Loss of P16 Protein Expression and Its Association with Epstein-Barr Virus LMP-1 Expression in Hodgkin's Lymphoma

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Abstract

Background: Expression of Epstein-Barr virus Latent Member Protein-1 (EBV LMP-1) and loss of P16 protein expression are documented in lymphoma, indicating a relationship between them, but this relationship is not clear and sometimes contradictory. Thus, this study was conducted to examine the relationship between the loss of P16 and EBV LMP-1 expression in Jordanian patients diagnosed with lymphoma.

Methods: Sections were made from archival formalin-fixed and paraffin-embedded blocks from 55 patients diagnosed with lymphoma. P16 expression and LMP-1 expression were detected by immunohistochemistry using monoclonal antibodies.

Results: In Hodgkin's Lymphoma (HL), the loss of P16 was higher in LMP-1 positive cases (61%) than LMP-1 negative cases (25%; P = 0.072). Conversely, in Non-Hodgkin's Lymphoma (NHL), none of LMP-1 positive samples showed loss of P16. Furthermore, among LMP-1 HL positive cases, the loss of P16 was more frequent in male (75%) than female (33%). Also, there was a significantly higher proportion of LMP-1 positive cases showing loss of P16 in HL (11:18), compared to those in NHL (0:8, P < 0.001), confirming a difference between HL and NHL, concerning the LMP-1/P16 relationship.

Conclusion: A trend for an association between loss of P16 and LMP-1 expression was observed in HL but not NHL patients. These findings suggest that there are molecular and clinical differences in the pathogenesis and development of different subtypes of lymphoma.

Keywords: Epstein-Barr virus infections; Immunohistochemistry; Lymphoma; Monoclonal antibodies

P16, a tumor suppressor protein, is known as a Cyclin-Dependent Kinase (CDK) inhibitor (also known as INK4a) that plays a critical role in the molecular mechanisms of cell senescence, regulation of the apoptosis pathway and G1 cell cycle arrest [9-11]. In the G1 cell cycle regulation, the binding of P16 to the Cyclin-Dependent Kinase 4 and 6 (CDK4/6) leads to arrest cell in the G1 phase which, in turn, prevents the cell cycle progression from the G1 phase to the S phase [9-11]. Therefore, the loss of P16 can release cell from the G-phase which, in turn, causes cells to progress from the G1 phase to the S phase.
phase, while, the increased expression of P16 can cause the human cells to undergo senescence [9-12]. Of further concern is the fact that different groups of investigators have linked the loss of P16 to an early event in the pathogenesis and progression of different types of human tumors, including lymphoma as well as to the presence of EBV-infected cells [8-14].

EBV is a gamma herpes virus that has been demonstrated to be necessary for driving infected B-cells toward proliferation, immortalization and malignant transformation in vitro [4-7]. This malignant transformation process was suggested to be due to the expressions of number of latent EBV viral oncogene such as Latent Membrane Protein-1 (LMP-1) and some nuclear antigens [4-7, 12-16]. LMP-1 is one of the most constitutively expressed viral oncogenes in many EBV-associated cancers. In addition, the expression of LMP-1 was also reported to be necessary for growth and provide survival signals that enhance tumor growth, and hence contribute to immortalization and malignant phenotype of B-cells [6-7, 12-18].

Concerning the relation between EBV and P16, LMP-1 was shown to block senescence via inhibition of the senescence-associated induction of P16 in human and mouse fibroblast infected cells [12]. This was explained by the ability of LMP-1 to induce genetic alterations in infected EBV cells or stimulate DNA methyl transferase which leads to hypermethylation of promoter region of P16 gene [6-7, 12-19]. Thus, it has been proposed that an inverse correlation might be existed between the presence of EBV LMP-1 and loss of P16 expression in lymphoma tumor cells. However, this relationship is not clear and sometime contradictory.

