Original Article
Epstein-Barr virus infection is inversely correlated with the expression of retinoblastoma protein in Reed-Sternberg cells in classic Hodgkin lymphoma

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Abstract: Classic Hodgkin lymphoma (cHL) is characterized by few neoplastic Hodgkin/Reed-Sternberg (H/RS) cells in a background of intense inflammatory infiltrate. Epstein-Barr virus (EBV) has been shown to affect cell cycle and regulation of apoptosis. In total, 82 cases of cHL were studied. Five- micrometer sections were prepared and stained with haematoxylin and eosin and immunohistochemical streptavidin-biotin methods for EBV-LMP-1, pRb, ki-67 and cleaved caspase-3. In-situ hybridization for EBV encoded RNA was used to confirm the detection of EBV in H/RS cells. There were 45 nodular sclerosis, 28 mixed cellularity, 4 lymphocyte-rich, and 5 lymphocyte depletion subtypes in this series of cases. EBV and pRb were detected in 55% (46/82) and 64% (50/82) of the cases respectively. EBV was detected in 78% (25/32) of pRb-negative cases and 81% (29/36) of EBV-negative cases are pRb-positive. A statistically significant inverse relationship was observed between the presence of EBV and expression of pRb (*P* = 0.001). In conclusion, EBV infection is inversely correlated with pRb in H/RS cells in cHL.

Keywords: Hodgkin lymphoma, Epstein Barr virus, pRb

Introduction

Classic Hodgkin lymphoma (cHL), a B cell related neoplasm in the majority of cases [1], is characterized by few neoplastic Hodgkin/Reed-Sternberg (H/RS) cells (1%-10%) with a background of abundant inflammatory infiltrate [2]. Most H/RS cells are in G1, S, G2 or M phases of the cell cycle [3] and have defective cell cycle and apoptosis regulation with alterations of retinoblastoma (Rb) tumor suppressor pathways [4]. Constitutive activation of the nuclear factor (NF)-κB pathway is believed to be involved in the proliferation and survival of these cells [5].

Epstein-Barr virus (EBV) is a human herpes virus type 4 that infects humans predominantly at an early age with more than 90% of the adult population infected with EBV worldwide [6]. EBV has a definitive causative role of both B lymphocyte and epithelial malignancies, which include; Burkitt lymphoma, cHL, nasopharyngeal carcinoma, and malignancies linked to immunosuppression, including post-transplant lymphoma and AIDS-associated lymphomas [7, 8].

Variable rates of detection of EBV in H/RS cells have been found in various parts of the world with a range of 30-96% of cHL cases [9]. EBV has been shown to affect the cell cycle and regulation of apoptosis [4, 10].

Latent membrane protein 1 (LMP1) is an essential membrane protein with a petite aminoterminal cytoplasmic tail, six membrane-spanning domains, and a cytoplasmic carboxy-terminal domain. LMP1 is considered the EBV oncoprotein and is expressed in many cancers associated with EBV [11]. LMP1 mimics an immanently active CD40 receptor, a signaling pathway leading to the activation of the NF-κB through the TRAF pathway, as oligomerization of LMP1

molecules via interactions of the transmembrane domains brings the carboxyl-terminal domains in close proximity to induce ligand-independent signaling. The activation of NF-κB leads to activation of several important signaling pathways, including the mitogen-activated protein kinase, c-Jun N-terminal kinase, and phosphatidylinositol 3-kinase pathways, which results in proliferation of H/RS cells [11-13].

pRb is a tumor suppressor protein that in its hypophosphorylated status, functions as a negative regulator of the cell cycle by forming complexes with a transcription factor; E2F1. E2F1 in its active form induces expression of target genes required for S-phase transition and progression of cell cycle. When pRb is phosphorylated by the cyclin D/cyclin dependent kinases complexes it becomes inactivated and releases E2F1, thus permitting entry into the G1-S phases [14].

In the present study we investigate the correlation of EBV infection with the expression of pRb in H/RS cells in cHL and its effect on H/RS cell proliferation and apoptosis.

Materials and methods

Review of cases

Archival paraffin blocks of 82 cases of previously diagnosed cHL were obtained from surgical pathology files of Tawam Hospital in Al-Ain city. Tawam Hospital is the main oncology center of the UAE. All haematoxylin and eosin (H&E) and immunohistochemical stained sections were reviewed to confirm the diagnosis and to classify the cases into histologic subtypes according to the WHO classification.

Research ethical approval

This research project is approved by Al Ain Medical District Human Research Ethical Committee-Protocol No. 05-45.

