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The Value of Immunohistochemistry of P53 and Ki67 in Non-Hodgkin's Lymphoma

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Abstract: ***Aims-To determine the frequency of P53 & Ki67 proteins, as detected by immunohistochemistry in lymph node biopsies of Iraqi NHL patients, to investigate their pattern of expression and to study the correlations and comparisons between P53 and Ki67 immunohistochemical detections and various clinical and histological parameters of NHL patients in order to specify their importance as prognostic factors in NHL patients.A total of 85 non-Hodgkin's lymphoma Iraqi patients including 54 males and 31 females cases, who has lymph nodes biopsies. Using the Working Formulation classification, the most common grade encountered was the Intermediate grade NHL constituted (65.9%) of the cases, while the lowest commoner was the Low grade with frequency of (15.3%). High grade lymphoma was encountered in (18.8%).The overall frequency of both P53 and Ki67 proteins positivity in NHL cases included was (63.5%) and (68.2%) respectively.Regarding the P53 and Ki67 results, no significant difference were found concerning age, sex, site of lymph node biopsy, and sclerosis which present in some morphological subtype of NHL. However there were statistically significant differences between P53 or Ki67 and different histological grade of NHL, whereby both P53 positivity and Ki67 expression were found to increase significantly (P<0.05, P<0.005 respectively) with increasing histological grade from low to intermediate and high grades, similarly both markers were significantly more commonly encountered in DLCL than other histological subtypes (P<0.002, P<0.01 respectively).Significant association (P<0.01) was demonstrated between the extent of Ki67 expression and the different histological grade.Ki67 immunostaining was significantly associated with Mitotic Index (MI) (P<0.0001).There was significant coexpression between both markers (P53 & Ki67) in NHL cases. P53 was significantly associated with high extent of Ki67 immunostaining. In conclusion, this study revealed that P53 and Ki67 expression are a common event in Iraqi NHL patients and that these two markers were significantly associated with the grade of this type of lymphoma suggesting that the combined IHC evaluation of P53 and Ki67 may provide a valuable means of assessing the prognosis in this disease.***

Keywords: Non-Hodgkin's lymphoma, immunohistochemistry, P53, Ki67

1. Introduction

Non-Hodgkin's Lymphoma (NHL): Malignant monoclonal proliferation of lymphoid /or macrophage system forming heterogeneous groups of lymphoproliferative malignancies with different pattern of behavior and responses to treatment [1]. Immune dysfunction both acquired and congenital has been associated with development of aggressive NHL [2][3]. NHL is caused by long-term stimulation of the immune system, when B-cells proliferate at high rate for many years, more mutations occur, some of these mutations cause cancer [4][5].The incidence and mortality rates of NHL have been increased steadily during the past few decades in several countries [4].Lymphoma has been commonly detected in developed countries [6], and this condition ranks sixth among cancer diseases affecting males and females in the USA. Although the improvement of the diagnosis, classification, and treatment for NHL results in a decreased death rate, non-Hodgkin lymphoma (NHL) remains the ninth leading cause of cancer-related deaths in the USA [7].In Iraq, NHL is considered to be one of the commonest malignancies [8]. According to the result of Iraqi Cancer Registry (2015), NHL was the seventh most common cancer constituting 4.3 percent of total malignancies in both sexes [9]. Much of the biology of lymphomas has been elucidated by studding their antigenic feature and immunologic properties. The understanding of lymphomas has been greatly enhanced by new reagents (such as monoclonal antibodies), new techniques (such as tissue section immunohistochemistry [IHC] and immunofluorescence), and new instruments (such as the epifluorescence microscope, flow cytometers and automated immunostainers) [4]. The major advantages of tissue section immunohistochemistry (IHC) are that the topography of the tissue is intact and that morphological and immunologic features can be integrated to allow analysis of tissue immunoarchitecture[10]. Lymphoma represents a single immortalized clone result from collective "malignant" aberrancies of neoplastic lymphoid cells and as such it is a paradigm of monoclonality[4][11]. These malignant properties are relevant to tissue immunohistochemical diagnosis such as abnormal expression of an oncogen or tumor suppressor gene e.g. P53 [1][13][14][15]. That led to loss of proliferative control [4].**P53** is a tumor suppressor gene located on the short arm of chromosome 17, at band 13.1 (17p13.1) [16]. It encodes a 53-Kd nuclear phosphoprotein, and hence the name (P53), which is made up of 393 amino acids [17], it normally involved in the regulation of cell cycle. P53 expression has been reported to be associated with high proliferation in human malignancies in general and in malignant lymphoma [18][19]. The overexpressed gene products are especially useful in immunohistochemical assessment of malignancies, [20][21][22][23][24][25][26][27], since in normal physiological lymph nodes, the wt. P53 is present at low level in the cells and has a short half-life [28]. The presence of mutation is thought to increase the half-life of the protein and make the detection possible by IHC, thus it could be an indirect method to detect mutation in the gene [19][28][29]. These abnormalities of cell cycle proteins seem to be an

important mechanism of tumorigenesis and may play a role in prognosis of aggressive NHL [30]. Another immunohistochemical feature secondarily related to altered oncogen status which is of diagnostic usefulness is the monoclonal Ki67 antibody. This antibody detects a nuclear proliferation antigen that has great utility in providing an estimate of proliferative rate of NHL that provides important prognostic information [4][18].

