

Loss of P16/INK4A Protein Expression is a Common Abnormality in Hodgkin's Lymphoma

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Abstract—P16/INK4A is tumor suppressor protein that plays a critical role in cell cycle regulation. Loss of P16 protein expression has been implicated in pathogenesis of many cancers, including lymphoma. Therefore, we sought to investigate if loss of P16 protein expression is associated with lymphoma and/or any specific lymphoma subtypes (Hodgkin's lymphoma (HL) and nonHodgkin's lymphoma (NHL)). Fifty-five lymphoma cases consisted of 30 cases of HL and 25 cases of NHL, with an age range of 3 to 78 years, were examined for loss of P16 by immunohistochemical technique using a specific antibody reacting against P16. In total, P16 loss was seen in 33% of all lymphoma cases. P16 loss was identified in 47.7% of HL cases. In contrast, only 16% of NHL showed loss of P16. Loss of P16 was seen in 67% of HL patients with 50 years of age or older, whereas P16 loss was found in only 42% of HL patients with less than 50 years of age. P16 loss in HL is somewhat higher in male (55%) than in female (30%). In subtypes of HL, P16 loss was found exclusively in all cases of lymphocyte depletion, lymphocyte predominance and unclassified cases, whereas P16 loss was seen in 39% of mixed cellularity and 29% of nodular sclerosis cases. In low grade NHL patients, P16 loss was seen in approximately one-third of cases, whereas no or very rare of P16 loss was found in intermediate and high grade cases. P16 loss did not show any correlation with age or gender of NHL patients. In conclusion, the high rate of P16 loss seen in our study suggests that loss of P16 expression plays a critical role in the pathogenesis of lymphoma, particularly with HL.

Keywords—B-cells, immunostaining, P16 protein, Reed-Sternberg cells, tumors.

I. INTRODUCTION

LYMPHOMA is a group of malignant neoplasms that commonly originate from cells native to lymphoid tissue. Classically, lymphoma can be classified as Hodgkin's lymphoma (HL) and Non-Hodgkin's lymphoma (NHL) [1], [2]. The HL is usually characterized by the presence of large and mononucleated and/or multinucleated cells known as Reed-Sternberg cells (RS cells). The RS cells usually very rare, and account for less than 3% of cell population in HL [1]-[3]. RS cells commonly originate from B-cells or in very rare cases from T-cells. On other hand, NHL is an extremely heterogeneous type in its molecular pathophysiology, histology, and clinical course as

compared to HL [1]-[5]. It occurs in all histological types and anatomical sites other than HL. NHL was also classified as B-cell and T-cell lineages according to Kiel classification [4].

Lymphoma is a life threatening diseases that occurs in all age groups worldwide [6]-[8]. According to Jordanian National Cancer Registry (JNCR), which is the major national source of data on cancer, a total of 4333 new cancer cases were reported in 2004 [9]. Of these 4333 cases, there were 206 cases of lymphoma, accounting for 5.7% of all types of cancer cases with a male to female ration of 1.6:1. About 60.7% of these lymphoma cases are NHL. The chance of survival rate for our patients appears to be very low. However, lymphoma is considered as one of the most curable types of cancers, if it is detected at early stage, and the patients receive a proper treatment [10], [11]. For instance, the chance of survival rate for lymphoma patients in United States appears to be quiet high compared to other types of cancer.

