higher than those with NHL B-cell type(31.6%) while the percentage of females with NHL T- cell type (36.4%) was higher than those females with T- cell lymphoma(22.7%) ( Table(2).

**III. Distribution of NHL according to their grade /differentiation:-**

The percentage of each grade in NHL B-cell type group were higher than their counter parts in NHL T-cell type group. Statistically, there were significant and highly significant differences on comparing NHL B-cell type of low and moderate – grades with their counter parts in the NHL T-cell type (P<0.05). However, there was no significant difference between NHL B-cell type with high-grade and its counterpart in T-cell lymphoma (P>0.05) .Within NHL B-cell type group, the highest percentage of grade was found in these with low grade (63.2%) followed by the moderate grade (54.5%) then the high grade (50%). Regarding NHL T-cell type group, the highest percentage(50%) was found with high and then followed by the moderate grade(45.5%) then the low grade (36.8%) (Table 3).Statistically, there were no significant differences among the studied groups (p>0.05).

**Table (1):** Distribution of NHL patients according to their age.

| Histopathological grouping | N | Mean (years) | S.D. (years) |
|---|---|---|---|
| NHL, B-cell type | 24 | 40.16 | 18.36 |
| NHL, T-cell type | 16 | 28.34 | 12.03 |
| Total NHL | 40 | 42.74 | 19.45 |
| Healthy- control tissues | 20 | 29.87 | 18.21 |

**Table (2): Distribution of the patients according to their gender**.

| | | | Histopathological Diagnosis | | | |
|---|---|---|---|---|---|---|
| | | | Non-Hodgkin´s lymphoma | | | |
| | | | Total | B cell-Type | T cell- Type | control |
| Gender | Male | Count | 27 | 15 | 12 | 11 |
| | | % within Gender | 67.5% | 39.5% | 31.6% | 28.9% |
| | | % within Diagnosis | | 65.2% | 70.6% | 55.0% |
| | Female | Count | 13 | 8 | 5 | 9 |
| | | % within Gender | 32.5% | 36.4% | 22.7% | 40.9% |
| | | % within Diagnosis | | 34.8% | 29.4% | 45.0% |
| Total | | Count | 40 | 23 | 17 | 20 |
| | | % within Gender | 100% | 40% | 26.7% | 33.3% |
| | | % within Diagnosis | | 100.0% | 100.0% | 100.0% |

**Table (3): Distribution of NHL according to their grade / differentiation.**

| | | Histopathological Diagnosis of NHL | | Total | P-value |
|---|---|---|---|---|---|
| Grades | | B cell- Type | T cell - Type | | |
| Low | Count | 12 | 7 | 19 | *1 |
| | % within Grade | 63.2% | 36.8% | 100.0% | 0.0226 |
| | % within Diagnosis | 52.2% | 30.4% | 47.5% | |
| Moderate | Count | 6 | 5 | 11 | *2 |
| | % within Grade | 54.5% | 45.5% | 100.0% | 0.00321 |
| | % within Diagnosis | 26.1% | 29.4% | 27.5% | |
| High | Count | 5 | 5 | 10 | *3 |
| | % within Grade | 50% | 50% | 100.0% | 0.121137 |
| | % within Diagnosis | 21.7% | 29.4% | 25% | |
| Total | Count | 23 | 17 | 40 | |
| | % within Grade | 72.5% | 27.5% | 100.0% | |
| | % within Diagnosis | 100.0% | 100.0% | 100.0% | |

P-value: 0.670

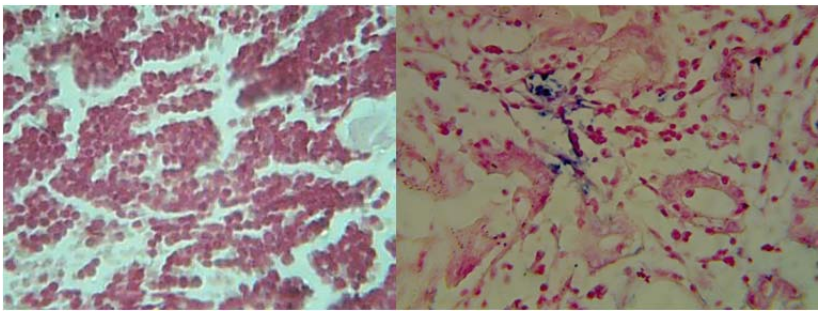
*1: S=Significant difference on comparing B-cell lymphoma with T-cell group.

