

Prognostic Significance of Cell Proliferation and Apoptosis-Regulating Proteins in Epstein-Barr Virus Positive and Negative Pediatric Non-Hodgkin's Lymphoma

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Abstract Apoptosis-related proteins and proliferation activity and their relationship with Epstein-Barr Virus (EBV) are contemporary issues in pediatric non-Hodgkin's lymphoma (pNHL). In this study prognostic or pathogenetic role of EBV latent infection, proliferating activity, and apoptosis-regulating proteins in pNHL were explored. EBV-EBER, *lmp-1*, *ki-67*, *bcl-2*, *survivin*, *bax*, *fas*, *c-myc*, *p53* and apoptotic index by TUNEL method were explored in 70 pNHL cases and evaluated statistically. Of the 70 cases evaluated, 24 were female and 46 were male. Seven cases

were stage I/II and 63 cases were stage III/IV. The mean age was 7.16 ± 3.72 (1–15). EBV was positive in (25.7%) cases. Overall survival was 82%, while event free survival was 75%. *Bax* was expressed in 40% of the cases, while the expression of *bcl-2*, was 50%, *survivin* 42.9%, *p53* 8.6%, *fas* 18.6% and *c-myc* in 45.7%. Mean apoptotic index was 131.29 ± 96.69 per 5,000 cells. Mean proliferation index was 55.97% (12–92%). *Fas* positivity was high in EBV positive cases ($p=0.0001$). EBV positivity was not related with prognosis. Apoptotic index was found to be an independent prognostic factor ($p=0.017$). Our results suggest that apoptosis-regulating proteins have a role in the pathogenesis of pNHL. EBV was correlated with apoptotic index and *fas*, *bcl-2*. No correlation was observed with proliferation index and studied factors. High apoptotic index was related with good prognosis.

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Introduction

Pediatric lymphomas account 12% of all newly diagnosed childhood cancer [1, 2]. Approximately 60% of them are pediatric non-Hodgkin lymphomas (pNHL) that are diffuse aggressive lymphomas and they disseminate widely [3]. Nowadays more than 90% of B-cell NHLs and 75–85% of lymphoblastic and ALCL are cured with intensive regimens. Better understanding of the pathogenesis of pediatric lymphomas may provide the basis for novel therapies that can improve cure rates and reduce toxicity.

PNHL mainly includes precursor lymphoblastic lymphomas (LL; mostly T cell) and mature lymphomas [(Burkitt/

Burkitt like lymphoma (BLL), Diffuse large B cell lymphoma (DLBCL), anaplastic large cell lymphoma (ALCL; T-cell), and rarely peripheral T-cell lymphoma (PTCL)]. The etiology and pathogenesis of pNHL are not clearly understood although much has been done about them. Apoptosis related genes and proteins and proliferation activity and their relationship with Epstein Barr Virus (EBV) are contemporary issues [4–12]. Apoptosis-related proteins might play an important role in the pathogenesis and prognosis in pNHL [13–16]. Bcl-2 prolongs cell survival through its ability to block apoptosis [17]. Bax is a member of the bcl-2 family of proteins and comprises agonist of apoptosis [18]. Survivin, a member of the inhibitor of apoptosis protein family can directly inhibit caspase-3 and caspase-7 [19–22]. CD95 (fas) is a transmembrane molecule that induces apoptosis. C-myc is an oncogene that plays a role in cell-cycle in the pass of G1 phase to S [23, 24]. Ki 67 is a proliferation marker expressed in G1, S, G2, M phases of cell-cycle. It is used for detecting growth fraction [25].

The aim of this study was to reveal the expression of proliferating activity, apoptosis-regulating proteins in pNHL and determine its prognostic role and relationship with EBV.