The immunohistochemical techniques used in the current study for P53 and Ki67 protein expression in NHL is easily applicable in any pathology laboratories familiar with IHC procedure and does not require expensive instrumentation or laborious tissue preparation [15][19][30], with high sensitivity and specificity, which could be applied in retrospective studies in the screening of large series of cases [30][31].

2. Materials and Methods

A total of 139 cases of NHL were diagnosed at the Dept. of Histopathology in Baghdad Medical City Teaching Hospital-Baghdad during period between January 2000 to December 2011, based on lymph node biopsies. Out of these cases only 85 had adequate biopsies for section on paraffin blocks and were included in this study. All patients were diagnosed by lymph node biopsies; they were fixed in formalin, embedded in paraffin wax and routinely stained with hematoxylin and eosin (H & E). Information's regarding the site of lymph node biopsy, histological subtype, age and sex, were recovered from the routine histological files. H & E slides for all cases were reviewed.

Classification was done for all cases according to Working Formulation (WF) as defined by National Cancer Institute (NCI) in 1982 [4]. Detailed histopathological subclassification according to grade criteria and morphological subtypes were performed. Cases that were not specified and those classified by other systems, we reclassified according to WF. With special attention on presence and absence of sclerosis in all cases.

In all specimens "Mitotic Index" (MI), was quantitated by counting the number of mitosis per 1000 nucleus in at least ten (X 100) oil immersion fields in areas of highest mitotic activity [32].

All NHLs presented with sclerosis which included within WF, was regarded as one group to evaluate their prognostic significance in relation to P53 and Ki67 immunohistochemical studies [4].
All morphological subtypes with diffuse large cells lymphomas (DLCL) in both intermediate and high grades were considered as single type in comparison to other NHL cases to assess the prognostic significance of both tumor suppressor gene "P53" and growth fraction detected by "Ki67"antibody in DLCL [33][34]. All specimens included were then evaluated for P53 and Ki67 presence by IHC.

Reagents used:

- Monoclonal antihuman P53 antibody, DO-7 (DAKO, Glostrup, Denmark). This antibody reacts with both the wild and mutant types of P53 proteins [23][31][35].
- Monoclonal antibody MIB-1 which is directed against Ki67 antigen, which was purchased from (BioGenex, laboratories, San Ramon, CA). This antibody was designed for the specific localization of nuclear Ki67 antigen in formalin-fixed, paraffin-embedded tissue in various types of malignancy including lymphoma and breast carcinoma [18][34].
- Immunoperoxide reagents and Diaminobenzidin-tetrahydrochloride (DAB) which used as a chromageneic substrate were also purchased from (BioGenex, laboratories, San Ramon, CA).
- Target Retrieval solution for pretreatment procedure, obtained from (DAKO, Glostrup, Denmark).
- Positively charged slides ("plus" slides; Fisher brand) from (BioGenex, laboratories, San Ramon, CA) were used in our study.

With each batch, control sections were included:

- For P53 antibody, about ten sections from three blocks of squamous cell carcinoma of the skin were stained for P53 expression, and the positive case with strong and high P53 expression was taken as a positive control [36].
- For Ki67 monoclonal antibodies a several pharyngeal tonsillar hyperplasia sections were stained for Ki67, out of these a strong and high Ki67 expression were served as positive control [37][38][39].
- For the negative control with each section, buffer (PBS) was added in place of primary antibodies [18][38][39].

Evaluation of P53 Immunohistochemical Results
Positive P53 protein immunohistochemical expression gives clear cut nuclear brownish staining, granular or homogenous without any cytoplasmic or background staining. The result of P53 positively in each individual specimen was analyzed according to 3 independent variables:

1) Intensity of staining [20][40]**:**
The intensity of staining of the brownish coloration was considered strong if it could be detected very clearly at even low magnification 10 X and moderate if it was detected with difficulty at low magnification, while of P53 positively could only be detected at high magnification 40X it was considered weak.

Interpretation	Stain intensity
Weak (W)	Barely detectable
Moderate (M)	Detectable (neither weak nor strong)
Strong (St)	Very dark brown

2) The pattern of staining[41]:
The pattern was considered diffuse if the positive cells were distributed through almost all the sections, while it was considered patchy if more than one area of the section showed large number of positive cells and if they were only very few cells positive in the section then a scattered pattern was considered present.

Interpretation	Stain pattern
Diffuse (D)	Homogenous distribution in all section
Patchy (P)	Patchy positivity
Scattered (S)	Very few positive cells in section

3) Extent of the staining (percentage):
The extent of P53 positivity was interpreted as high if more than 10% of the tumor cells/HPS showed nuclear brownish staining, while low extent where positive cells in between 5-10% using HPF.

Interpretation	Stain extent
Low expression (L)	> 5% up to 10%
High expression (H)	> 10%

Therefore negative cases are those with either complete absence of staining or fewer than 5% brown nuclear staining on oil immersion [42][43].

Evaluation of Ki67 Immunohistochemical Results:
Any nuclear staining of Ki67, regardless of its intensity, was considered to be positive.The percentage of positively stained cell was recorded as Ki67 Labelling Index (LI). The 26% of positive nuclear staining was used as the cut off value between high and low immunoreactivity level, as a border line between high and low proliferation capacity of a single positive case, a growth fraction of < 1 to 26% of Ki67 positive cells was considered as low extent, whereas a growth fraction greater than 26% (27-100%) was considered as high extent [38][44].