Various environmental factors have been implicated in pathogenesis of lymphoma, but findings are still inconclusive and not consistent for these factors [12]-[15]. Furthermore, genes involved in cell cycle regulation have been also implicated in lymphoma etiology and other tumors, [16]-[35]. One of these genes is the *p16* gene. This gene is located at 9p21 chromosome [21]. The P16 protein encoded by the *p16* gene is well known as inhibitor of cyclin-dependent kinase (INK4A). This protein is also known as tumor suppressor protein that plays a critical role in a G1 cell cycle arrest and regulation of the apoptosis pathway [18]-[21]. Binding of P16 protein to cdk4/6 might result in prevention of cyclin D-CDK4/6 complex to form as well as in inhibition of phosphorylation of the retinoblastoma protein (Rb) by cyclin D-CDK4/6 complex [22]. Thereby, these events lead to arresting the cell in the G1 phases, and prevent cell cycle progression from G1 phase to S phase [19]-[25]. A dysfunction in P16 protein in this pathway (P16-cyclinD-CDK4-Rb) keeps Rb protein phosphorylated and inactive, and also releases the G1 phase arrest imposed by Rb. This might also allow genetically defective cells to divide without control [20]-[25].

The P16 protein is considered as one of the major target in carcinogenesis, because it is frequently mutated or deleted in many human cancers [21]-[31]. For example, homozygous deletion of the P16 gene has been found in various human cell lines [26], [27]. Similarly, a homozygous deletion of P16 in mice might also develop B-cell lymphoma with a high incidence [29]. Moreover, absent or aberrant expression of the P16 protein is also linked to an early event in the pathogenesis

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and progression of human cancer, including lymphomas [21]-[28]. It is worth to note that the etiology of lymphoma in Jordanian patients is not defined yet, and possibility that P16 protein could be altered, mutated and/or deleted in lymphoma might also be existed. Based on these facts, we decided to investigate if loss of P16 protein expression is associated with lymphoma and/or any specific lymphoma subtypes.

II. MATERIALS AND METHODS

A. Patient population:

Fifty-five patients diagnosed with lymphoma were selected from the archive of laboratory of pathology at the King Hussein Medical Centre, Amman, Jordan, during a two-year period from January 2006 to December 2008. This hospital is one of the best known and largest government-based hospitals in Jordan. Patients from all sectors and regions (central, north, northeast, and south) of the country can gain an access to this medical facility. The hospital is considered as the most referral center for hematological and cancer diseases in Jordan. Information about the age and gender of these patients were used to obtain from hospital records. Of these 55 lymphoma cases, there were 32 males (58.2%) and 23 females (41.8%), aged from 3 to 79 years (mean age 36.9 years).

B. The hematoxylin-eosin Staining and classification of lymphomas:

Appropriate formalin-fixed and paraffin-embedded tissue blocks were retrieved for all 55 lymphoma patient. These tissue blocks were sectioned at 4 μm thick, and new slides were made. These new slides were stained with hematoxylin-eosin as described previously [16]. The hematoxylin-eosin slides were also assessed for presence of tumor cells in at least three areas of the tissue. Histologic diagnosis and phenotypic evaluations were also carried out for all lymphoma cases by experience hematopathologist Dr. Raji Hadeth (faculty of medicine, department of pathology, University of Science and Technology, Irbid, Jordan). He reclassified all fifty-five lymphoma cases according to WHO and international working formulation of the National Cancer Institute [1]-[3]. HL cases were further subdivided into subtypes, including MC, NS and LD, LP and unclassified (UN). NHL cases were also subdivided into high, intermediate and low grades.

C. Analysis of the expression of P16 protein using immunohistochemistry:

Fifty five specimens of formalin-fixed and paraffin embedded tissues were examined for loss of P16 protein expressions. The paraffin embedded lymphoma tissue corresponding to the particular hematoxylin-eosin slides were then selected and analyzed for the expression of P16 protein. Briefly, tissue section slides were deparaffinized and rehydrated. Then, slides were immersed in 3% hydrogen peroxidase in methanol for 10 min and rinsed in running water. Subsequently slides were treated under pressure in the Decloaking chamber (Biocare Medical, CA, USA). Then, slides were allowed to cool at room temperature for 20 min, and incubated with phosphate buffer saline (PBS) (diluted at 1:10) for 15 min. Slides were then incubated with primary mouse monoclonal antibody against P16 protein for one hour (diluted at 1:50, F-12, Santa Cruz Biotechnology). One drop of biotinylated goat anti-mouse (diluted at 1:10; from