Materials and Method

This study included 70 cases of pNHL diagnosed, treated and followed between 1989 and 2005 with an age range of 2–15 in Dr. Behcet Uz Children Research Hospital, Izmir, Turkey. Forty six of the cases were male, while 24 were female. Seven cases were stage I or II, 63 cases were stage III or IV. The mean age was 7.16 ± 3.72 . Twelve of the cases were precursor T LL. Mature lymphomas included 35 cases of BLL, 11 cases of DLBCL, 9 cases of ALCL (T-cell), and three cases of peripheral T-cell lymphoma. The cases having paraffin blocks available for immunohistochemistry (IHC) were included in the study. One presentable paraffin block was selected for each case. All blocks were from pre-treatment tissues. Clinical data were obtained by retrospective chart review. Survival was determined from the date of diagnosis. All cases were included in survival analysis. The cases were staged according to St. Jude systems. The cases diagnosed before 1994 underwent Modified Ziegler or COMP protocol. BFM-90 treatment protocol was applied for the cases diagnosed after 1994. EBV positivity (LMP-1 and/or EBER expression) was investigated by IHC for LMP-1 and by in situ hybridization for EBER. The expression of ki-67, Bcl-2, survivin, Bax, fas, c-myc and p53 was detected by IHC. Cytoplasmic immunoreactivity for lmp-1, survivin, bax, fas and nuclear for bcl-2, c-myc, ki-67, p53 was considered positive. Scoring was done as

positive or negative for all parameters. Tumor cells stained more than 10% were classified as positive. Apoptotic Index (AI) was detected by TUNEL method and scored as number in 5,000 cells. Proliferation Index (PI) was scored as % of positive cells among 5,000 neoplastic cells on ki-67 stained slides [15, 17, 33].

In IHC method 5 μ m thick sections were cut from each of the paraffin-embedded block and mounted onto polylysine glass slides. After being deparaffinised with xylene for an hour at room temperature and progressively dehydrated in decreasing concentrations of alcohol (70%, 80%, 95%, 99%; each 5 min), the sections were immersed in 3% H₂O₂ in distilled water for 10 min at room temperature to block endogenous peroxidase activity. The sections were incubated in microwave at 400 W for 20 min in citrate buffer (pH 6.0) for antigen retrieval. PBS was used for all subsequent washes. After non-specific binding was blocked with blocking reagent for 5 min, primary antibodies were applied at 1:100 dilutions at room temperature for 60 min [monoclonal antibodies to bcl-2 (Lab Vision), survivin (Lab Vision), bax (Lab Vision), p53 (Neomarkers), fas (Neomarkers), ki-67 (Neomarkers), c-myc (DBS), LMP (Novocastra, NCL-EBV S1–4)]. Universal kit for IHC was used by rabbit/mouse streptavidin biotin technique, (ScyTek) according to the manufacturer's instruction. Visualization of the bound primary antibodies was performed using diaminobenzidine solution (Sigma Chemical Co., St. Louis, MO, USA) as a chromogen and counterstained with Mayer's hematoxylin. Tissues were incubated with PBS without the primary antibody as negative control and a positive control side, which was shown previously to be strongly positive, was used. The scientists who performed the IHC analysis were blinded to pathological features.

FISH analysis for EBER was done by EBV-EBER (Novocastra) using ISH detection kit (ISH detection kit Novocastra NCL-ISH-D) according to the instruction of the company. After being deparaffinised with xylene and progressively hydrated in decreasing concentrations of alcohol, sections were incubated with Proteinase K for 30 min at 37°C. After washing in distilled water and dehydrating, the air dried sections were incubated with EBV-EBER (mixture of small mRNA conjugated with fluorescein isothiocyanate) for 2 h at 37°C.