*2: H.S= highly significant difference on comparing B-cell lymphoma with T-cell group.

*3: N.S= Non significant difference on comparing B-cell lymphoma with T-cell group.

**Table (4):Distribution of HCMV-ISH results in Non-Hodgkin´s lymphoma Tissues.**

| HCMV | | NHL*1& *2 (N=40) N | % | Apparently Healthy Control (N=20) | | P-value |
|---|---|---|---|---|---|---|
| HCMV | Negative | 16 | 40% | 20 | 100% | *1= among NHL= Z test P=0.636 Non sign. (P>0.05) *2= between NHL and control= P=0.00 Highly sign. (P<0.01) |
| | Positive | 24 | 60% | 0 | 0 | |
| HCMV Scoring | Negative | 16 | 40% | 0 | 0 | $\chi^2$ test P=0.00 Highly sign. (P<0.01) |
| | Low | 8 | 33.3% | 0 | 0 | |
| | Moderate | 6 | 25% | 0 | 0 | |
| | High | 10 | 41.7% | 0 | 0 | |
| HCMV Intensity | Negative | 16 | 40% | 0 | 0 | $\chi^2$ test P=0.00 Highly sign. (P<0.01) |
| | Weak | 11 | 45.8% | 0 | 0 | |
| | Moderate | 8 | 33.3% | 0 | 0 | |
| | Strong | 5 | 20.9% | 0 | 0 | |



**A** **B**

**Figure(1):** In situ hybridization for detection of HCMV in NHL; NBT/BCIP stained(blue) and counter stained by nuclear fast red(red); A. NHL with negative HCMV-ISH reaction(40x); B. Moderate score and strong intensity of positive HCMV -ISH reaction(40x).

**Table (5): The results of HCMV signal scoring &intensity among T&B cell types of NHL.**

| HCMV | | **B** cell **type**- NHL (N=23) N | % | **T** cell **type**- NHL (N=17) N | % | P-value |
|---|---|---|---|---|---|---|
| HCMV | Negative | 6 | 26.1% | 8 | 47.1 | Highly sign. For (P<0.01) |
| | Positive | 17 | 73.9% | 9 | 52.9 | |
| HCMV Scoring | Negative | 6 | 26.1% | 8 | 47.1 | $\chi^2$ test P=0.00 Highly sign. (P<0.01) |
| | Low | 4 | 23.5% | 3 | 33.3 | |
| | Moderate | 6 | 35.3% | 4 | 44.4 | |
| | High | 7 | 41.2% | 2 | 22.2 | |
| HCMV Intensity | Negative | 6 | 40% | 8 | 47.1 | Highly sign. (P<0.01) |
| | Weak | 8 | 47.1% | 3 | 33.3 | |
| | Moderate | 5 | 29.4 | 5 | 55.6 | |
| | Strong | 4 | 23.5% | 1 | 11.1 | |

**IV. Distribution of HCMV-DNA-CISH in Non-Hodgkin´s lymphoma Tissues:**

The **HCMV-DNA-CISH** was detected as a blue discoloration at nuclear localization (Figure 1). Table (4) shows that the HCMV has non-significantly associated among Non-Hodgkin´s lymphoma at 5 percent level **(P>0.05)**, however, on comparing the percentage of positive results of HCMV- ISH reaction in HCMV - infected cases (60%:24 out of 40 cases) with those in the control group which none have showed HCMV- ISH reaction, significant association with Non-Hodgkin´s lymphoma at 5 percent level was observed (Table 4).

It was found that the highest percentage of HCMV-ISH reaction was in those with was with high score (10 cases; 41.7%) then followed by low score (8 cases; 33.3%) and moderate score (6cases; %25). HCMV- negative ISH reactions were noticed in 16 cases(40%).While, the highest percentage of HCMV-ISH reactions was found in the weak intensity category(11cases;45.8%) followed by the group of moderate intensity (8cases;33.3%) then by strong intensity(5 cases;20.9%). The results of scoring as well as intensities of ISH reactions for HCMV-DNA show significant associations with Non-Hodgkin´s lymphoma at 5 percent level (Table 4).