BioGenex, San Ramon, CA, USA) was added to each slide, and washed with PBS. After that, slides were incubated with streptavidin peroxidase (diluted at 1:10, from BioGenex) for 20 min at room temperature and washed with PBS. Slides were then incubated at room temperature with 3,3-Diaminobenzidine chromogen (from BioGenex), and counterstained with hematoxylin and then stained with eosin before dehydration and mounted with DPX. Finally, slides were examined under light microscope (Nikon, Japan). Six cases of non-neoplastic reactive tonsils and spleens were used as positive controls for P16 protein and a previously well-known negative case for P16 was used as negative control. In addition, specificity for immunostaining was examined by replacing primary mouse monoclonal antibody against P16 protein with nonimmune serum.

D. Immunostaining assessment and evaluation:

In this study, the P16 immunohistochemical staining data for all lymphoma cases were interpreted as follows: any cytoplasmic staining without nuclear staining was judged as negative for both HL and NHL cases. For HL, specimen was considered negative, if positive immunohistochemical staining in both nuclear and cytoplasmic compartments is present in less than 10% tumor cells. Whereas, in all NHL specimens, the expression of P16 protein was considered negative, only if positive staining in both nuclear and cytoplasmic compartments is present in less than 20% tumor cells.

III. RESULTS AND DISCUSSION

This current study is continuation for our recent study provided some evidence for role of EBV in pathogenesis of lymphoma. Based on our study, 55 lymphoma patients were classified and evaluated for LMP-1 expression [15]. There were 32 males and 23 female with a mean age of 36.9 years. Of these, there were 30 patients HL and 25 patients NHL. HL were sub-divided into five subtypes, there were 60% (18/30) mixed cellularity (MC), 23.33% (7/30) nodular sclerosis (NS), 6.66% (2/30) lymphocyte depletion (LD), 4.33% (1/30) lymphocyte predominance (LP) and 6.66% (2/30) unclassified cases (UN). Similarly, NHL is also sub-divided into high, intermediate and low grades. There were 48% (12/25) high grade, 20% (5/25) Intermediate and 32% (8/25) low grade.

In this work, immunohistochemistry staining was also carried out to evaluate the expression of P16 protein in HL. Cytoplasmic and nuclear P16 staining were seen in about half of HL samples (Fig. 1). Similarly, both cytoplasmic and nuclear P16 staining were also detected in most of NHL samples (Fig. 2). Based on our qualitative analysis, the tumor cells are classified as positive for P16 protein expression if P16 expression was detected in both nuclear and cytoplasmic compartments of these cells. This is because staining of cytoplasmic compartment only is considered as nonspecific by some investigators [17, 32].