In our previous study, we reported that the loss of P16 expression was observed in 33% of all lymphoma cases [20]. The loss of P16 expression was also seen in 47.7% of HL cases, whereas, only 16% of NHL showed loss of P16. We also demonstrated that the presence of EBV occurred more frequent in patients with HL (60.0%) than in patients with NHL (32.0%) [16]. Based on these data, we suggest that an addition work is required to clarify whether there is a correlation between the loss of P16 expression and presence of EBV among our patients diagnosed with lymphoma. To address this question and to continue our recent study, the goal of our current study is to investigate if there is a relationship between the loss of P16 expression and expression of LMP-1 of EBV in patients with lymphoma, and to correlate the results with clinicopathological parameters.

Materials and Methods

Patient Population
A total of 55 frozen lymphoma biopsies were used for this study. These lymphoma biopsies were taken from the Department of Pathology, King Hussein Hospital, Amman, Jordan, between January 2006 and December 2008. 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). Information about the age and gender of these patients were obtained from hospital records.

All lymphoma cases were classified according to the WHO and international working formulation of the National Cancer Institute by an experience hematopathologist [1, 2]. The fifty five lymphoma biopsies were classified as 30 cases HL and 25 cases NHL. HL cases were also classified as (18:30) Mixed Cellularity (MC), (7:30) Nodular Sclerosis (NS), (2:30) Lymphocyte Depletion (LD), (1:30) Lymphocyte Rich (LR) and (2:30) Unclassified (UC). NHL cases were also classified as (12:25) of high grade, (5:25) of intermediate and (8:25) of low grade. It is worth to mention that the law does not require ethical approval for this work, because the experiments performed in this research actually did not relate to patient’s privacy, confidentiality, or treatment.

Hematoxylin-Eosin Staining and Classification of Lymphomas
Appropriate formalin-fixed and paraffin-embedded tissue blocks were retrieved for all 55 lymphoma samples. These tissue blocks were sectioned at 4 μm thick, and new slides were made. These new slides were stained with hematoxylin-eosin and mounted with Distrene dibutylphthalate-Xylene (DPX) and examined under light microscope (Nikon, Japan) as described previously [20].

Immunohistochemistry Study
The 55 lymphoma specimen tissues were examined for loss of P16 expression and LMP-1 expression. The streptavidin biotin-peroxidase labeling method was used on 4 μm thick consecutive sections of formalin fixed and paraffin-embedded tissue specimens as previously described [16, 20]. To detect the presence of EBV, all slides were incubated with primary mouse monoclonal antibody against LMP-1 (Biocare Medical, Pike Lane Concord, CA, USA), diluted 1:50, for 30 min. To detect the presence of P16, all slides were incubated with primary mouse monoclonal anti-p16/INK4a antibody, diluted 1:50, for one hour (F-12, Santa Cruz Biotechnology). Specificity for immunostaining was examined by replacing primary mouse
monoclonal antibody against LMP-1 and/or P16 with non-immune serum.

**Immunostaining Assessment and Evaluation**

After the immunohistochemical staining procedure performed, all the 55 lymphoma biopsies were assessed if each specimen was LMP-1-positive or negative as well as if P16 expression was lost or present. Briefly, in HL, the specimen is considered LMP-1-positive if any Reed–Sternberg (RS) cell was membrane or paranuclear staining positive. This is because the HL is usually characterized by the scarcity of the neoplastic RS cell (1%-10%). Whereas in NHL specimens with less than 5% LMP-1-positive tumor cells were judged as negative and specimens with more than 5% LMP-1-positive tumor cells were interpreted as positive. It is worth to mention that the percentage of LMP-1-positive staining whether membrane or paranuclear were taken into consideration. For the P16 immunohistochemical staining data, 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 was present in less than 10% tumor cells. Whereas, in all NHL specimens, the expression of P16 was considered negative only if positive staining in both nuclear and cytoplasmic compartments is present in less than 20% tumor cells.

**Statistical Analysis**

Means and standard deviations were calculated. Associations between loss of P16 expression and LMP-1 expression in patients with lymphoma was determined by using Fishers exact chi-square test. Statistical analysis was performed using the Statistical Package for Social Science for Windows version 11.0 (SPSS, Chicago, IL, USA). P-values less than 0.05 were considered statistically significant.