**V. Results of ISH of HCMV in T&B cell types of NHL:-**

The percentage of HCMV –ISH reaction results in NHL,B-cell type was (73.9%),while in NHL,T-cell type (52.9%). None of the control group showed HCMV -ISH reaction. The statistical analysis shows significant difference between the patients and control groups (p<0.05). The high percentage of signal scoring of HCMV-ISH in B-cell NHL was found in high signal score for test was (**41.2**%) while, T-cell NHL cases with moderate score signal was (44.4%). The weak intensity of HCMV-ISH reactions was (47.1%) in B-cell NHL and in T-cell NHL, the high percentage of signal intensity in moderate intensity was found **55.6%** (Table 5).

**VI. The results of P21 IHC signal scoring & intensity among studied groups:**

The P21 protein was detected as a brownish discoloration at nuclear &cytoplasmic localization (Figure 2 ) where P21 was detected by IHC at high power field examination in 23 cases (57.5%) of studied NHL cases. The highest positive results of P21- IHC reaction was showing low score (12 cases; 52.2 %) then those showing moderate and high score was 30.4%& 17.4%, respectively. While, the highest percentage of P21-IHC reactions was found in the moderate intensity category (11cases;47.8%) followed by the group of weak intensity (7cases;30.4%) then by strong intensity(5 cases;21.7%). Seventeen cases (42.5%) showed negative IHC reactions. None of control group showed positive IHC test (Table 6).

**VII. The results of expression of p21-IHC protein in (T&B) cell types of NHL:-**

The highest percentage of p21- expression was shown in NHL,B-cell type with low score(8 cases out of 15; 53.3%) followed by moderate score (4 cases out of 15; 26.7%) and lastly the percentage of P21 score within high score( 3out of 15; 20% ),while the highest(4; 50%) within NHL,T-cell type was of moderate score followed by low score (3 cases out of 8; 37.5%) and lastly the percentage of p21 expression IHC within high score( 1 case out of 8; 12.5%). However on comparing the group of the NHL, B-cell with its T-cell counterpart group, regarding their negative, low, moderate and high p21 scoring , significant as well as highly significant differences($p<o.o5$) were found regarding weak and moderate scoring, respectively while non-significant differences ($p>0.05$) were found between (B&T) Lymphomas with negative scoring. The weak intensity of P21-IHC reactions was (46.7%) in B-cell NHL and this was higher than the moderate & strong intensity was 40%& 13.3%, respectively. While in T-cell NHL, was found the high percentage of signal intensity in weak intensity **50 %** followed by moderate & strong intensity was 37.5 % & 12.5 %,respectively (Table 7)

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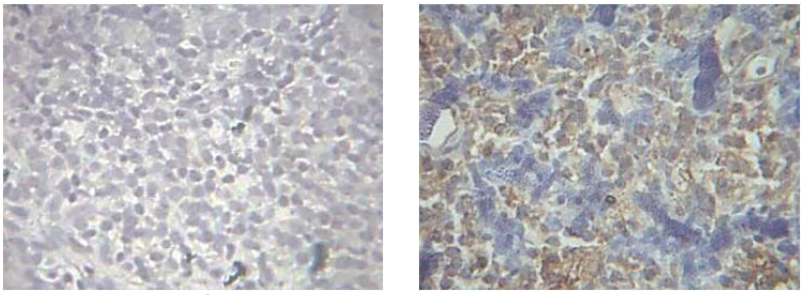
**Table (6): The results of P21- IHC scoring & intensity in NHL Tissues.**

| P21 | | NHL (N=40) N | % | Apparently Healthy Control (N=20) N | % | P-value |
|---|---|---|---|---|---|---|
| P21 | Negative | 17 | 42.5% | 20 | 100 | Z test P=0.431 Non sign. (P>0.05) |
| | Positive | 23 | 57.5% | 00 | 00 | |
| P21 Scoring | Positive | 23 | 57.5% | 00 | 00 | $\chi^2$ test P=0.002 Highly sign. (P<0.01) |
| | Low | 12 | 52.2% | 00 | 00 | |
| | Moderate | 7 | 30.4% | 00 | 00 | |
| | High | 4 | 17.4 | 00 | 00 | |
| P21 Intensity | Positive | 23 | 57.5% | 00 | 00 | $\chi^2$ test P=0.001 Highly sign. (P<0.01) |
| | Low | 7 | 30.4 | 00 | 00 | |
| | Moderate | 11 | 47.8 | 00 | 00 | |
| | Strong | 5 | 21.7 | 00 | 00 | |