Interpretation	Stain extent
Low Labelling Index (L)	≤ 26%
High Labelling Index (H)	> 26%

3. Results

A total of 85 patients with NHL were included in this study. Their ages range between (3-75) years with a mean (± SD) of 47.51±17.63 years. Males formed 54(63.5%) of cases, while females formed 31(36.5%) of cases, with a male:female ratio of 1.7:1 . Cervical lymph nodes were the commonest site of involvement constituting 44(53%), followed by Axillary lymph nodes (14.5%), generalized lymphadenopathy (12%), submandibular (4.8%), and supraclavicular (4.8%). The classification of these non-Hodgkin's lymphoma cases according to the working formulation. Intermediate grade NHL constituted (65.9%) of the cases, followed by high grade NHL (18.8%), while low grade NHL constituted only (15.3%) of the cases.

Immunohistochemical staining for P53:
P53 protein staining was confined to the nuclei of the malignant lymphoid cells and revealed a partial, diffuse or granular pattern. P53 positive staining was detected in 54 of 85 NHL cases (63.5%), while 31 cases were negative for P53 protein because of complete absence of any nuclear staining or less than 5% positive cells. There were no significant differences demonstrated in P53 positivity in relation to age, sex, and site of lymph node biopsies.

P53 positive staining was observed in 30.8% of low grade NHL, (67.9%) of intermediate grade NHL and (75%) of high grade NHL. This difference in P53 expression was statistically significant (p<0.05), table (1), (figure 1).

Table 1: Results of P53 immunostaining in different histological grades of NHL

Histo-pathological grade	P53 Positive N (%)	P53 Negative N (%)	Total N (%)	*P*
Low grade	4(30.8)	9(69.2)	13(100)	<0.05
Intermediate grade	38(67.9)	18(32.1)	56(100)	
High grade	12(75.0)	4(25.0)	16(100)	
Total	54(63.5)	31(36.5)	85(100)	

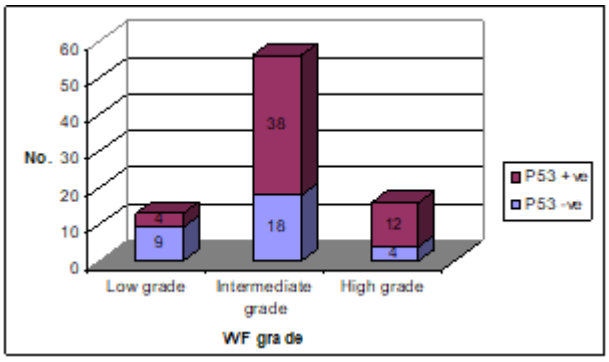

Figure 1: Histogram of P53 staining in different histological grades of NHL

P53 positive staining was commoner in DLCL (88.5%), compared to other NHL (52.5%), the difference being statistically significant (P<0.002), table (2).

Table 2: Comparison of P53 immunostaining between diffuse large cell lymphoma (DLCL) and other subtypes

	P53 Positive N (%)	P53 Negative N (%)	Total N (%)	*P*
DLCL	23(88.5)	3(11.5)	26(100)	< 0.002
Non-DLCL	31(52.5)	28(47.5)	59(100)	
Total	54(63.5)	31(36.5)	85(100)	

Sclerosis may be seen in some of morphological subtypes of NHL such as follicular, diffuse mixed and diffuse large cell lymphomas. P53 positivity was encountered in (75%) of cases with sclerosis compared to (72.5%) in cases without sclerosis, however, the difference between two groups was insignificant statistically (P>0.05), table (3).

Table 3: Comparison of P53 immunostaining between cases with or without sclerosis

Sclerosis	P53 Positive N (%)	P53 Negative N (%)	Total N (%)	*P*
Present	9(75.0)	3(25.0)	12(100)	NS
Absent	29(72.5)	11(27.5)	40(100)	
Total	38(73.1)	14(26.9)	52(100)	

Interpretation of P53 positivity
The extent, intensity and pattern of nuclear staining were examined and specified for each of the 54 P53 positive cases. High extent of positivity has predominated and was seen in 53 cases (98.1%), while it was low in only 1 case (1.9%). The intensity was strong in 14 cases (25.9%), moderate in 29 cases (53.7%) and weak in 11 cases (20.4%).

The most frequent pattern was diffuse staining which was seen in 48 cases (88.9%). (table 4).

Table 4: Characteristics of P53 positive staining

Interpretation	N (%)
Extent:	
High	53(98.1)
Low	1(1.9)
Intensity:	
Strong	14(25.9)
Moderate	29(53.7)
Weak	11(20.4)
Pattern:	
Diffuse	48(88.9)
Scattered	1(1.9)
Patch	5(9.2)

The most frequent combinations included 26 cases (48.1%) of high extent, moderate intensity and diffuse pattern; 11 cases (20.4%) had high extent, strong intensity and diffuse pattern, and 11 cases (20.4%) were of high extent, weak intensity and diffuse pattern, (table 5).

Table 5: Variable combinations of extent, intensity and pattern of P53 positivity

Positivity	N (%)
High, Moderate, and Diffuse	26 (48.1)
High, Moderate, and Patchy	2 (3.7)
High, Strong, and Diffuse	11 (20.4)
High, Strong, and Patchy	3 (5.5)
High, Weak, and Diffuse	11 (20.4)
Low Moderate, and Scattered	1 (1.9)

Mitotic Index (MI):
The MIs in various grades and morphological subtypes of NHL are shown in (figure 20). In the 13 low grade NHL, the MIs were generally low with a mean (±SD) of 5.54±9.01 while in the intermediate grade NHL (56 cases) a mean of 15.98±17.2 was observed, and reaching 29.75±22.47 in the 16 cases of high grade NHL. The correlation between the MI and the grade of NHL was statistically significant (P<0.002), (table 6)

Table 6: Correlation between MI and histological grade

MI	Low grade N=13	Intermediate grade N=56	High grade N=16
Mean ±SD Range	5.54±9.01 0-25	15.96±17.52 0-105	29.75±22.47 7-79
P*	<0.002		

*ANOVA test (analysis of variant).