Immunohistochemistry

Five-μm sections were prepared and mounted on aminopropyltriethoxysilane (APES) coated slides. After dewaxing with xylene and rehydrating with graded alcohol, slides were placed in a 0.01 M citrate buffer solution (pH = 6.0) and pre-treatment procedure to unmask the anti-

gens was performed in a water bath at 90°C for 1 hour. Then, sections were treated with peroxidase block for 30 minutes followed by protein block for 30 minutes. Sections were then incubated for one hour at room temperature with EBV-latent membrane protein-1 (LMP1) (mouse monoclonal, clone CS.1-4, DAKO, Glostrup, Denmark, 1:100), pRb (mouse monoclonal, clone Ab-5, Thermo Scientific, USA, 1:50), and ki-67 (mouse monoclonal, clone MIB-1, DAKO, Glostrup, Denmark, 1:50). After conjugation with primary antibodies and 3 times washing with phosphate buffered saline with Tween 20 (PBST) 5 minutes each, sections were incubated with biotin-labeled secondary antibody (Thermo Scientific, USA) for 20 minutes at room temperature then washed for 3 times with PBST; 5 minutes each. Finally, sections were incubated with streptavidin-peroxidase complex for 20 minutes at room temperature (Thermo Scientific, USA) followed by 3 times washing with PBST, then DAB chromogen (Thermo Scientific, USA) was added for 5 minutes and brown color was developed in positive cells. Sections from tonsillar tissue were used as positive controls for ki-67. For pRb, sections from a known colonic carcinoma were used as a positive control. For LMP-1, known positive control sections of cHL were used. For negative control, the primary antibody was not added to sections and the whole procedure carried out in the same manner as mentioned above.

Double labeling was done following the same above procedure. The first primary antibody (anti-pRb) was detected as above and following the development of brown nuclear staining, sections were washed with PBST for 3 times; 5 minutes each. Then the second primary antibody (anti-LMP1) was added for one hour at room temperature followed by 3 washes with PBST 5 minutes each, followed by adding alkaline phosphatase-conjugated polymer (Thermo Scientific, USA) for 20 minutes at room temperature. Then the sections were washed for 3 time in PBST followed by adding the alkaline phosphatase substrate (Thermo Scientific, USA) for one minute and adding the Fast red chromogen (Thermo Scientific, USA) on it and leave the mixture for 10 minutes until appropriate cytoplasmic red color developed. Sections were dehydrated, cleared and then mounted with DPX.

Table 1. Expression of EBV and pRb in H/RS cells in cHL

Histologic type	Number	LMP-1/ EBER	pRb
Nodular sclerosis	45	25 (55%)	31 (69%)
Mixed Cellularity	28	17 (61%)	12 (43%)
Lymphocytic- rich	4	3 (75%)	3 (75%)
Lymphocytic depletion	5	1 (20%)	4 (80%)
Total	82	46 (55%)	50 (64%)

Staining method for apoptosis

Cleaved casapase-3 IHC detection kit (Cell Signaling Technology, Boston, USA) was used to detect the activation of caspase-3 using standard avidin-biotin immunoperoxidase method to detect intracellular cleaved casapase-3 protein. Staining was performed by standard technique using rabbit anti-human polyclonal antibody. A known positive control sections for apoptosis from breast carcinoma were used. For negative control, primary antibody was replaced with normal rabbit serum and the whole procedure was carried out as in the standard procedure.

In situ hybridization

In situ hybridization (ISH) was performed by standard techniques using a specific oligonucleotide probe (Novocastra-LEBV-K, UK) which hybridizes to EBV encoded RNA (EBER) transcripts concentrated in the nuclei of latently infected cells. With each batch of studied cases, positive and negative control slides were also run. The positive control slide was a known case of EBV positive HL to which a specific EBER oligonucleotide probe was added. The negative control slide was another section of the same case of known EBV positive HL to which a random probe consisting of fluorescein labeled oligonucleotide cocktail was added. In addition, for each case studied, two sections were used; the EBER oligonucleotide probe was added to one section, and the random probe was added to the other. Using this random probe as a negative background control alongside the EBV probe contributes to the validation of staining obtained by the EBV probe. If this negative control slide showed significant background staining in a particular case, the slide having the EBER probe was considered non-interpretable and the test was repeated.

Interpretation of results

Two pathologists have reviewed the slides independently. Using the H&E and immunohisto-

chemical stains, cases were classified into cHL histologic subtypes. For EBV detection, slides stained by IHC and ISH were reviewed independently to prevent any bias.

For IHC, H/RS cells are the only counted cells in this study. Cases were labeled as positive for EBV-LMP-1 expression if the neoplastic cells were reactive in a membrane, cytoplasmic and/or Golgi staining pattern in H/RS cells. For ISH, cells with a dark blue precipitate in the nucleus were identified as positive for the presence of EBER when the negative control slide showed a clean background. EBV expression was considered positive if the neoplastic cells were positive by both methods. For pRb IHC, cases were labeled positive if H/RS cells were reactive in a nuclear staining pattern.

