EBV promotes tumorigenicity and worsens the prognosis of Hodgkin lymphoma through the expression of survival enhancing proteins by activating the JAK / STAT and NF-kB signaling pathways. (A comparative study between EBV positive and negative Hodgkin lymphoma)

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Article

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Abstract

Background

Epstein-Barr virus (EBV) is involved in 40% of all Hodgkin's lymphoma (HL) cases. EBV produces epigenetic alterations in the host genome that decrease the expression of proapoptotic proteins and activate the expression of survival immortalization genes.

Methods

According to EBNA-1 detection by nested PCR, 64 patients with HL were classified into two groups: EBV positive and negative HL. The expression levels of JAK/STAT and NF-kB pathway mRNA molecules (JAK2, STAT1, IRF-1, PD-L1, IFN-, NFkB, Bcl-xL, and COX-2) by both RT-PCR and qRT-PCR, and the results proteins PD-L1, Bcl-xL, and COX-2 by immunohistochemistry were analyzed and correlated with each other,

Results

Positive mRNA expression of either (JAK/STAT) or (NF-kB) pathways: (JAK2, STAT1, IRF-1, and PD-L1) and (IFN-, NFkB, Bcl-xL, COX-2) respectively are highly significantly correlated with each other (p 0.001) and with EBV positive HL patients (p 0.001). EBV positive HL cases had a higher incidence of relapse (p = 0.008), poor DFS (p = 0.013), higher mortality (p = 0.015), and low OS rates (p 0.028)

Conclusion

EBV in HL promotes the expression of survival immortalization signals by activating the JAK/STAT and NF-kB pathways and is associated with poor clinicopathological criteria, a higher incidence of disease progression, relapse, and poor overall survival. Therefore, targeting the JAK/STAT and NF-kB pathway members could be valuable in the management of EBV-associated Hodgkin's Lymphoma cases.

Introduction

Hodgkin's lymphoma (HL) is an uncommon B-cell lymphoid malignancy that affects 10% to 15% of all lymphoma patients. Currently, regular chemotherapy and radiation treatment can cure 80% to 90% of HL patients.On the other hand, tumour cell features control the disease's activity and receptivity to treatment. Large multinucleated Reed Sternberg cells in an inflammatory background are the main feature of identifying HL.(1). Several risk factors are related to the development of HL, such as autoimmune diseases, high socioeconomic status, families with congenital and acquired immunodeficiency, a positive family history of HL or other lymphoid neoplasms, and those with latent Epstein-Barr virus (EBV) infection (2).

Understanding how EBV activates cellular signal transduction pathways could aid in the development of new therapeutics for HL related to EBV. Cellular signalling adaptor proteins bind to LMP1 and cause LMP1 complexes to cluster, constructing binding sites for cellular signalling adaptor proteins that result in constitutive activation of several pathways. EBV tumorigenicity is linked to six different types of cell signalling pathways, including NF-B, JAK/STAT, PI3K/Akt, (MAP) kinases, (TGF-), and Wnt/-catenin, that are required for malignant cell growth and survival.(3, 4), However, PI3K/AKT, MAPK, TGF, and Wnt/catenin signalling pathways are mostly involved in the mechanism of epithelial-mesenchymal transition (EMT) and tumour angiogenesis of EBV-associated nasopharyngeal carcinoma.(4)

The NF-B and JAK/STAT pathways are the primary signalling pathways involved in EBV-associated lymphoma. At the same time, latent membrane protein 1 (LMP1) promotes tumorigenesis by activating different mechanisms that result in overexpression of different survival-promoting proteins such as immunochekpoint PD-L1, anti-apoptotic proteins Bcl-xL, and COX-2. LMP1 stimulates the transcription factor AP-1 production, a direct promoter of PD-L1. LMP1 mediates its immortalization signalling through IFN-, an active secretion of IFN (5). When IFN binds to its receptor, it causes a rapid and dramatic increase in the creation of the IFN receptor (IFNGR) heterotetrameric complex on tumour cell membranes, which is composed of IFNGR1 and IFNGR2, which then activates the JAK/STAT and NF-kB pathways.