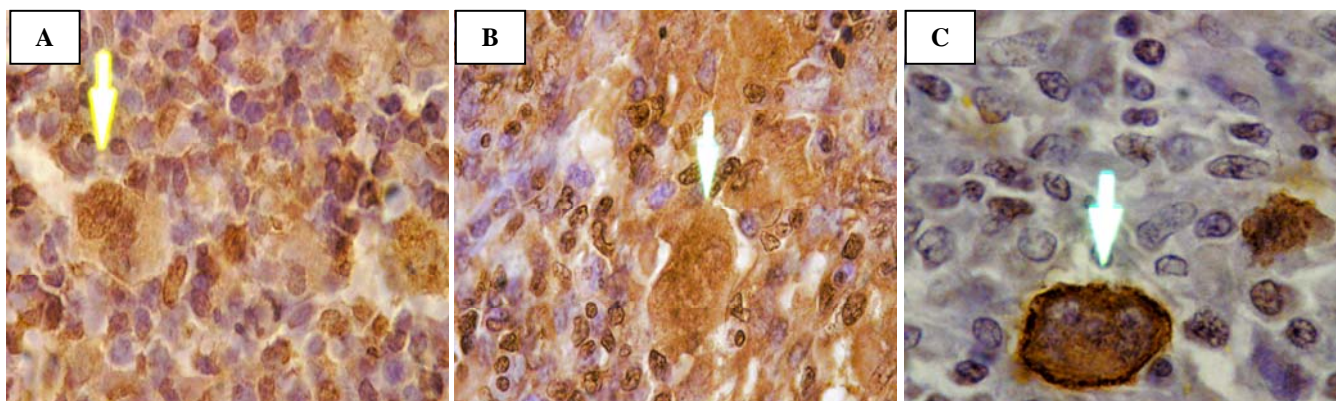


Fig. 1. Expression of P16 in Reed-Sternberg (RS) cells and many bystander lymphocytes of representative samples of Hodgkin's lymphoma (HL). A. HL patient with mixed cellularity. B. HL patient with nodular sclerosis. C. HL patient with mixed cellularity (negative control). For all slides, original magnifications were 100X. Positions of RS cells are indicated by arrows.

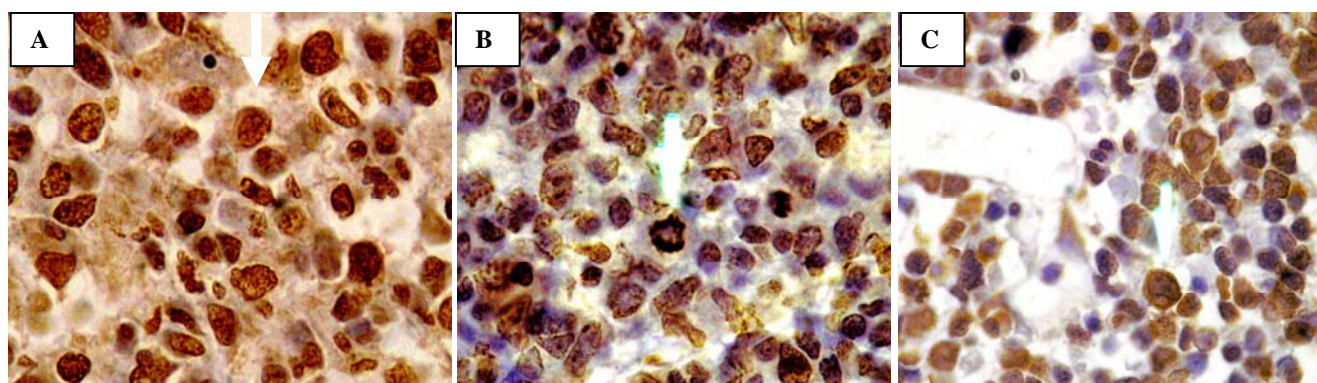


Fig. 2. Expression of P16 protein in representative samples of non-Hodgkin's lymphoma (NHL). A. NHL patient with low grade. B. NHL patient with intermediate grade. C. NHL patient with high grade. For all slides, original magnifications were 100X. Positions of positive P16 cells are indicated by arrows.

According to Table I, of the 55 cases studied, the overall loss of P16 protein expression in all lymphoma cases examined was found to be 33% (18/55). Of these 18 cases, loss of P16 protein expression was observed in 47.7% (14/30) of HL cases as compared to 16% (4/25) of NHL cases. The absence of P16 protein expression seems to occur more frequent in HL than in NHL. Our result agrees with a previous study where they found that 50.8% of all 66 HL cases showed no expression for P16 protein [23]. Other investigators have also reported that the loss of P16 protein expression was as high as 82.5% of all HL cases examined (30 out of 37 cases of HL), supporting the observation that loss of P16 expression is frequently reported in HL [26]. In addition, loss of P16 protein expression was seen in only 14% of all NHL patients. Our result is clearly lower than what was reported in previous studies, which reported that loss of P16 protein expression was observed in 53-78% of all NHL cases examined [33], [34]. The difference in these observations might be due to difference in cutoff point or criteria used in determining positivity, immunostaining approach or technique, size of sample examined, heterogeneity or variability of the disease,

race and ethnicity of selected patients as well as difficulty in classification of some cases of lymphoma as NHL or HL.