Apoptotic index

The apoptotic index (AI), which was based on cleaved caspase-3 labeling, was determined by counting the number of H/RS cells in 10 randomly selected fields without sclerosis or fibrosis. For cleaved caspase-3 labeling, H/RS cells were considered apoptotic when there was a cytoplasmic and nuclear staining pattern. The labeling index for H/RS cells was expressed as the percentage of labeled H/RS cell against the total number of H/RS cells enumerated.

Ki-67 index

The Ki-67 index, which is based on Ki-67 protein labeling, was determined by randomly counting the number of H/RS cells in ten randomly selected fields without sclerosis or fibrosis. For Ki-67 labeling, H/RS cells were considered positive when they showed a nuclear staining pattern. The Ki-67 index for H/RS cells was expressed as the percentage of labeled H/RS cell against the total number of H/RS cells enumerated.

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS) (version 20) statistical program (SPSS, Chicago, IL). Standard cross-tabulation, the Chi-square and Mantel-Haenszel test were used to explore associations. Paired student t test was used to compare ki-67 and apoptotic indices in relation to the expression of EBV and pRb.
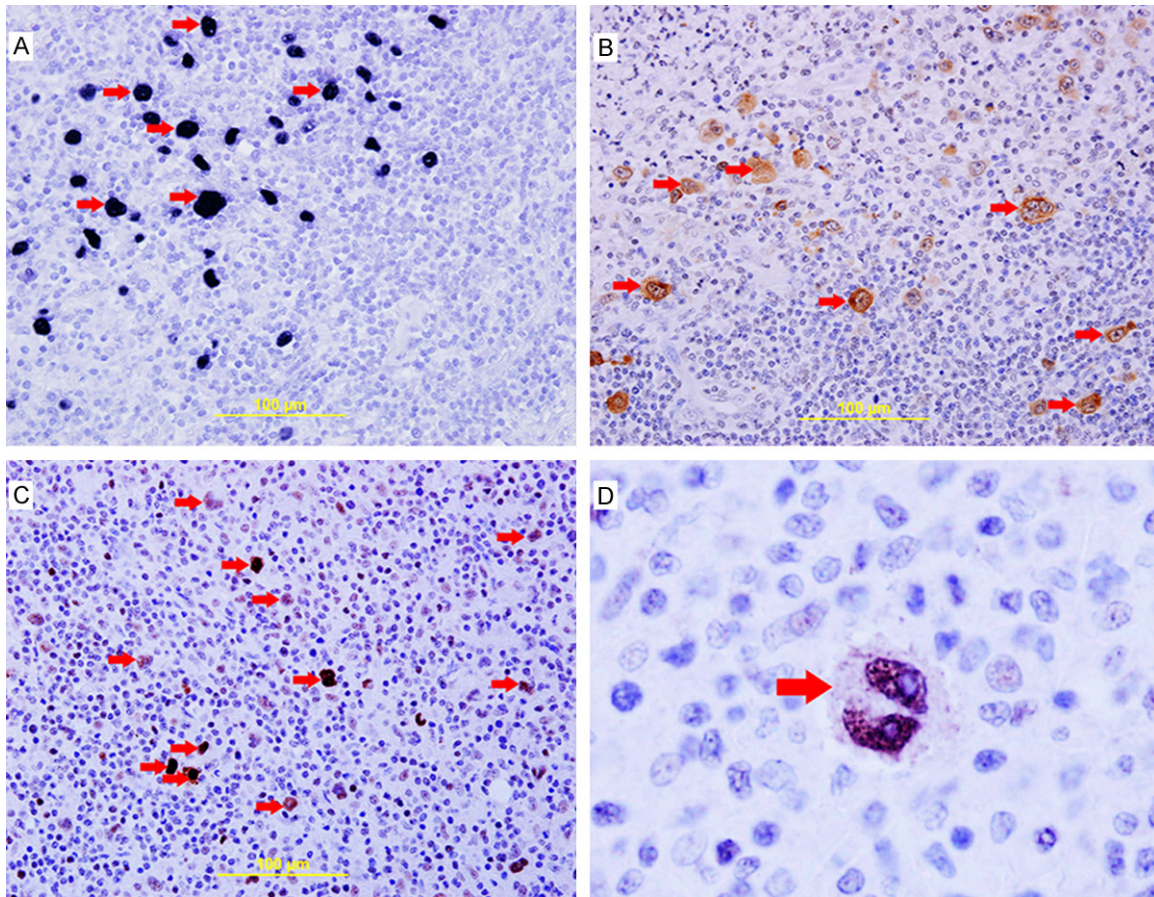
Figure 1. Expression of EBV and pRb in cHL. A. Representative section of cHL showing dark blue nuclear staining of H/RS cells (thick arrow) for EBER, In situ hybridization method. B. Representative section of cHL showing brown membranous and cytoplasmic staining of H/RS cell (thick arrow) for LMP-1, Immunoperoxidase streptavidin-biotin method. C. Representative section of cHL showing brown nuclear staining of H/RS cells (thick arrow) for pRb, Immunoperoxidase streptavidin-biotin method. D. A binucleated RS cell showing strong nuclear staining (thick arrow) for pRb, Immunoperoxidase streptavidin-biotin method.