Bcl-xL, and COX-2 expression mediated by NF-kB signaling pathway in EBV (+ve) HL

LMP1 has two distinct functional domains. CTAR1 and CTAR2 bind to factors linked to the TNF receptor and activate the NF-kB pathway by increasing IFN expression. IFN-, through the canonical pathway, activates Ik-B kinases (IKKs) that rapidly phosphorylate IkBa and release the NF-B active NF-B NF-B molecule. Free NF-Kb enters the nucleus and promotes transcription of regulatory genes involved in cell survival. Bcl-xL and COX-2 are immortalization survival genes regulated by NF-Kb (4, 6, 7). Nf-kb also improves the expression of genes that encode antioxidant enzymes, such as the sod2 gene, which encodes the antioxidant enzyme (MnSOD), Overexpression of MnSOD inhibits the accumulation of reactive oxygen species (ROS) and rescues cells from necroptosis (7). IFN-, on the other hand, is an NF-B pathway potential activator.When NF-B signalling is compromised, IFN instead activates the RIP1-dependent cell death pathway. This results in increased accumulation of reactive oxygen species (ROS) and, eventually, loss of mitochondrial membrane potential, which makes NF-B targeting a focal point that triggers necroptosis in immortalized, proliferating EBV-infected RS cells (4).

PD-L1 expression is mediated by the JAK2 / STAT1 / IRF-1 signaling pathway in EBV (+ve) HL.

IFN-, promotes the activation of the JAK/STAT signalling pathway through activation of JAK2. Jak phosphorylates the cytokine receptor on tyrosine (Tyr) residues that serve as docking activating sites for STAT1 phosphorylation and, consequently, STAT1 activation occurs. Activated STAT homodimers or

heterodimers leave the cytoplasm towards the nucleus where they bind to different promoter sequences of survival regulatory genes(3). PD-L1 helps tumour cells to evade immune surveillance by having a promoter that is responsive to STAT1 signalling through the putative IRF-1 site that results in overexpression of PD-L1PD-L1 helps tumour cells to evade immune surveillance by having a promoter that is responsive to STAT1 signalling through the putative IRF-1 site that results in overexpression of PD-L1(4); STAT activity is tightly regulated in normal cells, and activation is only transitory. On the other hand, EBV-associated HL is characterized by dysregulation of the Jak/STAT system; inhibiting negative regulators results in more signaling for PD-L1 production. (8)

Apoptosis is a type of intentional cell death in which old, damaged, and a succession of processes discard useless cells. Many tumors have the potential to inhibit apoptosis. (9). The two primary apoptotic pathways are the extrinsic or death receptor pathways and the intrinsic or mitochondrial pathways. The extrinsic route is activated when TNF receptor 1 or FAS is ligated with specific death ligands. Since it has a crucial impact on the etiology and the resistance of various hematologic malignancies to treatment, the mitochondrial or intrinsic apoptotic pathway has been extensively studied and therapeutically targeted. Many cellular stresses, such as DNA damage, hypoxia, or oxidative stress, activate the mitochondrial pathway (10), (11).

Bcl-xL belongs to the Bcl-2 family of anti-apoptotic proteins. It is a transmembrane protein found in mitochondria and has a potent inhibition of Bax, safeguarding the mitochondrial outer membrane's integrity from deterioration and subsequent inhibition of the cytoplasmic release of cytochrome to stop the following apoptotic cascade; cytochrome, Apaf-1, caspase-9 complex constitutes the apoptosome that activates caspase-3, This stimulates cytoplasmic endonucleases (CAD/ICAD) and proteases, resulting in chromosomal DNA breakdown, chromatin condensation, cytoskeletal remodeling, and cell disintegration. Up-regulation of Bcl-xL expression in tumor cells can lead to resistance to chemotherapy by enhancing tumor cell survival signaling of the tumor cells (12, 13).