Loss of P16 expression in HL cases corresponding to the two variables age and sex is shown in Table II. Loss of P16 protein expression was observed in 67% (4/6) of HL patients with 50 years of age and above, whereas loss of P16 was seen in 42% (10/24) of patients with below 50 years of age. The obtained data showed that there were tendency toward slight increase in loss of P16 expression in HL cases as the age of our patients increase. Concerning the effect of gender, loss of P16 protein expression occurred in 55% (11/20) of male cases with HL compared to only 30% (3/10) of female cases with HL. Our data suggest sex-related difference might exist in HL cases. These unexpected findings should be interpreted cautiously. This sex-related difference might be related to nature of our sample. In fact, our recent study revealed that HL occurs more frequently in male than in female with a male to female ratio 2:1 [15].

On other hand, loss of P16 protein expression was found in 16.7% (2/12) of NHL cases with below 50 years of age, and a similar frequency (15.4% (2/13)) was seen in NHL cases with 50 years of age and above (Table III). Interestingly, similar

result was also obtained with regard to gender, loss of P16 protein expression was observed in 16.7% (2/12) of male cases and 15.4% (2/13) of female cases. This result suggests that absent or loss of P16 protein expression was not correlated with age or gender of our NHL patients.

TABLE I
IMMUNOSTAINING RESULTS FOR EXPRESSION OF P16 PROTEIN IN LYMPHOMA

Type of Lymphoma	P16 expression		
	Total	Positive N %	Negative N %
HL	30	16 (53.3)	14 (46.7)
NHL	25	21 (84.0)	4 (16.0)
Total N (%)	55	37 (67.3)	18 (32.7)

TABLE II
IMMUNOSTAINING RESULTS FOR EXPRESSION OF P16 PROTEIN IN HL IN
RELATION TO GENDER AND AGE

Characteristics	P16 expression		
	Total	Positive N %	Negative N %
Age groups			
< 50 yrs	24	14 (58.3)	10 (41.7)
≥ 50 yrs	6	2 (33.3)	4 (66.7)
Gender			
Male	20	9 (45.0)	11 (55.0)
Female	10	7 (70.0)	3 (30.0)

TABLE III
IMMUNOSTAINING RESULTS FOR EXPRESSION OF P16 PROTEIN IN NHL IN
RELATION TO GENDER AND AGE

Characteristics	P16 expression		
	Total	Positive N %	Negative N %
Age groups			
< 50 yrs	12	10 (83.3)	2 (16.7)
≥ 50 yrs	13	11 (84.6)	2 (15.4)
Gender			
Male	12	10 (83.3)	2 (16.7)
Female	13	11 (84.6)	2 (15.4)

TABLE IV
IMMUNOSTAINING RESULTS FOR EXPRESSION OF P16 PROTEIN IN SUBTYPES OF
HL

Subtype of HL	P16 expression		
	Total	Positive N %	Negative N %
MC	18	11 (61.1)	7 (38.9)
NS	7	5 (71.4)	2 (28.6)
Others	5	0 (0.0)	5 (100)
Total N (%)	30	16 (53.3)	14 (46.7)

TABLE V
IMMUNOSTAINING RESULTS FOR EXPRESSION OF P16 PROTEIN IN SUBTYPES OF
NHL

Subtype of NHL	P16 expression		
	Total	Positive N %	Negative N %
Low	8	5 (62.5)	3 (37.5)
Intermediate	5	5 (100)	0 (0.0)
High	12	11 (91.7)	1 (8.3)
Total N (%)	25	21 (84)	4 (16)