The AI was detected by Colorimetric TUNEL System (Promega, G7360) end-labeling the fragmented DNA of apoptotic cells was detected by using a modified TUNEL assay. Biotinylated nucleotide is incorporated at the 3'-OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinant, (rTdT) enzyme. Horseradish peroxidase-labeled streptavidin is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen,

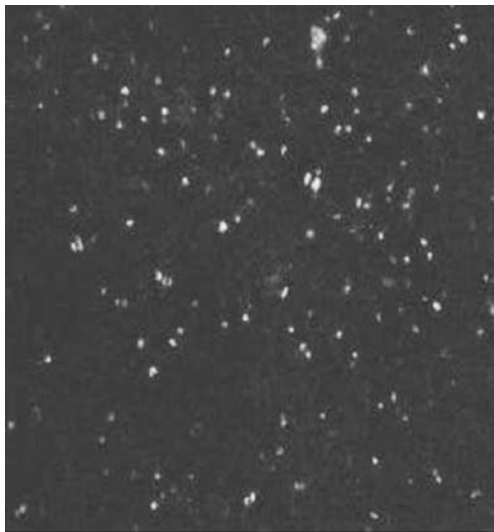


Fig. 1 EBV positivity shown by FISH EBER

DAB. Using this procedure, apoptotic nuclei are stained dark brown.

In statistical analysis Chi square test and Spearman correlation analysis was used to assess the relationship between ordinal data. Survival analysis was estimated according to Kaplan Meier method and Log Rank Analysis in SPSS software version 9.05. $P < 0.05$ was considered statistically significant.

Results

EBV (LMP-1 and/or EBER) was positive in 18 cases (25.7%; Fig. 1). Exitus was observed in ten cases, while relapsing was observed in four cases. Overall survival was 82%, while event free survival was 75%. Bax was expressed in 28 cases (40%; Fig. 2), bcl-2 in 35 cases (50%), survivin in 30 cases (42.9%), p53 in six cases (8.6%), fas in 13 cases (18.6%;

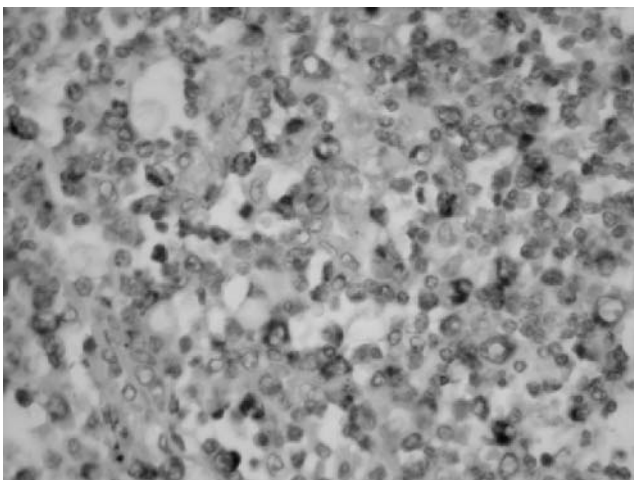


Fig. 2 Diffuse cytoplasmic bax positivity (DAB $\times 100$)

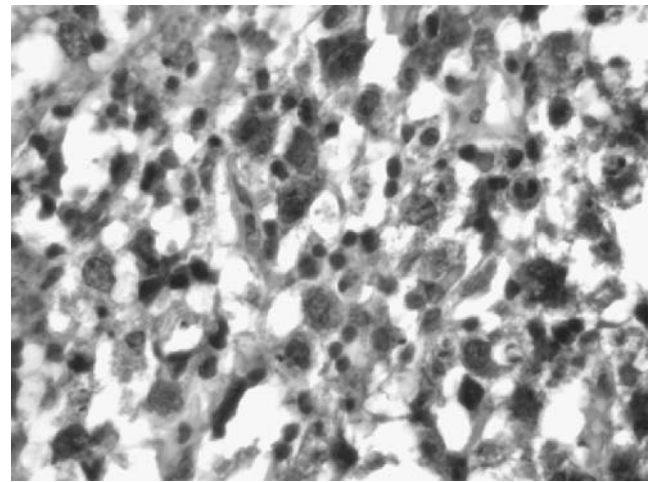


Fig. 3 Cytoplasmic fas positivity in anaplastic large cell lymphoma (DAB $\times 200$)

Fig. 3) and c-myc in 32 cases (45.7%; Fig. 4). Mean AI was counted as 131.29 ± 96.69 (20–450) per 5,000 cells (Fig. 5). Mean PI was 55.97% (12–92%; Fig. 6).