**A** **B**

**Figure (2)**: Immunohistochemistry for detection of p21 in NHL; DAB chromogen stained(brown) and counter stained by Mayers hematoxyline (blue);A. Negative IHC-reaction for p21 reaction(20x).B. High score and moderate intensity of p21 IHC- reaction(20x).

**Table (7). The results of expression of p21-IHC protein in (T&B) cell types of NHL.**

| P21 | | B Type- NHL (N=23) N | % | T Type- NHL (N=17) N | % | P-value |
|---|---|---|---|---|---|---|
| P21 | Negative | 8 | 34.8% | 9 | 52.9 | Highly sign. For (P<0.01) |
| | Positive | 15 | 65.2% | 8 | 47.1 | |
| P21 Scoring | Negative | 8 | 34.8% | 9 | 52.9 | $\chi^2$ test P=0.00 Highly sign. (P<0.01) |
| | Low | 8 | 53.3% | 3 | 37.5 | |
| | Moderate | 4 | 26.7% | 4 | 50 | |
| | High | 3 | 20% | 1 | 12.5 | |
| P21 Intensity | Negative | 8 | 34.8% | 9 | 52.9 | Highly sign. (P<0.01) |
| | Weak | 7 | 46.7% | 4 | 50 | |
| | Moderate | 6 | 40% | 3 | 37.5 | |
| | Strong | 2 | 13.3% | 1 | 12.5 | |

**Table (8): Correlations among studied markers HCMV, P21, within Grade and Age in patients with Non-Hodgkin lymphoma.**

| Assay | | | | |
|---|---|---|---|---|
| **Spearman's rho** | | **Age groups/Year** | **Grade** | **HCMV** |
| Grade | r. | .063 | | |
| | P-value | .697 | | |
| HCMV | r. | .009 | .209 | |
| | P-value | .956** | .196* | |
| P21 | r. | -.027 | .093 | -.066 |
| | P-value | .867** | .566* | .685* |

*Correlation is significant (P<0.05). **Correlation is highly significant (P<0.01).

**VIII. Correlations among studied markers (HCMV, P21) within Grade and Age in patients with Non-Hodgkin lymphoma**:

There is a strong positive relationship (with highly significant correlation) between the results of HCMV and P21 markers (p <0.01).Also, there are significant correlations among the results of HCMV and grade of Non-Hodgkin lymphoma (Table 8).

## DISCUSSION

As the number of cases included in this study was 40 and this fact is noticed on reviewing other Iraqi studies (55 cases)[39]do not really reflect the actual age and gender of patients with NHL, since the sample size of these cases was small, too. However, in this study it was found that the mean age of patients with NHL was in their forties(40.2 years) as shown in table(1). This result was closely comparable to an Iraqi study done by [39] who found that the mean age of their NHL patients was (39.70) years. Also this study was in agreement with Jordanian study done by Al-Masri *et al.,* (40) who found that the median age of their NHL patients was 43.5 years.

Our study also in agreement with Pakistanian studies done by Lal *et al.,*(41) and Naz *et al.*(42) who found the median age of their NHL patients was (48.0±13.3) years and (42.5 )years, respectively. Also this study is in agreement with the study of Naz *et al.,* (42) who found the mean age of NHL patients was (41.3) years .The present results are consistent with the results of Manipadam *et al,.* from Iran [43], who found the mean age of their NHL patients was 41.3years .Regarding T and B cell types of NHL of this study, it was found that the mean age of those patients with NHL, B-cell type (41.6 years) was higher than the mean age of their counterpart patients with NHL, T-cell type (28.34years).

Our study running with another study in Pakistan done by [42] who also found that the mean age ( 43.5±16,9 years)of their patients with NHL, B-cell type was higher than the mean age of their counterpart patients with NHL, T-cell type (28.4±15.9 years).