Immunohistochemical staining for Ki67:

Ki67 immunoreactivity was clearly evident as diffuse or dot like nuclear staining with nucleolar accentuation making it easy to decide whether a cell was positive or not. Strong Ki67 staining was also observed on the chromosomes in mitotic cells.

In the current study positivity for Ki67 expression was demonstrated in fifty eight out of 85 NHL cases reaching a frequency of (68.2%).

No significant difference was observed in Ki67 staining in relation to age, sex, and site of lymph node involvement. The distribution of Ki67 staining in different grades of NHL are presented in table (7), (figure 2), the frequency of positivity has increased with increasing histological grade, and the difference was statistically significant (P<0.005).

Table 7: Results of Ki67 immunostaining in different histological grades of NHL

Histo-pathological grade	Ki67 Positive N (%)	Ki67 Negative N (%)	Total N (%)	*P*
Low grade	4(30.8)	9(69.2)	13(100)	< 0.005
Intermediate grade	40(71.4)	16(28.6)	56(100)	
High grade	14(87.5)	2(12.5)	16(100)	
Total	58(68.2)	27(31.8)	85(100)	

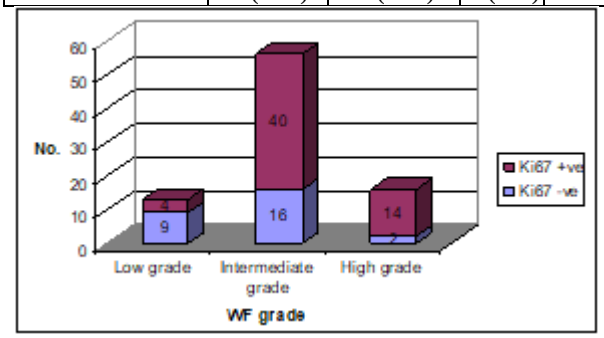

Figure 2: Histogram of Ki67 staining in different histological grade of NHL

Ki67 positivity was encountered in (88.5%) of DLCL compared to 59.3% of non-DLCL cases, and this difference was statistically significant (P<0.01)(table 8).

Table 8: Comparison of Ki67 immunostaining between diffuse large cell lymphoma (DLCL) and other subtypes

	Ki67 Positive N (%)	Ki67 Negative N (%)	Total N (%)	P
DLCL	23(88.5)	3(11.5)	26(100)	< 0.01
Non-DLCL	35(59.3)	24(40.7)	59(100)	
Total	58(68.2)	27(31.8)	85(100)	

No statistically significant difference in Ki67 protein expression was observed between NHL cases with and without sclerosis, (P>0.05), (table 9).

Table 9: Comparison of Ki67 immunostaining between cases with or without sclerosis

Sclerosis	Ki67 Positive N (%)	Ki67 Negative N (%)	Total N (%)	*P*
Present	9(75.0)	3(25.0)	12(100)	NS
Absent	27(67.5)	13(32.5)	40(100)	
Total	36(69.2)	16(30.8)	52(100)	

Comparison of the MI in Ki67 positive and negative cases showed that Ki67 positive cases had a higher mean MI of 21.76±20.06, compared to a mean of 6.67±10.12 in Ki67 negative cases. The difference between the two groups was statistically significant, (P<0.0001), (table 10).

Table 10: Correlation of Mitotic Index and Ki67 positivity

	Ki67 Positive N=58	Ki67 Negative N=27	*P*
Mitotic Index Mean ± SD Range	21.76 ± 20.06 0 - 105	6.67 ± 10.12 0 - 45	< 0.0001

The evaluation of P53 immunostaining versus Ki67 LI:
Coexpression of P53 and Ki67 was found in 47 cases (87%), while 7 (13%) of P53 positive cases had negative Ki67 immunosataining. This association between P53 and

Ki67 immunostaining was statistically significant, (P<0.0001), (table 11).

Table 11: Association between P53 and Ki67 immunostaining in 85 cases of NHL

	P53 Positive N (%)	P53 Negative N (%)	Total N (%)	P
Ki67 Positive N (%)	47(87.0)	11(35.5)	58(68.2)	< 0.0001
Ki67 Negative N (%)	7(13.0)	20(64.5)	27(31.8)	
Total N (%)	54(100)	31(100)	85(100)	

4. Discussion

Recently, a variety of studies all over the world have focused on the role of P53 tumor suppressor gene and Ki67 antigen in the pathogenesis of human malignant tumors and its correlation with various pathological and clinical parameters. Among the pathological and hematological malignancies evaluated in relevance to P53 and Ki67 is the NHL.

No studies on P53 and/or Ki67 in lymphomas have been reported from our country. Thus the current study attempt to address the IHC expression of P53 and Ki67 in NHL Iraqi patients.