Statistical Analysis

Between these parameters AI was found to be an independent prognostic factor ($p = 0.017$). EBV positivity was correlated with fas ($p = 0.001$) and bcl-2 ($p = 0.030$) expression. Fas positivity was high in EBV positive cases ($p = 0.0001$). Bcl-2 expression was also positively correlated with PI. The expression of bax, survivin or c-myc did not correlate with age, sex, stage of disease, T or B cell phenotype, histological type, EBV positivity, and response rate to therapy in correlation analysis. AI was positively correlated with PI ($p = 0.012$) and EBV ($p = 0.033$) in correlation analysis, but in survival analysis—the Log Rank Test—AI is not found to be a statistically significant prognostic determinant (Fig. 3).

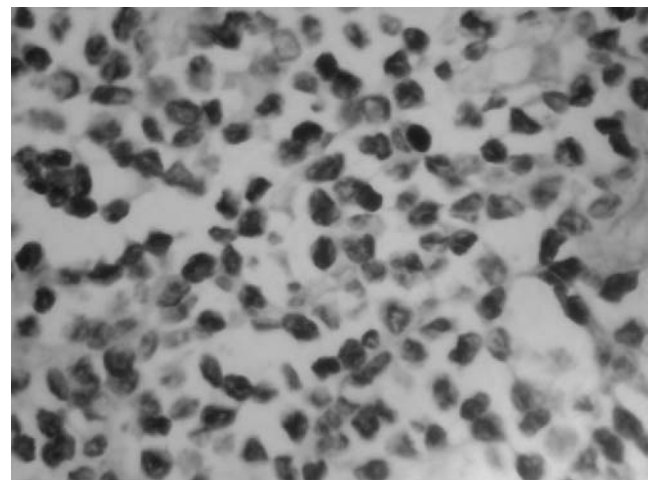


Fig. 4 Nuclear c-myc positivity in Burkitt lymphoma (DAB $\times 200$)

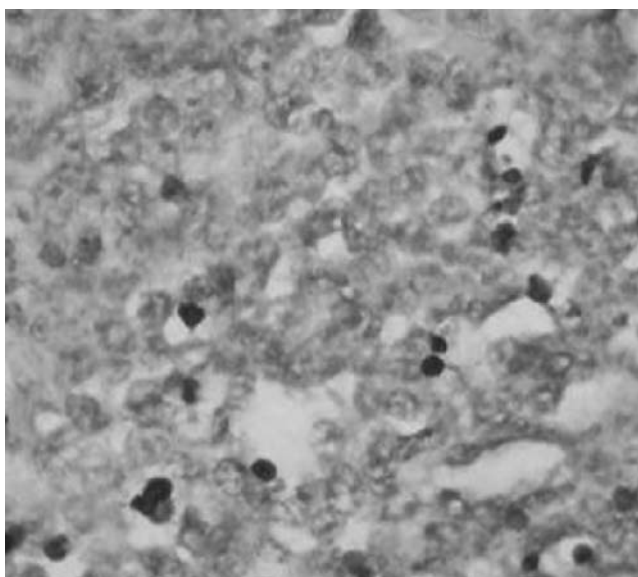


Fig. 5 Apoptotic cells stained dark with TUNEL method (DAB ×200)

Relationship of the studied parameters according to EBV status and histologic types are shown in Table 1 and Fig. 7 respectively. None of the cases of LL and PTCL were positive with EBV. Only one of nine ALCL cases was positive. Four of 11 DLCL cases were positive. Instead, 22 of 35 BBLL cases were EBV positive expressing EBER and/or LMP-1.