The present result of M:F ratio is consistent with Al-Masri *et al.* [40] in Jordan who found males to females ratio 1.4:1**.**The present result are compatible with these results reported by Hashemi&Parwaresh (2001)in Iran(M:F ratio was 1.5:1).Also, the present M:F predominance result is consistent with two other studies done in Pakistan[41&42] who found M:F ratio of 2:1 and 2.6:1, respectively) and a study done in India by Manipadam *et al.*[44] who found M:F ratio of 3:1 .

The predominance of male gender as documented in our study and many other series of studies is contrasted by the majority of females in relation to males was reported by another researchers in Turkey[45]and Germany[46].However, still there is need to generate more data regarding variation in gender predominance in our population for better studies.

Our results of high and low NHL grades are compatible with these results reported by Draggassky *et al.*[47] in Argentina (39.7% and 38.25%for high and low grades, respectively).Also the high grade result of the present study is consistent with the result 34%for high grade NHL of Castella *et al.*, [48] in United Arab Emirates (UAE).

However, their results of moderate- and low- grades (59% &7%, respectively) were higher and lower, respectively than those documented in the present study (35% for each grade). Although a similar mean age of both native UAE and Iraqi populations was documented in both Castella *et al*.,[48] and the present study and the since low-grade lymphomas are seen most frequently in older age groups, the explanation in one hand, for small proportion of low- grade lymphomas found in the Emirates study has been attributed partially to the younger age distribution of UAE patients population with NHL (who were of mean age of the general UAE patients with low grade are seen mostly frequently in older ages groups). On other hand, the present results could mark for the occurrences of low grade malignant lymphomas in Iraqi patients at earlier age than that expected worldwide. This discrepancy could be attributed or as the result of small sampling in the present study, as compared to other abroad studies. These results also call for more research works into the reasons for the prevalence of this low grade lymphomas in our country.

Regarding (B&T)all types of Non-Hodgkin´s lymphoma, considering the degree of malignancy , the majority were with low grading of NHL, B-cell 63.2% & high grading for T-cell types of the present study was 50% . These results are disagreement with the result of Hashemi and parawersh [45] in Iran who also found the majority of their case with B- cell & T-cell NHL were of higher grading, too(76% & 83%) for B-cell & T-cell, respectively. Since these and the present result are fronted by the results in western countries, who had documented a majority of lymphomas in these countries of low-grade typed(54.5%). One reason for this difference could be due to the low mean age of the population in Iran & Iraq compared to the western countries. Inadequate screening of the patients might have also contributed to the differences. In addition, the patients often present themselves to the medical care system at much later stages of the diseases where the low grade lymphomas have evolved into secondary type of high grade once. For a more precise understanding of this phenomenon, it is necessary to examine the etiology and the epidemiology of the lymphomas in Iraq as well as Iran because there is the possibility that higher grade lymphomas are of the primary type and some important factors may be involved in high occurrences that is a subject that need further investigation, since there has been little research done in this area [45].

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 present total CMV-positivity rate in NHL specimens of this study was 60 %, where CMV- rate in NHL, B-cell type cases was 73.9 % whereas in NHL, T-cell type tissues was 52.9 % . The different types of NHL included in this study as well as the limited numbers of NHL tissues tested made it difficult to conclude as to what extent the present results could reflect the actual association between CMV and the NHL group of diseases studied here in.

Our results are incompatible with another study in U.S.A done by Roy F *et al*., (2003)who found the percentage of CMV in NHL was 89%.These results reveals that the difference between the positivity rates in (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 lymphoma and occurrence of CMV s infection in our country.

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 cohort studies certainly influences the significance of these results [27].

The negativity of healthy control tissues used in this study for CMV nucleic acids support the suggestion that this virus might not represent an epiphenomenon of CMV- positive cancers and / or tumors, and that these observations rather might directly aiding in tumor initiation and progression as well as cancer development [51,52]. In these studies, most neoplastic cells in sentinel lymph nodes of > 90% of breast cancers were CMV- positive[51] and 98% of brain metastases from cancers in colon and breast revealed CMV proteins and/or nucleic acids[52]. However some researchers have failed to detect CMV in their studied tumor tissues [53-58].