P53 Expression

The prevalence of P53 expression in Iraqi NHL patients as demonstrated by IHC in the current study was 63.5%. This prevalence of 63.5% is higher than the frequency reported in various studies from Western countries using IHC which ranged from 16% - 46.8%. [15] [28][29][30][45][46][47][48]. (table 12).The reasons for the increased level of P53 expression in current study may be related to many factors:

The type of antibody: The type of antibody used and its sensitivity is an important determinant of the detection level of P53 protein by IHC. A variety of primary antibodies are available commercially. Said et al 1992 used in his study (PAb 240, PAb 1801, PAb 2401)[15], Du M et al 1995 use (CM 1) polyclonal antibody[12], Chilosi et al 1996 [31],

Table 12: Prevalence of P53 abnormalities in NHL in different studies utilizing different methods

Author (years)	Total No. of cases	% of P53 by IHC	% of P53 by PCR-SSCP
Said J W et al 1992	89	35%	
Du M et al 1995	60	23%	24%
Chilosi M et al 1996	253	36%	36%
Villuendas et al 1997	57	26.3%	21%
Martinez D et al 1997	87	18%	7.4%
Koduru et al 1997	173	46.8%	10.5%
Manukhani M et al 1997	92	32%	31.5%
Preuhome C et al 1998	35	23%	33.3%
Apage P et al 1999	37	16%	
Moller M et al 1999	199	21%	20.5%
Paganano K et al 2001	62	30%	
Current study 2003	85	63.5%	

*PCR: Plymerase chain reaction.
*SSCP: Single strand conformation polymorphism.
*IHC: Immunohistochemistry.

- Koduru et al 1997 [45], andMansukhani et al 1997 [29], used Do-1 monoclonal antibody. The antibody used in current study was Do-7 (DAKO) which is one of the most sensitive ones [49], and showed nuclear specific staining of the highest intensity [23], further more it was shown to be more specific for m.P53 than wt.P53 [42][50], and it has thus been considered by many as the primary antibody of choice [51], and this is the reason for its choice as the antibody to detect P53 in the current study.
- **Fixation:** Another technical aspect of P53 IHC in relevant to formalin fixation, which was proposed by some workers to cause difficulties in IHC, since it may affect a wide variety of tissue antigens [52], however, such a difficulty was not encountered in current study, which is most probably related to effective antigen retrieval procedure, in which DAKO Target Retrieval solution for pretreatment procedure employed in the current study that was based on heating by water bath or at 95^{o}C for 30 minutes, while microwave oven using was avoided, since some researchers suggest that its use does not significantly enhance sensitivity of P53 immunohistochemical stain as proposed by some [25][53] but may in fact lowers it [42].
- **Other reasons:** the other reasons for the difference of P53 expression is that some studies were dealt with specific or selected NHL entities. Koduru et al 1997, work only on B-cell NHL [45], while in study by Du M et al 1995 the number of low grade cases were more than high grade cases [12] and this may lower the percentage of P53 expression.

The different interpretation criteria for the evaluation of the staining and the optimal cutoff value for P53 positivity that vary among different laboratories are among the factors that may contribute in the differences in the reported frequencies as well, [28][29][30][31][56][45][48].

P53 abnormalities in lymphoma could be detected also by using molecular methods (i.e. studying P53 gene), PCR-SSCP (Polymerase Chain Reaction–Single Strand Conformational Polymorphism) [12][28][29][31] [45][46][48] and Southern blot analysis [45][54].

Several studies have shown a direct correlation or a concordance between IHC detection of P53 protein and P53 gene mutation detected by molecular methods [42][45][55][56], and such a concordance were not only documented in NHL [45][48][56], but also in hematological malignancies e.g. CLL, CML, AML, MDS, and ALL, [27][45][51][55][56][57] and many other solid tumors like colorectal, gastric, breast, endometrial, and bladder carcinomas [21][23][42][49][58][59]. IHC has shown a sensitivity of 79% and specificity of 98% in detecting P53 mutations [30]. However a small percentage of P53 genetic defect are not detectable by IHC. Two possibilities were proposed for this discordance; first, the accumulation of mutant P53 may not reach the level detected by IHC, since extensive deletion that lead to complete loss of P53 gene or silent P53 mutations such as frame shift mutation will result in complete absence of P53 protein or production of unstable protein undetectable by IHC [12][31][60]. Secondly, the antibody used may not recognize all mutant P53 [12]. P53 staining as implied in current work using DO-

7 (DAKO) antibody have been shown to be a highly sensitive staining method [23][49][51].

Despite the reported advantage of P53 IHC and its general concordance with molecular methods, however some investigators have documented immunohistochemically detected P53 overexpression in NHL without detectable P53 gene mutations [29][45][51][61]. The explanation of this discordance between IHC and molecular methods of detection may be related to the fact that some mutations may have been missed by routine PCR-SSCP or the possibility that mutation occur outside the exons been tested [14][45]. Moreover some cases of P53 immunoreactivity may not indicate mutation, but overexpression and/or stabilization (and inactivation) of wt. P53 protein by other mechanisms as malfunction of P53 protein by its binding to viral oncogene's products (large T-antigen or E6 and E7 antigens or E1 b55) [62][63], and cellular proteins (mdm-2) [64][65].

Such mechanisms have been seen in NHL, CLL and PLL [29][46][51] and may actually be an added advantage of IHC over molecular methods, since they may reveal P53 protein inactivation by complexing with other proteins despite absence of mutations of P53 gene [51][53].