Results

There were 45 nodular sclerosis (NS), 28 mixed cellularity (MC), 4 lymphocyte-rich (LR), and 5 lymphocyte depletion (LD) subtypes in this series of cases (**Table 1**).

Expression of EBV in H/RS cell in cHL

EBV-positive cases show brown cytoplasmic staining of LMP-1 and dark blue nuclear staining for EBER in H/RS cells (**Figure 1A** and **1B**). EBV was detected in 55% (46/82) of the cases (**Table 1**, **Figure 1A** and **1B**). The median percentage of EBV-positive H/RS cells is 35%. EBV was detected in 55% of NS, 61% of MC, 75% of LR and 20% of LD (**Table 1**, **Figure 2**).

A significant difference was found between the frequencies of EBV-positive and EBV-negative

cases of MC subtype of cHL (P = 0.0029), while in NS subtype of cHL, although we found a higher frequency of EBV-positive cases, it did not reach statistical significance (P = 0.2). The number of LR and LD cases was few, hence differences in EBV expression cannot be evaluated.

Expression of pRb in H/RS cell in cHL

There was brown nuclear staining of pRb in H/RS cells in pRb-positive cases (**Figure 1C** and **1D**). pRb was detected in 64% (50/82) of the cases (**Table 1**, **Figure 1C** and **1D**). The median percentage of pRb-positive H/RS cells is 42%. pRb was detected in 69% of NS, 43% of MC, 75% of LR and 80% of LD (**Table 1**, **Figure 2**).

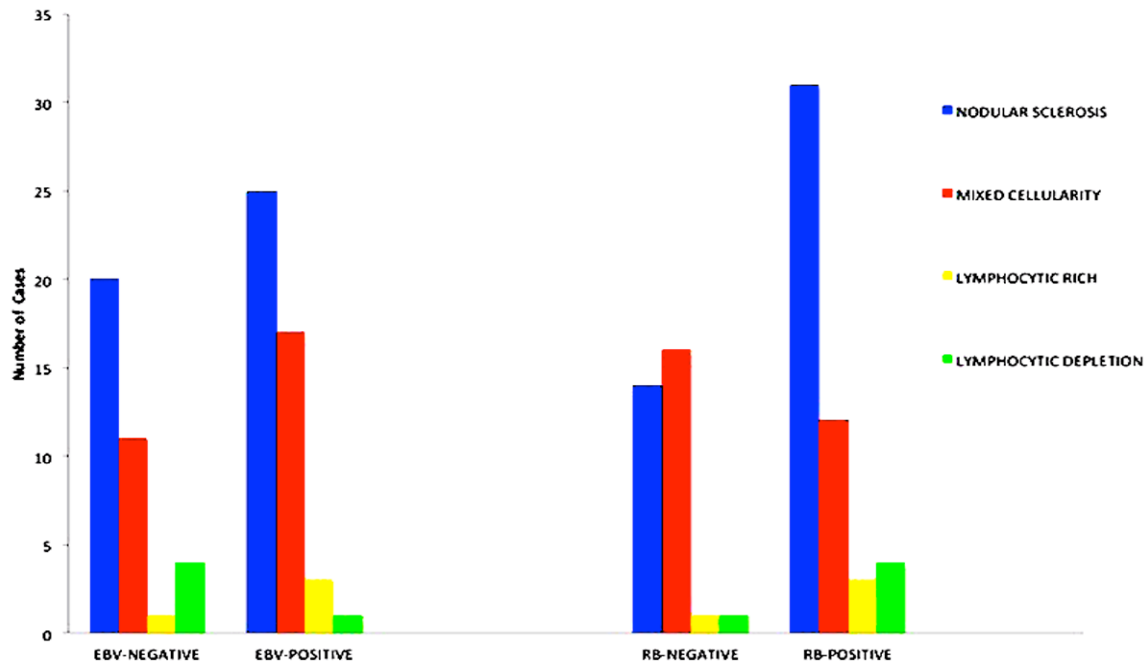A significant differences between the frequencies of pRb-positive and pRb-negative cases of

Figure 2. Bar graph showing expression of EBV and pRb in histologic subtypes of classic Hodgkin lymphoma.