**Results**

Using immunostaining technique, the expression of P16 and LMP-1 were determined from thin sections which were made from the 55 biopsy specimens derived from patients that were diagnosed with lymphoma. The effects of some known clinicopathological parameters, such as age, gender, and type of lymphomas as well as their subtypes were also examined. Table 1 shows the relationship between the loss of P16 expression and expression of EBV LMP-1 in patients with HL and other clinicopathologic variables. It can be seen that the loss of P16 expression in HL biopsies was higher in the LMP-1-positive group (11 of 18, 61%) than the LMP-1-negative group (3 of 12 (25%), P = 0.072), but the difference was not statistically significant. Our data also indicated that there was a numerically higher proportion of P16 negative in the LMP-1 positive (6 of 13, 46.2%), compared to LMP-1 negative (3 of 12, 25%), in both MC and NS subtypes. Furthermore, for HL patients aged 49 years or younger, the loss of P16 expression was significantly greater in LMP-1-positive cases (8 of 15, 53%) than in LMP-1-negative cases (2 of 9 (22%), P > 0.05). Whereas, for HL patients aged 50 years and older, the loss of P16 expression in LMP-1 positive cases (33.3%) was similar to that found in LMP-1 negative cases (33.3%).

In female with HL, the loss of P16 expression was slightly higher in LMP-1 positive cases (2 of 6, 33%) than in LMP-1 negative cases (1 of 4, 25%), but the difference was not statistically significant (Table 1). In addition, male with HL, LMP-1 positive cases showed more loss of P16 expression (9 of 12, 75%) when compared to LMP-1 negative cases (2 of 8, 25%). This difference was statistically significant (P < 0.05).

Our data in Table 2 also indicated that in all NHL cases with LMP-1 positive (8:2, 25.6%), the loss of P16 expression was not detected at all. Furthermore, the loss of P16 expression was detected in 4 of 25 (16%) NHL cases. Statistical analyses also revealed that no significant association was detected between the loss of P16 expression and expression of LMP-1 in all NHL cases when adjusted for clinical factors such as subtypes of NHL, age and gender.

The immunostaining data combined in Tables 1 and 2 revealed that there was a trend for a difference in the number of lymphoma biopsies showing loss of P16 between the LMP-1 positive (11:26, 42.3%) and LMP-1 negative (7:29 (24.1%), P > 0.05). In addition, there was a significantly higher proportion of LMP-1 positive biopsies showing loss of P16 in HL (11:18, 61%), compared to that in NHL cases (0:8 (00%), P = 0.0074).

**Discussion**

In the present study, the relationship between the loss of P16 expression and expression of LMP-1 of EBV in malignant lymphoma among Jordanian patients was investigated. Apparently, our study revealed a moderate, but not statistically significant association between the loss of P16 and LMP-1 positive cases in HL. This finding is consistent with previous published reports which revealed that an inverse relationship was observed between the...
expression of P16 and the presence of EBV, confirming further this finding [13,14, 17,18].

To address the question of whether an association between the loss of P16 expression and expression of LMP-1 could be a result of variations of clinical variables such as subtypes, age and gender, these lymphoma cases were divided and examined according to subtypes, age and gender. Our immunostaining data showed that there was a numerically higher proportion of loss of P16 in the LMP-1 positive, compared to LMP-1 negative, in the MC and NS subtypes. For LD, LR and UC subtypes, unfortunately, the sample number were too small to comment on the relationship between LMP-1 and P16. Perhaps more importantly is that none of these samples were LMP-1 negative to allow a comparison. It is reasonable to conclude that all LD, LR and UC biopsies were P16 negative, independent of LMP-1 expression. Moreover, it is also worth to mention that the association between loss of P16 expression and expression of LMP-1 in HL group might be driven mainly by the LD/LR/UC group. This is because all LD/LR/UC cases were found to be both P16 negative and LMP-1 positive.