The International Working Formulation (IWF) has served as a good prognostic variable in NHL. It divides NHL into 10 major subtypes, grouped into three grades [4]. The current study have revealed that P53 was significantly associated with histological grades of NHL, and the highest incidence of P53 immunoreactivity was found in high grade NHL, in which (75%) of cases showed strong nuclear staining while the incidence of P53 in the intermediate grade was (67.9%), and the low grade lymphoma showed the lowest incidence of (30.8%). The proportion of P53 positivity significantly ($P<0.05$) increased from low grade through intermediate to high grade NHL. This finding is consistent with the finding of Said et al 1992, and Burra et al 2000 [15][19]. The former identified the correlation of P53 expression with the grade of malignancy implies that P53 positivity has an adverse prognostic value. Melachrinou et al 1995 observed that biopsies with less than 5% cells positive for P53 were much more in low grade NHL [66], and hence a higher percentage of P53 positive cells in any individual might reflect the aggressiveness of the tumor, confirming the prognostic significance of its value in NHL, [19][28][66].

The correlation of P53 positivity with the grade of NHL led to the hypothesis that P53 mutation probably occurs relatively late in the evolution of lymphoid neoplasia, and that other oncogenes may play a more decisive role in the initiation, and early progression of these neoplasms [52]. The absence of P53 mutation in some cases expressing P53 protein also supports this view [19]. The study of Said et al 1992, shows that P53 immunoreactivity was not demonstrated in either low grade lymphoma or chronic lymphocytic leukemia (CLL), but by the use of sensitive molecular techniques, P53 mutation are detected in cases of CLL [15]. In the current study the findings indicate that about one third of low grade NHL cases were positive P53 expression, there were a proportion which is low comparing with other grade of NHL. In CLL, one out of 4 cases show positive P53 expression, which was demonstrated mainly in scattered large (paraimunoblast) cells, and this might herald progression to high grade malignancy [15][43]. The detection of P53 expression in low grade NHL in the present study may be due to sensitive P53 antibody (DO-7, DAKO) used.

Diffuse large cell lymphoma (DLCL) are aggressive tumors of about 60% of B-cell origin [4]. In present study it has been observed that significantly higher expression of P53 protein was in DLCL and this finding is similar to the study of Pagnanoet. al. 2001, in which there was a significant association between P53 abnormalities and aggressive lymphoma (197). This hypothesis is supported by the higher incidence of P53 mutation in DLCL tumors and DLCL cell lines, which may represent particularly aggressive lymphomas [54].

Many studies of P53 expression in NHL has shown a negative influence on survival and that P53 positive patients have significantly short survival and poor response to therapy compared to those negative for P53, therefore it can serve as prognostic indicator [43][45][55][48]. Disruption of P53 dependent apoptosis could contribute significantly to the poor prognosis of NHL with P53 protein expression. This observation is related to the fact that antineoplastic therapy acts predominantly through the induction of apoptosis in malignancies [48],and many studies have shown an association between resistance to both chemo-radiotherapy and low capacity to undergo apoptosis, thus susceptibility to apoptosis is an important determinant of response to antineoplastic therapy and certainly central to this response are the proteins that modulate apoptosis including P53 protein [47][48], an exception from this pattern is provided by antimitotic agents, the apoptosis-inducing capacity of which is not affected by functional status of P53 protein [30][48]. Therefore, wt. P53 protein is important for inducing response of tumors to therapy as it is required for induction of apoptosis by both chemo and radiotherapy [12][48]. So, evaluation for the expression of P53 protein may give some indication of the susceptibility of a specific tumor to anticancer treatment [43][67] as cells lacking wt. P53 protein are more likely to be resistant to apoptosis induction by treatment than tumors with normal P53 protein [67]. Thus the association between short survival and P53 positivity is more likely to be due to treatment resistance rather than tumor aggressiveness only [51][67] and that tumors which are generally susceptible to treatment show low rates of P53 positivity [68], such a correlation has been shown in non-Hodgkin's lymphoma [45][68], follicular lymphoma [31][56], mantle cell lymphoma [56], adult T-cell leukemia [69] CLL q[51][55], Hodgkin disease [70], and bronchogenic carcinoma [23].

Therefore it is now becoming important to specify the state of P53 in each tumor to predict sensitivity to antineoplastic therapy and try to perform restoration for P53 function by new forms of conventional therapy or by transferring P53 gene into malignant cells [68]. Furthermore, by passing the P53 apoptotic pathway by the use of new therapeutic approaches will be of interest, especially in patients with lymphoma in whom P53 is over expressed [47].

Ki67 expression

Resent reports indicate that measurement of the proliferative activity in lymphoma yield useful objective data that will help to guide therapy and predict the prognosis of individual patients [18][67]. The pathologist must choose among several methods used to measure this parameter. These include; counting mitotic figure [19][71], measuring of labeling index after incorporation of ^{3}H-thymidine [73], or bromodeoxyridine[72], cell cycle analysis by flow cytometry [73][74][75], and Ki67 immunostaining of a nuclear protein associated with cell proliferation [18][33][75]. With each technique having its advantages and disadvantages. The final decision on which method is used is based on:

1) What is the best method of measuring proliferative activity?
2) Is the proliferative activity related to clinical, classification, grade, and whether it give additional information independent of that obtained from the pathological classification?

Most authors agree that proliferative indices of malignant NHL are useful prognostic indicators and provide information independent of other histological and clinical variables [75][76][77][78][79].

The proliferative activity of NHL in the present study was estimated by using 2 different methods: mitotic count (Mitotic Index "MI"), and Ki67 immunostaining.