Table 2. Cross tabulation of EBV expression and pRb expression in H/RS cells in HL

Expression		pRb		Total
		Negative	Positive	
EBV	Negative	7	29	36
	Positive	25	21	46
Total		32	50	82

(Chi-Square: 10.339, df = 1, P = 0.001).

NS subtype of cHL (P = 0.0001), while in MC subtype of cHL there was a higher frequency of pRb-negative cases over pRb-positive cases with a statistical significance (P = 0.05) (**Figure 2**). The number of LR and LD cases was few, hence differences in pRb expression cannot be evaluated.

Double labeling of LMP1 and pRb in H/RS cells

LMP1-positive H/RS cells show negative staining for pRb in 78% (25/32) of pRb negative cases (**Figure 3A-C**). pRb-positive H/RS cells show negative staining for LMP1 in 81% (29/36) of EBV-negative cases (**Figure 3D-F**). LMP1-positive H/RS cells show positive staining for pRb in 45% (21/46) of EBV-positive cases (**Figure 3G-I**). LMP1-negative H/RS cells show negative staining for pRb in 19% (7/36) of EBV-negative cases (Data not shown).

Correlation of EBV with pRb in H/RS cell in cHL

EBV was detected in 78% (25/32) of pRb-negative cases and 81% (29/36) of EBV-negative cases are pRb-positive (**Table 2**, **Figure 3**). A statistically significant inverse relationship was observed between the presence of EBV and expression of pRb (Chi-Square:10.339, df = 1, P = 0.001) (**Table 2**).

Cell proliferation and apoptosis

Ki-67 positivity ranged from 34% to 92% of the H/RS cells, with variable staining intensity from case to case (**Figure 5**). The mean Ki-67 index in EBV+ve HL was 77% but only 64% in EBV-ve HL (**Figures 4A**, **5A** and **5B**). There was a significant association between the expression of EBV and the Ki-67 index (P < 0.0001) (**Table 3**). The mean Ki-67 index in pRb+ve HL was 65.3% but only 79.2% in pRb-ve HL (**Figures 4A**, **5C** and **5D**). There was a significant inverse association between the expression of pRb and the Ki-67 index (P < 0.0001) (**Table 3**). There was good correlation between apoptotic morphology and cleaved caspase-3 staining (**Figure 5**). The AI of the H/RS cells in the cases ranged from 5.2 to 10.3%, with a mean of 8.1% in EBV+ve HL, while in EBV-ve HL, the mean AI was 8.77% (**Figures 4B**, **5E** and **5F**). Although the analysis of the EBV expression in combina-
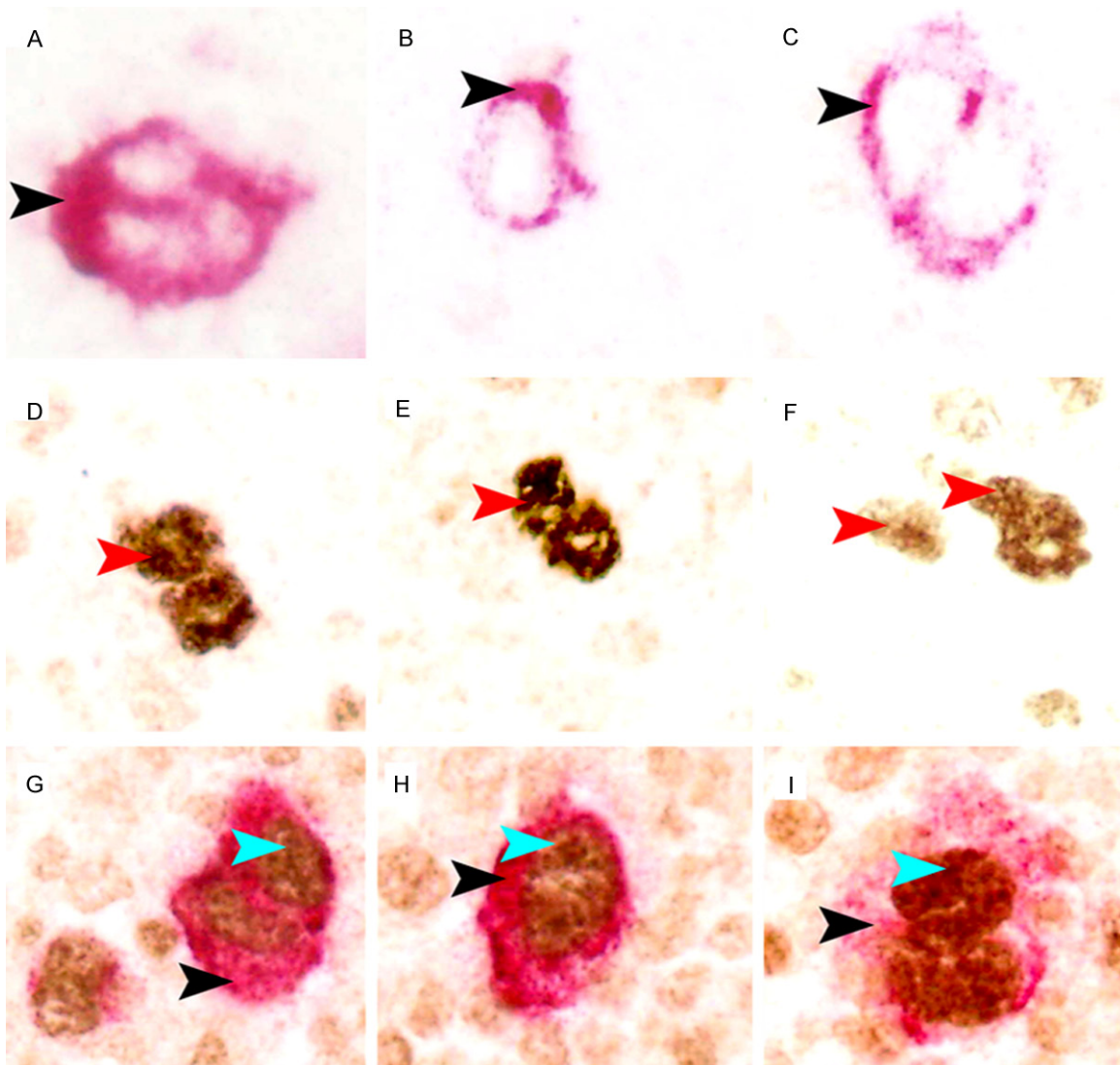
Figure 3. Double labeling of LMP1 and pRb in H/RS cells in cHL. A-C. Representative sections of cHL showing red membranous and cytoplasmic staining of H/RS cell (arrow head) for LMP1. No nuclear staining for pRb. This is seen in 30.5% of the cases. D-F. Representative sections of cHL showing brown nuclear staining of H/RS cells (arrow head) for pRb without cytoplasmic staining for LMP1. This is seen in 35% of the cases. G-I. Representative sections of cHL showing red membranous and cytoplasmic staining of H/RS cell (green arrow head) for LMP-1 and brown nuclear staining for pRb (black arrow head). This is seen in 25% of the cases. Double labeling Immunoperoxidase streptavidin-biotin-DAB and alkaline phosphatase-Fast Red method.