Result of loss of P16 protein expression in relation to the histologic subtypes of HL is listed in Table IV. The loss of P16 protein expression was seen in 100% (5/5) of LD, LP and

UC cases. By contrast, loss of P16 protein expression was reported in 39% (7/18) of MC cases and 29% (2/7) of NS cases. These findings are difficult to explain. The reason is that the size of the sample of these subtype cases was too small (2 LD, 1 LR and 2 UN cases) to conclude whether loss of P16 protein expression was associated with specific subtypes. In addition, there was great diversity of lymphoma subtypes and misclassification of some of lymphoma or ambiguity in some of these cases; these factors also affect the results. Nonetheless, these observations provide further support to the notion that loss of P16 protein expression might play role in the development of some subtypes of HL [19]. In addition, risk factors other than P16 protein seem to play role in development and progression of this disease, particularly in MC and NC cases.

The loss of P16 protein expression in relation to subtypes of NHL cases is presented in Table V. Based on immunohistochemical staining results, absence of P16 protein expression was observed only in one high grade cases (1/12) 8%, and no loss of P16 protein expression was reported in intermediate grade cases. In addition, loss of P16 protein expression was seen in 38% (3/8) of low grade cases. This could be interpreted to mean that the contribution of loss of P16 protein expression to the progression of NHL appears to be not necessary, particularly in those cases that showed aggressive proliferation. In addition, our data also suggest that loss of P16 protein expression is potentially associated with early initiation events in NHL progression. These results appear to correlate well with previous result reported by Liu et al [33]. In contrary, a recent study reported that deletion of P16 gene or loss of dog chromosome 11 was restricted to high grade in naturally occurring canine T-cell NHL [31]. Moreover, there are major differences in the incidence of subtypes of NHL in different geographical locations and among different racial and ethnic populations [4]. Taken together, our and these findings combined suggest that additional unknown potential risk factors or mechanisms must be account for this progressive transformation in NHL cases. This notion is supported by the observation that lymphoma can be originated from B-cell and T-cell precursors, and each type might be susceptible to different kinds of mutations that could contribute to the origin and progression of these diseases. We suggest that further study looking for some selected genes markers such as p15, Rb and p53 must be carried out.

There are some possible limitations in our current study. First, the differences we observed in this study might be coincidence, due to the fact that our selected sample is small in size. Small size sample might affect the result of the study. Therefore, we suggest conducting another study with larger sample size. Second, our approach of evaluating the loss of P16 protein expression is based on immunohistochemistry staining technique only. In light of these results, for future study, we suggest the use of PCR-based technique and in situ hybridization technique to evaluate the mRNA expression of P16 gene as well as Real Time PCR as way to quantify the amount of mRNA transcript of P16 gene.

Furthermore, it is also important to mention that deletion, mutation and promoter hypermethylation of *p16* gene have been reported in various types of cancers, including lymphoma

[21]-[31]. It is possible to suggest that the lack or loss of P16 protein expression that we have seen in our study is presumably due to mutation or deletion in P16 gene. Therefore, to explore possible genetic alteration in P16 gene or to clarify the underlying genetic mechanism of loss of P16 protein expression in our lymphoma cases, we suggest further study at gene level to eliciting the exact mechanism that responsible for such loss.

In conclusion, loss of P16 protein expression was seen in 18 of 55 lymphoma cases, accounting for 33% of all cases. Fourteen of these cases were from patients with HL and 4 cases from patients with NHL. Although this is a small study, we believe that the findings of our study provide an additional evidence for the role of P16 protein in pathogenesis of lymphoma, particularly in HL. Our data also suggest that NHL occurring in our patients appears to develop along a different pathway, with a different pattern of risk factors. For future study, we suggest that further work with larger sample size of lymphoma cases must be carried out to verify our above observations and speculations. Moreover, understanding the potential risk factor in our lymphoma cases can be very beneficial for therapeutic development and might lead to better therapeutic strategies that will reduce both the human and social burden of malignant lymphoma.

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