Because clinical factors such as gender and increasing age may affect individuals' likelihood of developing lymphoma [2, 6, 16-22], effects of age and gender on the relationship between loss of P16 and presence of LMP-1 were investigated. With respect of age, unfortunately, the number of HL cases in patients aged 50 years and older was too small to comment on the relationship between EBV LMP-1 and P16. On the other hand, a correlation was found between the expression of EBV LMP-1 and loss of P16 expression in HL patients below 50 years old. This result suggests that P16 expression may be down regulated early in HL patients. With respect to gender, the loss of P16 expression was more frequent in male cases than in female cases. The reasons for variations in relationship between the loss of P16 expression and LMP-1 of EBV expression in examined clinical variables such as age and gender are unclear. Nonetheless, it might be possible to suggest that these variations might be related to

| Parameters | Total (N) | EBV LMP-1 | P16 protein expression |
|------------|-----------|-----------|------------------------|
|            |           | Positive  | Present N (%)          | Loss N (%)  |
| Total      | 30        | 18 (60%)  | 7 (23.3%)              | 11 (36.7%)  |
|            |           | Negative  | 12 (40%)               | 9 (30%)     |

| Subtype of HL | Parameters | Total (N) | EBV LMP-1 | P16 protein expression |
|---------------|------------|-----------|-----------|------------------------|
|               |            | Positive  | Present N (%) | Loss N (%) |
| MC            | 18         | 11 (61%)  | 6 (33.3%)  | 5 (27.8%)  |
|               |            | Negative  | 7 (39%)    | 5 (27.8%)  | 2 (11.1%)  |
| NS            | 7          | 2 (28.6%) | 1 (14.3%)  | 1 (14.3%)  |
|               |            | Negative  | 5 (71.4%)  | 4 (57.1%)  | 1 (14.3%)  |
| LD            | 2          | 2 (100%)  | 0 (0%)     | 2 (100%)   |
|               |            | Negative  | 0 (0%)     | 0 (0%)     | 0 (0%)     |
| LR            | 1          | 1 (100%)  | 0 (0%)     | 1 (100%)   |
|               |            | Negative  | 0 (0%)     | 0 (0%)     | 0 (0%)     |
| UC            | 2          | 2 (100%)  | 0 (0%)     | 2 (100%)   |
|               |            | Negative  | 0 (0%)     | 0 (0%)     | 0 (0%)     |

| Age (years) | Parameters | Total (N) | EBV LMP-1 | P16 protein expression |
|-------------|------------|-----------|-----------|------------------------|
|             |            | Positive  | Present N (%) | Loss N (%) |
| <50         | 24         | 15 (62.5%) | 7 (29.2%)  | 8 (33.3%)  |
|             |            | Negative  | 9 (37.5%)  | 7 (29.2%)  | 2 (8.3%)   |
| ≥50         | 6          | 3 (50%)   | 1 (16.7%)  | 2 (33.3%)  |
|             |            | Negative  | 3 (50%)    | 1 (16.7%)  | 2 (33.3%)  |

| Gender      | Parameters | Total (N) | EBV LMP-1 | P16 protein expression |
|-------------|------------|-----------|-----------|------------------------|
|             |            | Positive  | Present N (%) | Loss N (%) |
| Male        | 20         | 12 (60%)  | 3 (15%)   | 9 (45%)   |
|             |            | Negative  | 8 (40%)   | 6 (30%)   | 2 (10%)   |
| Female      | 10         | 6 (60%)   | 4 (40%)   | 2 (20%)   |
|             |            | Negative  | 4 (40%)   | 3 (30%)   | 1 (10%)   |

EBV, Epstein-Barr virus; LMP-1, latent membrane protein-1; MC, mixed cellularity; NS, nodular sclerosis; LD, lymphocyte depletion; LR, lymphocyte-rich; UC, unclassified cases
some unknown age and gender-linked genetic or environmental factors.

In this part of study, the association between the loss of P16 expression and EBV LMP-1 expression in malignant cells of NHL was also examined. With respect to all clinical factors examined such as subtypes of NHL, age and gender, our data revealed that no association was found between the expression of EBV LMP-1 and loss of P16 expression in all NHL cases. These observations suggest that the loss of P16 expression may be an independent event and not necessarily associated or linked with EBV LMP-1 expression in NHL malignant lymphoma. Our finding is not in agreement with previous studies which demonstrated that the loss of P16 expression was more frequently observed in EBV LMP-1 positive carcinomas than EBV-LMP-1 negative carcinomas [6, 7, 12-14, 18-24]. Thus, our data revealed that the status of P16 expression in EBV-positive NHL cases is differing from that for EBV-positive HL cases and other carcinomas. Taken together, our findings combined with other findings suggest that EBV has different molecular biological effects upon infection and transformation of different cell types.