tion with the AI showed a statistical significant correlation (P < 0.0001) (**Table 3**), this difference is likely of little biological meaning (8.1% vs. 8.77% AI).

The mean AI of the H/RS cells in pRb+ve HL was 8.2%, while in pRb-ve HL, the mean AI was 8.34% (**Figures 4B**, **5G** and **5H**). Analysis of the pRb expression in combination with the AI did not show any statistically significant correlation (P = 0.199) (**Table 3**).

Discussion

There have been strong evidences that H/RS cells are associated with intense disturbance of the cell cycle and regulation of apoptosis [4]. The constitutive activation of the NF-κB pathway is considered to be involved in the proliferation and survival of H/RS cells [5]. Moreover, substantial evidence that H/RS cells have defective cell cycle and apoptosis regulation has been provided by studies showing that
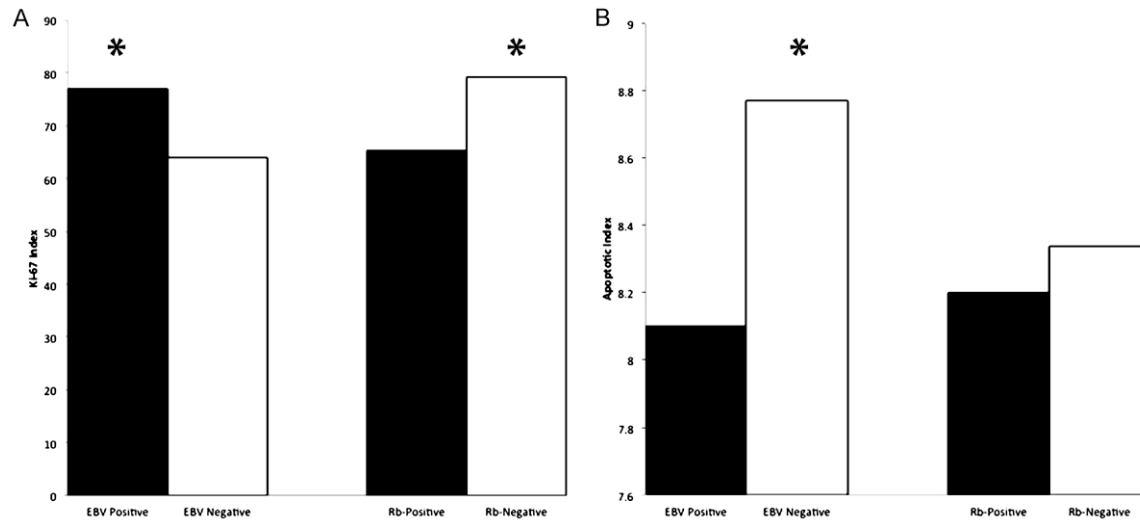
Figure 4. Bar graph showing Ki-67 proliferative index and apoptotic index in relation to EBV and pRb expression in H/RS cells in cHL.