An infection with CMV is usually reported in those immunocompromised hosts such as organ transplants recipients, AIDS patients or those treated with immunosuppressive drugs [59]. In addition, this infection might occur in those with hematological malignancies [60,61]. However, and apart from those in the transplant milieu, such infection is uncommon in patients with hematologic neoplasms. Moreover, and published clinical issues have pointed, over time, to a trend of increasing incidence of this viral infection in both lymphomas and leukemias [62,63].These higher incidences might be referred to an increased attention to either the serious sequels of CMV infection (and therefore, increased rates of diagnosis, among such patients) or more serious immunosuppression of immune chemotherapies. As such, CMV infection became an important problem among clinical issues of hematological malignancies.

The incidence of CMV infection differs between sexes. It was reported that CMV pneumonia episodes have been found among 27 male and 9 female patients out of total 36 patients with lymphomas [62].In addition, 82 episodes of CMV infection were reported by Torres in 71 patients with lymphoma where 62% patients were men. Such gender difference in relation to CMV infection risk has been reported among either immunocompetent hosts or liver transplantated patients [62,63] Regarding the field of viral carcinogenesis and/ or tumorigenesis, the variability of detection results of CMV in the studied tumor specimens among laboratories have raised that the presence of CMV in these tumors could be false results or based on artefact data. However, they might likely be referred to the differences in the preparations of these samples, and sensitivities as well as specificities of the employed methods for the localization of CMV nucleic acids and / or proteins where the techniques should be developed and specified to work in such tumor specimens.

Expression of p21 gene was observed in 57.5% of total NHL tissues where its percentage in NHL of B-cell type was higher (62.2%) than in NHL of T-cell type (47.1%) (Table 6&7). Our results are compatible with another study done by Chilosi *et al*., (1996) who found the percentage of p21 in NHL.

It could speculate, and as Chilosi *and coworkers in* [64] proposed, a possibility that the p2 1 WAF' overexpression observed in these neoplastic cells in the studied tissues is a consequence of the physiologic activation of the WAF1 gene, a downstream target of p53 phosphoprotein . This speculation is in line with the fact that all of the studied NHL cases had p53 gene in germ line configuration.However, our results are incompatible with another study done by Gong JZ *et al*., [65] who found absence of somatic changes in p21 gene in non-Hodgkin's lymphoma.

Herein, it was found that in mice with spontaneously developed experimental thymic lymphomas, those lymphomas which were lacking p21 had higher apoptotic rates, suggesting that p21 could possess anti-apoptotic activity, either in the context presence of a p53-triggered response or in absence of p53 [66,67].

In p21-null mice but have their p53, 70% of the animals developed tumors at the time of death while p21- deficient mice developed autoimmunity [68]. The mechanisms which have been reported to explain the p53-independent antiapoptotic activity of p21 [67],were particularly in relevance to the ability of p21 to bind (and inhibit) caspase-3 [69]

The inability of p21 to induce growth arrest are related to p21 gene structural abnormalities, however, these are rare in human tumors and rather absent in lymphomas . Alternatively, the amount of p21 protein may be insufficient to inhibit the activity of the cdk4/cyclin D complex, since low p21 protein promoted assembly of active kinase complexes, while a higher concentration inhibited the kinase activity . Abnormalities in the cell cycle downstream of regulation of p21 could also be involved in melanomas . Interestingly, concomitant p53/p21 overexpression also showed mdm2 overexpression . Despite that, mdm2 did not inhibit p53-mediated transactivation, because of the p53+/p21+ phenotype [70].

To assess the importance of CMV in relation to the expressed p21 protein in lymphogenesis in patients with NHL, we compared the percentage of the latent CMV and expression of p21 in lymphomas arising in patients with NHL.

T-cell–mediated immunity was found to be the host's major defense against CMV.2,22 Patients with either Hodgkin's disease or NHL have marked decrease in CMV-directed cellular immunity[71,72]. Similarly, patients with NHL of B-cell or T-cell types might have impaired cellular-mediated immunity[73].

By analogy, this possibility is supported by the results of Salloumn et al.,[74] who reported that the simultaneous presence of latent EBV and p53-tumor suppressor gene expression in lymphomas were arising in connective tissue disorders(CTD) patients who were immune suppressed with methotrexate (MTX).

In conclusion, these findings could indicate that the noted cell cycle deregulation and CMV-related transformation are both important in the pathogenesis and /or carcinogenesis of this series of NHL studied herein.

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