Furthermore, based on previous investigations, EBV infection can cause the loss of P16 expression and/or inactivation of p16 gene through hypermethylation of its promoter region and these changes are attributable to the gene product of the LMP-1 of EBV [10-14, 18,19]. In our set of lymphoma sample, there were a significantly higher proportion of LMP-1 positive biopsies showing loss of P16 in HL, compared to that in NHL, confirming a difference between HL and NHL. Thus, concerning the LMP-1/P16 relationship, the loss of P16 expression in HL apparently correlates very well with EBV LMP-1 expression, suggesting that LMP-1 expression or EVB infection might be responsible for the loss of P16 expression or silencing the p16 gene in HL but not in NHL. This finding also suggests that lymphoma represents a heterogeneous group of diseases in terms of the LMP-1 expression and loss of P16 expression and their association, being mainly related to the HL. This finding apparently lends further support to notion that, the role of EBV infection in the loss of P16 expression is sometimes contradictory or controversial [25-27].

In conclusion, our results demonstrated that there was a positive correlation between expression of the EBV LMP-1 oncoprotein and loss of P16 tumor suppressor protein in HL biopsies but not in NHL biopsies. This finding suggests that EBV LMP-1 expression appears to play a role in the loss of P16 expression in HL but not in NHL malignancy.

Table 2. Loss of P16 protein expression in EBV LMP-1 positive and negative Non-Hodgkin’s lymphoma (NHL) patients according to the clinicopathological parameters

| Parameters      | Total (N) | EBV LMP-1 | P16 protein expression |
|-----------------|-----------|-----------|------------------------|
|                 |           | Present N (%) | Loss N (%) |
| Total           | 25        | 8 (32%)    | 0 (00%)               |
|                 |           | 17 (68%)   | 4 (16%)               |
| Grade of NHL    |           |           |                       |
| Low             | 8         | 2 (25%)    | 0 (00%)               |
|                 |           | 6 (75%)    | 3 (37.5%)             |
| Intermediate    | 5         | 3 (60%)    | 0 (00%)               |
|                 |           | 2 (40%)    | 0 (00%)               |
| High            | 12        | 3 (25%)    | 0 (00%)               |
|                 |           | 9 (75%)    | 1 (8.3%)              |
| Age (years)     |           |           |                       |
| <50             | 12        | 2 (16.7%)  | 0 (00%)               |
|                 |           | 10 (83.3%) | 2 (16.7%)             |
| ≥50             | 13        | 6 (46.2%)  | 0 (00%)               |
|                 |           | 7 (53.8%)  | 2 (15.4%)             |
| Gender          |           |           |                       |
| Male            | 12        | 3 (25%)    | 0 (00%)               |
|                 |           | 9 (75%)    | 2 (15.4%)             |
| Female          | 13        | 5 (38.5%)  | 0 (00%)               |
|                 |           | 8 (61.5%)  | 2 (15.4%)             |

EBV, Epstein-Barr virus; LMP-1, latent membrane protein-1
Therefore, the present study offers further evidence for the notion that lymphoma is a heterogeneous malignancy, and the pathogenesis and progression of lymphoma subtypes might be linked to several molecular alterations and environmental factors. In addition, we believe that further understanding of the molecular and environmental mechanisms in the pathogenesis and development of lymphoma can aid for the development of treatment for lymphoma.

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Conflict of Interest
The authors declare no conflict of interest.

Authors' Contribution
Fawzi Irshaid conceived the study, analyzed the data, wrote the manuscript and revised it. Khaled Tarawneh participated in its design and coordination, and helped to draft the manuscript. Aisha Alshdefat and Fatihah Dilmi contributed to the data gathering, literature review and performed experiments. Adnan Jaran, Raji Al-Hadithi and Ahad Al-Khatib, helped to draft the manuscript and data analysis. All authors read and approved the final manuscript.

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