The number of mitosis has been assessed in biopsy material from patients with NHL classified according to WF classification. The assessment of a MI may give good information on proliferative activity of lymphoma and it has a good prognostic value [71]. It was considered advantageous that the histopathologic diagnosis and MI can be determined on the same occasion [77]. The current study revealed an association between MI and histopathological grading of malignancy i.e. low grade malignant lymphomas had significantly lower MI than intermediate and high grade lymphomas. In general the mitotic rate of various type of NHL is thought to correlate with neoplasm's grade [33]. Other study found that proliferative activity measured by mitotic count correlates with prognosis and indicate an association between high mitotic activity and poor prognosis in NHL [71]. Thus it is possible that the accurate measurement of proliferation is equally or more indicative of tumor behavior than morphological diagnosis [80]. Low MI with only small differences among patients in same subgroup were recorded in this study in different subtypes of low grade NHL, but there was a striking variation in MI within the various subgroups of high grade NHL. These finding are in good agreement with previous studies by Ackerman et. al. 1987, and Weiss et. al. 1987 [33][77].

The other method for estimation of proliferative activity in present study was the Ki67 (MIB-1) immunostaining. Ki67 antibody provides a useful alternative to other methods of measuring cell cycle activity. This antibody distinguishes all actively proliferating cells (G_1, S, and G_2 M phases) from resting (G_0 phase) cells [80]. This method is practical and has obvious advantages over the counting of mitosis [33]. MI in malignant lymphoma is time consuming and often difficult to measure especially when pyknotic nuclei are abundant and reflect only one component of the cell cycle (M-phase). In addition MI often varies from field to field, and has typically analyzed the area of highest proliferation, and this selection will ignore lower proliferation zone [33][80][81]. Late or insufficient fixation of tumors, especially in the center of nodes, that have not been sectioned by surgeon result in a lower mitosis rate than does early fixation [78]. However, in the current study there was a highly significant correlation between MI and Ki67 immunostaining in which the highest MI was found in positive Ki67 cases of NHL. The relationship between mitotic count and Ki67 staining has been studied by several investigators. Our findings are in line with the results of previous studies [33][82][83].

In the current study Ki67 protein expression in Iraqi NHL patients was demonstrated in (68.2%) of cases, and the Ki67 LI in 58 positive cases had a mean of (29.96% ± 16.39).

A significant correlation could be demonstrated between the proportion of Ki67 positive cells and the histological classification into low, intermediate and high grade NHL according to the WF. A trend of increasing Ki67 positivity is noted among the WF subtypes. In general, low grade (indolent) types of lymphoma show the least Ki67 staining and high grade (aggressive) types the greatest. These results are in broad agreement with of Weiss et. al. 1987 and Schwartz et. al. 1989 in which they measures the area of greatest proliferation in NHL by Ki67 staining and they found good correlation with WF grades [33][80].

Regarding the growth fraction (Extent) of Ki67 in this study, it is evident that a significant correlation between the extent of Ki67 and the grade of NHL, in which low extent (<1 to 26%) Ki67 positive cells is more common in low grade, whereas the extent of greater than (26%) is related to intermediate and high grades lymphoma. These finding are consistent with the results presented by Geredeset. al. 1984, which demonstrate that there is a highly significant correlation between the proportion of Ki67 positive cells in NHL and the histologic classification into high grade and low grade malignancies. Using the growth fraction as the discriminating parameter, (93.8%) of high grade NHL and (88.5%) of low grade malignancies would have grouped correctly into these main categories of Kiel classification [84].These finding are in line with kinetic studies performed on cell suspensions of NHL in which ^{3}H-thymedine incorporation and/or flow cytometric DNA analysis where used as a parameter of proliferation [77][80].

Although there was a slight increase in mean overall Ki67 LI in WF, low grade (22.5%), intermediate (29.8%), and high (32.5%) and the range values tend to be wider in intermediate and high grades, there was no significant difference as measured by ANOVA test (analysis of variant). Schwartz et. al. 1989, demonstrated significant difference among mean overall Ki67 staining value in WF grades, although the difference between intermediate and high grades tumors is less highly significant [77]. Korkolopoulouet. al. 1998 suggested that such fining are not surprising as some malignant lymphoma belonging to the intermediate category (i.e. large non-cleave cell NHL) are actually included in high grade group of Update Kiel

classification [18]. In previous study, a high correlation was found between Ki67 LI and low and high grade malignancies as classified in the Kiel classification [79][84].

The current study have revealed that Ki67 positivity was significantly associated with DLCL, in which (88.5%) of DLCL cases were gave Ki67 positive staining. Previous studies by Weiss et. al. 1987 and Grogan et. al. 1988 found a high proliferative index in DLCL, and Ki67 staining to be independent predictor of survival by multivariate analysis [33][34]. In contrast the lowest percentage of Ki67 immunostaining was seen in CLL and follicular small cleaved cell lymphoma and that intermediate values were seen in diffuse small cleaved cell and diffuse mixed cell lymphoma. These facts together with the much higher Ki67 staining seen in follicular predominantly large cell lymphoma, support the finding that small lymphoid cells are predominantly in resting (G_0) phase of cell cycle [80], as well as the absence of this marker in some cases could be the evidence for the presence of cells with a lower proliferative rate and probably lower malignancy [84]. Another explanation for the Ki67 negative cells is that some cells with a relatively long G_1 phase gradually lose their ability to express Ki67 during G_1 and G_2, therefore a population of G_1 Ki67 negative cell that can still proliferate. Thus, it is possible that relatively low Ki67 index or negative cells are not sensitive to chemotherapy [81], or that chemotherapy would fail to eliminate some cells which are in resting phase of the cell cycle. Relapse would occur, should these cells subsequently replicate and act as stem cells [79].