these cells are characterized, in a large proportion of cases, by alterations of Rb tumor suppressor pathway [4]. The high rate of positive ki-67 staining in the present study supports the fact that H/RS cells are undergoing active proliferation and have left the G0 phase of the cell cycle [4].

We have shown LMP1 expression in H/RS cells in 55% of cHL in this study.

We have shown the mean Ki-67 index in EBV+ve HL is significantly higher than the mean Ki-67 index in EBV-ve HL (**Figure 4A**, **Table 3**). This indicates that EBV infection has an influence on cell cycle control leading to proliferation of H/RS cells. We also show a statistically significant decrease in the rate of apoptotic cells in EBV-infected cHL (**Figure 4B**, **Table 3**), indicating that EBV has an influence on apoptotic pathways.

One of the distinct biological characteristics of EBV is establishment of latency [15].Three types of latency have been defined, each having its own distinctive pattern of gene expression [15]. Type II latency is typically seen in cHL, where EBNA1, LMP1, LMP2A and LMP2B proteins are expressed [15]. EBNA2, LMP1, and EBNA3C, have been shown to be absolutely essential for EBV transformation of human B cells and establishment of latency in vitro [16-19].

We have also shown, in **Table 2** and **Figure 2**, a statistically significant inverse relationship bet-

ween LMP1 expression and pRb expression in H/RS cells, as we show 78% of pRb-negative cases are LMP1-positive and 81% of LMP1-negative cases are pRb-positive, suggesting that EBV infection is associated with a decreased expression of pRb leading to cell cycle progression and cell proliferation.

Studies on rodent fibroblasts have shown LMP1 can inactivate Rb gene through down regulation of p27 and increased levels of cyclin dependent kinase-2 leading to increased phosphorylation of Rb gene [20, 21].