Discussion

In this study we have analyzed the PI, AI and apoptosis related proteins in pNHL cases. We correlated this data with several clinical parameters and EBV status. EBV was correlated with AI and fas, p53, bcl-2 among apoptosis related proteins but

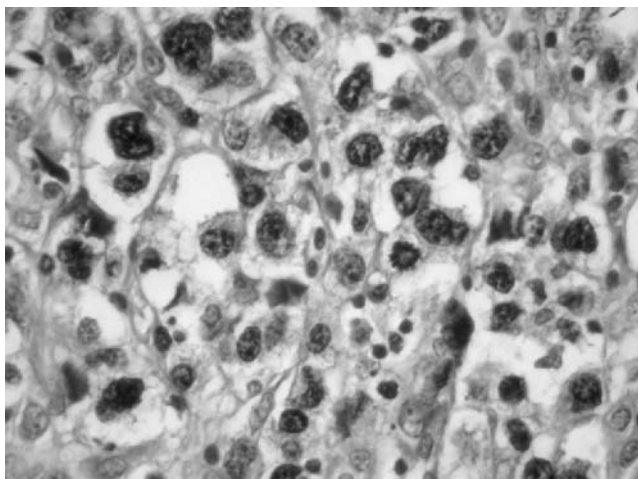


Fig. 6 Nuclear Ki-67 positivity in anaplastic large cell lymphoma (DAB ×200)

Table 1 Relationship of the parameters according to EBV status

EBER	-(n)	+(n)	p
Fas-	48	9	
Fas+	4	9	0.0001
P53-	49	15	
P53+	3	3	0.158
Bax-	34	8	
Bax+	18	10	0.121
Bcl-2-	22	13	
Bcl-2+	30	5	0.030
Survivin-	31	9	
Survivin+	21	9	0.481
c-myc-	30	22	
c-myc+	8	10	0.242

not with bax, survivin or c-myc. No correlation was observed with PI and studied factors. The prognostic significance of bax expression has been previously investigated in DLBCL, with conflicting results [17]. We hypothesized that high proapoptotic and low antiapoptotic factors would promote chemotherapy-induced apoptosis. Their regulation would be affected by latent EBV infection.

Our results indicate that EBV could have been included in lymphomagenesis affecting apoptosis especially on intrinsic pathway. On intrinsic pathway bcl-2 and p53 seems to be included in pathogenesis of EBV. On extrinsic pathway the positive correlation of fas expression with EBV seems to be an effect of LMP-1 acting as a member of tumor necrosis factor receptor family, starting signaling pathway without a ligand and finally inducing apoptosis. LMP-1 does not downregulate fas expression. In lmp-1

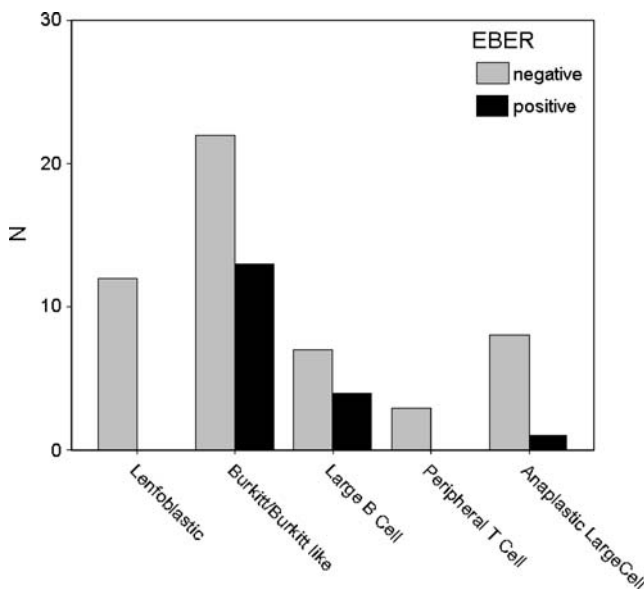


Fig. 7 Relationship of histologic subtypes according to EBV status. The most frequent EBV positivity was observed in BBLL group while all of the cases of LL and PTCL were negative