There are conflicting reports regarding the effect of tumor proliferation on clinical outcome in NHL; Gorganet. al. 1988 have previously found a correlation between high proliferation and poor prognosis in both high grade and large cell lymphomas and patients with Ki67 of (> 60%) is an independent poor prognostic factor which is comparable to the prognosis of DLCL patients with no treatment, suggesting that Ki67 may identify DLCL patients requiring alternative therapy [34]. Miller et. al. 1994 reported that tumor proliferation (>80%) was associated with poor survival in previously untreated patients with aggressive NHL, and those with low grade lymphoma who had a relatively high Ki67 index (>5%) had a worse survival than those with an index of (<5%) (151). Whereas Hall et. al. 1988 found that patients who achieved a good response to chemotherapy were less likely to relapse if they has a tumor proliferation of (>80%) [79]. Wilson et. al. 1997 suggests that tumors with low proliferation may be less sensitive to chemotherapy and should receive more prior regimens than rapidly proliferating tumors [67].

The major disadvantage for the use of Ki67 antibody in previous studies is that can be used on frozen sections only [38][79]. While in our study MIB-1 antibody was used and this kind of antibody resist formalin fixation and the immunostaining can thus be performed on paraffin wax embedded sections after antigen retrieval [18][38].

Possible errors which might arise in calculation of non-neoplastic cells, which is often variable, and large number of these cells present in NHL. Although the possibility that a minority of non-tumor cells in a tumor may show nuclear Ki67 immunoreactivity was not excluded, this is unlikely to have a major influence on the result. Sampling errors were minimized by (selection of fields), counting a sufficiently large number of cells and carefully examining Hematoxylin and Eosin (H & E) stained serial sections [79][84].

There was no significant association in present study between sclerosis and P53 or Ki67 immureactivity, which was not mentioned in any previous studies on review of references.

Regarding age, sex, and site of lymph node biopsies in current study, there were no statistical significant differences between these clinical variables and P53 or Ki67 immunostaining was found. This finding will correspond to other studies applied these markers [12][18][30][34][45] [48] [67].

In this study, there was a highly significant correlation between P53 and Ki67 immunohistochemical expression in NHL, in which 47 cases having coexpression of both markers, this finding is consistent with finding of Korkolopoloulouet. al. 1998 and that of Miraccoet. al. 1995, who demonstrated a significant correlation between P53 percentage and Ki67 LI in gastric dysplasia. The higher positivity of both markers was found to be characteristic for tumor with bad outcome [86]. Thus, the combined assessment of proliferation rate by Ki67 and P53 expression in NHL may provide important prognostic information independent of otherclinicopathological parameter [18], and the combined use of P53, Ki67 immunostaining and MI is a useful adjunct to clinicopathological parameters in order to identify patient with more aggressive tumor where possibly more prone to relapse or non-responding to chemo- and radiotherapy [18][86].

5. Conclusions

1) Immunohistochemical technique was easy to done and it does not require sophisticated expensive instrumentation and can analyze large number of samples.
2) The frequency of P53 protein positivity detected by IHC in NHL Iraqi patients is (63.5%) and it is more than the frequency reported from other countries. While the frequency of Ki67 protein expression was (68.2%), which has great utility in providing an estimate of proliferative rate of NHL by IHC, and it is useful alternative to other methods of measuring cell cycle activity.
3) The results in this study demonstrate that there is a significant correlation between P53 protein positivity and Ki67 expression with the histological grade according to WF, and it shows that there positivity of these markers increased significantly as the histological grade advance, also there is a significant correlation between these two markers and DLCL, and the percentage of large cells in mixed subtypes of NHL.
4) The assessment of mitosis in NHL gives prognostic information in addition to histopathologic classification. The proliferative activity and histopathologic diagnosis can be ascertained routinely on the same occasion. On the other hand a significant correlation was proved

through the study between Ki67 (MIB-1) positivity and the MI in NHL cases.

5) A significant correlation proved by this study between the extent of Ki67 LI and the grade of histology of NHL in which the high extent was more in high grade lymphoma.
6) Highly significant correlation was found between the coexpression of both P53 and Ki67 immunostaining, furthermore, there is a significant correlation between P53 positivity and the high extent of Ki67 LI.
7) Our findings strongly suggest that the combined imunohistochemical evaluation of P53 and Ki67 may provide a valuable means of assessing the prognosis of NHL.

6. Recommendation

1) Further prospective studies with clinical follow up of the patients to confirm the prognostic significance of these markers.
2) Showing a correlation between the proliferative state of NHL as determined by Ki67 protein expression, and their clinical behavior would have important implications in terms of treatment selection, response to treatment and overall survival, data from several independent studies will be required if such a correlation to be proved.
3) Additional cell cycle relevant studies may have additional value if studies beside P53 protein, namely P21 protein, mdm-2, BCL-2, and Bax….. etc., in evaluating prognosis in Iraqi NHL patients.
4) Further research on the molecular biology of the P53 gene status for any mutations.
5) IHC interpretation requires standardization, since there is controversy among papers in relevance to classification of pattern, extent, and intensity of positivity, such a task requires collaboration studies between several centers interested in IHC detection of P53 and Ki67.

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