Studies on B lymphocytes have also shown that LMP1 can decrease p27 and inactivate pRb by increasing phosphorylation of Rb gene [22]. Cannel et al have shown EBV drives the hyper-phosphorylation of pRb by altering the expression of several components of the signal transduction pathway that normally regulates the phosphorylation status of Rb gene, including the up regulation of a number of cyclins and cyclin-dependent kinases and the down regulation of a subset of cyclin-dependent kinase inhibitors, which result in the formation of active cyclin-cyclin dependent kinase complexes that are capable of phosphorylating and inactivating Rb gene [23]. Faqing et al have also shown that upon induction of LMP1 expression, the level of phosphorylated Rb, the number of cells in S stage of cell cycle, and the cell colony formation rate were higher than those without LMP1 induction [24]. Although type 2 latency is associated with cHL, some studies on cHL have
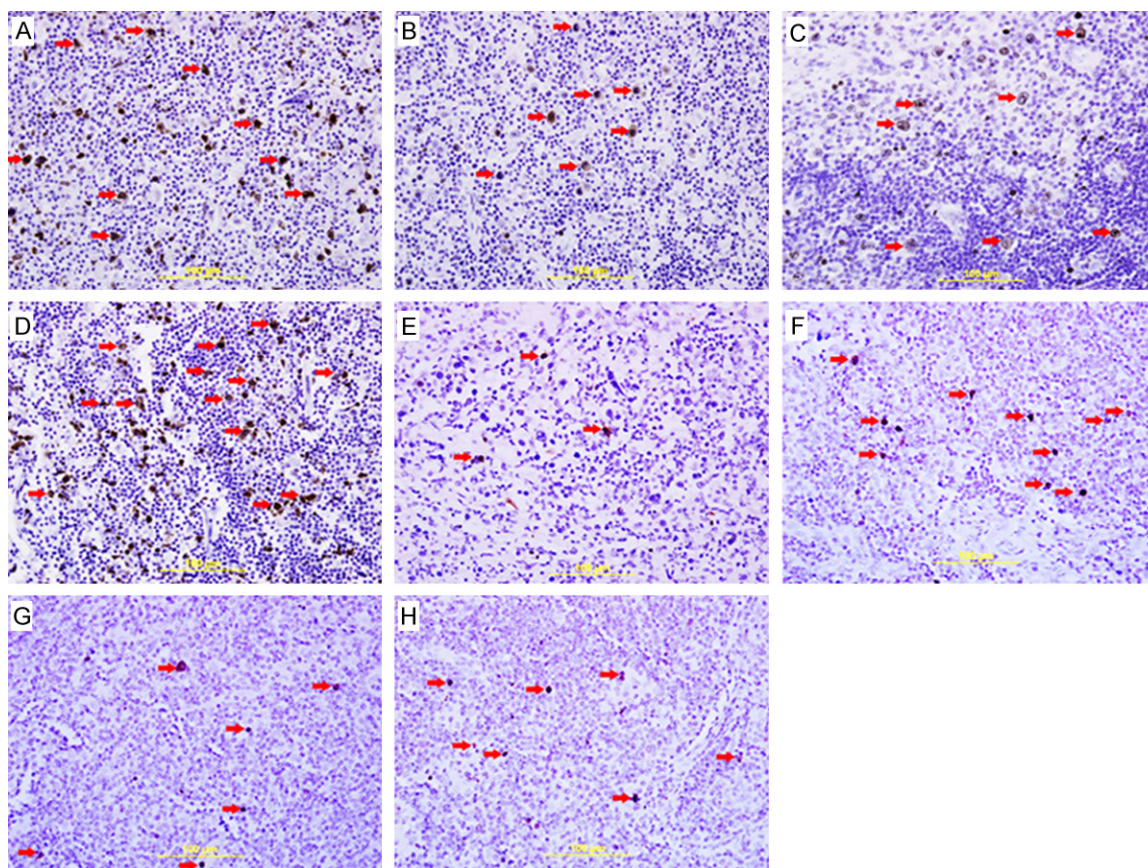
Figure 5. Ki-67 and cleaved caspase-3 immunohistochemical staining in cHL. A&B. Showing nuclear immunore-activity of H/RS cells (thick arrows) to ki-67 in EBV+ve and EBV-ve HL respectively. There is significant increase in ki-67 positive H/RS cells (thick arrows) in EBV+ve HL. Streptavidin-biotin immunoperoxidase method. C&D. Show-ing nuclear immunoreactivity of H/RS cells (thick arrows) to ki-67 in pRb+ve and pRb-ve HL respectively. Ki-67 index is higher in pRb-negative cases. There is a significant difference in ki-67 positive H/RS cells (thick arrows) between pRb-ve and pRb+ve HL. Streptavidin-biotin immunoperoxidase method. E&F. Showing cytoplasmic and nuclear immunoreactivity of H/RS cells (thick arrows) to cleaved-caspase in EBV+ve and EBV-ve HL respectively. There is significant increase in cleaved-caspase-positive H/RS cells (thick arrows) in EBV-ve HL. Streptavidin-biotin immunoperoxidase method. G&H. Showing cytoplasmic and nuclear immunoreactivity of H/RS cells (thick arrows) to cleaved-caspase in pRb+ve and pRb-ve HL respectively. There is no significant difference in cleaved-caspase-positive H/RS cells (thick arrows) between pRb+ve and pRb-ve HL. Streptavidin-biotin immunoperoxidase method.

Table 3. Ki-67 index and apoptotic index in relation to the expression of EBV and pRb in H/RS cells

Procedure	EBV			pRb		
	+ %	- %	p value	+ %	- %	P value
Ki-67 index	77	64	0.0001	65.3	79.2	0.0001
Apoptotic index	8.1	8.77	0.0001	8.2	8.34	0.199

shown that EBNA3C can also be expressed in EBV- associated cHL [25-27].

Knight et al. have shown EBNA3C can mediate degradation of pRb through SCF cellular ubiqui-tin ligase [28], and Maruo et al have also shown EBNA3C can increase phosphorylated pRb [19]. These reports [19-24, 28] support our

findings that EBV infection can decrease the expression of pRb in H/RS cells, overcoming cell cycle control and leading to their proliferation.

It is noteworthy to mention here that in MC subtype of cHL, the rate of pRb-negative cases are significantly higher than pRb positive cases, and EBV-positive cases are significantly higher than EBV-negative cases while in NS subtype of cHL, there was no significant difference between EBV-negative and EBV-positive cases but there was a signifi-cant higher rate of pRb-positive than pRb-nega-tive cases (**Figure 2**). These interesting findings

support the well-known favorable prognosis of NS over MC subtypes of cHL and suggest that down regulation of pRb is partly mediated by EBV.

In conclusion, EBV infection is associated with decreased expression of pRb in H/RS cells in cHL.

Disclosure of conflict of interest

None.

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