transfected cell lines fas expression is 20-fold increased [18]. It is known that it acts as CD40 receptor and activates B cell growth signaling pathway. This mechanism is mainly restricted to post transplantation lymphomas in immunosuppressed patients. Survivin is not related with EBV. This data shows that EBV does not affect inhibition of apoptosis during caspase activation. LMP-1 is a main transforming protein acting as a classical oncogene. It is an integral membrane protein. Some antiapoptotic proteins like bcl-2 are upregulated by lmp. PI and c-myc seems to keep their role in differential diagnosis of lymphomas, not a role in EBV pathogenesis or prognosis. Lmp-1 included in both apoptotic and antiapoptotic pathway is not suitable to be a target of new therapeutic approaches in pNHL [26–29].

In apoptosis; the intrinsic pathway that is initiated within the cell in response to cellular signals resulting from DNA damage, cell cycle defect, detachment from the extracellular matrix, hypoxia, loss of cell survival factors, involves release from the mitochondria of pro-apoptotic proteins that activate caspase enzymes, controlled by bcl family proteins. The extrinsic pathway is controlled by death receptors on the cell surface. There is crosstalk between these two pathways. The mitochondrial pathway after apoptosis induction through death receptors, can be amplified by intrinsic apoptotic signals [28–32]. In our series both intrinsic (bcl-2) and extrinsic (fas) pathways were related with EBV.

We expected a survival advantage in EBV positive cases. It is hypothesized that EBV-association provides a survival advantage by providing a more responsiveness to chemotherapy and radiotherapy associated with DNA damage. Naresh et al reported a 10 year relapse free survival of EBV positive and negative patients as 60% and 44% respectively, and a 10 year overall survival 85% and 64%, respectively [2]. Our data show that EBV does not determine prognosis. In our pediatric series the overall and event free survival rates are high. That might have caused a statistical disadvantage to calculate the prognostic role of EBV. AI is found to be a prognostic factor. It is also negatively correlated with stage. An increased AI on the tissues before chemotherapy is a good predictor for prognosis.

Subtyping of pNHL is important in planning treatment regimens. pNHL are all high grade lymphomas. Among them BBLL has the highest PI. We did not grade PI. We take it as percentage for each case for statistical evaluation. We also explored the prognostic role of AI: PI ratio because this ratio is a better determinant of tumor growth. In especially Burkitt lymphoma, the clinical development of the tumor is a result of a shift in the equilibrium between life and death. The fraction of the cells proliferating and surviving is high than the cells dying [18]. In our series AI: PI ratio is smaller in BBLL subtype than LBL and DLBCL subtypes. AI:PI value was not related with EBV.

Bcl-2 protein family is the most studied proteins in tumors involved in apoptosis. Bax is a 21-kd protein with significant homology clustered in the (Bcl-2 homology) BH-1 and BH-2 regions. The prognostic significance of bax expression has been previously investigated in DLBCL with conflicting results. In our series bax is expressed in 40% of the cases, but it is not related with EBV latency or prognosis or any other studied factor. Bax expression is very low in LBL, while it is expressed in half of the cases in other types [29–32].

In our center pediatric Hodgkin lymphoma cases were studied with the same parameters. In that series, the proliferation index was positively related with EBV but not with prognosis. None of the parameters were related with prognosis. EBV was negatively related with the apoptotic index. There were no relationships between bax, bcl-2, survivin, p53, fas, and c-myc with EBV [33]. In that series all of the EBER positive cases were also LMP-1 positive. In this NHL study there are cases which are lmp-1 negative but EBER positive, opposite to the literature suggesting an important role in viral latency and oncogenesis for LMP2 protein acting as an B cell receptor [34, 35].

Our results indicate that apoptosis-regulating proteins have a role in the pathogenesis of pNHL. EBV was correlated with apoptotic index, and fas and bcl-2 expression. No correlation was observed with proliferation index and studied factors. High apoptotic index was related with good prognosis.

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