COX-2 is an inflammation-associated enzyme that catalyses the synthesis of prostaglandins (PGs) from arachidonic acid. COX-2 is a cytoplasmic protein and is not detectable in most normal tissues and is activated by inflammatory and mitogenic stimuli. The COX-2/PGE-2 pathway inhibits the two major apoptosis pathways by inhibiting signalling through death receptors and mitochondria. Furthermore, increasing the expression of the anti-apoptotic protein BCL-2 by activating the Ras-MAPK/ERK pathway (14). Several epidemiologic studies have revealed that people who take nonsteroidal anti-inflammatory drugs (NSAIDs) the classic inhibitor of COX-2, regularly had a lower risk of HL than people who don't take them or use them seldom (15). The best-known target of NSAIDs is the cyclooxygenase (Cox) enzyme which is considered a major angiogenic stress response protein involved in the carcinogenesis and tumor progression of several solid (colorectal, breast, ovarian, lung, and others) and hematologic malignancies (16, 17).

Lymphoma is the sixth most frequent cancer, so developing novel therapeutic modalities in clinical trials is essential to achieve prolonged remission and improved survival (18, 19).

Tumor cells express PD-L1 as an "adaptive immunological mechanism" to avoid immunosurveillance in the tumour microenvironment (TME), acting as a "brake" on immune functions. Furthermore, multiple types of host cells in the TME, such as fibroblasts, T cells, dendritic cells, and macrophages, increase the expression of PD-L1 to provide a further enhanced niche for immune escape from cancer. By connecting to its receptors and activating proliferative and survival signalling pathways, PD-L1 acts as a pro-tumor factor in cancer cells. As a result, multiple studies have shown that inhibiting the PD-L1/PD-1 signalling axis with an antibody reactivates exhausted immune cells in the tumour microenvironment and eradicates cancer cells (20-22).

Following the success of immune checkpoint blockade therapy in the treatment of refractory solid tumors, it has recently gained traction as a promising method for the treatment of relapsed lymphoma. The immune checkpoint (PD-L1), modulates T-cell activation intensity and duration (23). Several PD-1/PD-L1 blocking antibodies have been developed as up-and-coming drugs in the treatment of solid tumors, such as bladder, kidney, melanoma, and lung cancers. (24).

This work aims to evaluate the expression of the mRNA molecules of the JAK/STAT and NF-kB pathways mRNAs in positive and negative patients with HL and to correlate the expression of the end-result proteins PDL1, COX-2, and Bcl-xL with the pathological and prognostic parameters of patients with positive and negative HL with EBV to check their validity as an immunotherapy target.

Patients And Methods

In this prospective cohort research, 64 new Hodgkin's lymphoma cases were included. They were admitted to the Department of General Surgery, Zagazig University Hospital, for biopsy, and then underwent histopathological diagnosis in the Department of Pathology. Patients were then referred to the clinical oncology and medical oncology departments, where they received their standard treatment regimen according to stage and guidelines. Follow-up was done every three months in the first two years, then every six months in the following three years, through physical examination, radiology, and CT and PET-CT scans.

Sixty-four samples were obtained after obtaining consent from the ethical committee and the patients or their legal representatives. A part of each sample was freshly frozen in liquid nitrogen for the detection of EBNA-1 by nested PCR and for the detection of the mRNAs of the JAK/STAT and NF-kB pathway (JAK2, STAT1, IRF-1, PD-L1, IFN-, NFkB, Bcl-xL, COX-2) detection by RT-PCR and qRT-PCR, while the remaining portion was treated with 10% formalin and processed for paraffin blocks were cut into 5-m thick slices and stained with hematoxylin and eosin (H&E) and immunohistochemistry for (PD-L1, COX-2, and Bcl-xL) and immunohistochemistry for (PD-L1, COX-2, and Bcl-xL) antibodies for light microscope examination. According to WHO classification, the histologic subtypes (25).

Treatment

Treatment was given according to international guidelines. Patients received chemotherapy alone or a combined modality protocol, depending on stage and risk factors. Most patients received ABVD (adriamycin, bleomycin, vinblastine, and dacarbazine) in standard doses every 2 weeks (one cycle = 4 weeks) with dose modification or delays depending on toxicity. Other regimens such as DHAP (dexamethasone, AraC, cisplatinum) or ICE (ifosfamide, carboplatin,

etoposide) regimens were delivered in the majority of first relapsed or refractory cases. Radiotherapy was given with megavoltage energies using an involved field technique to tumor doses of 30–36 Gy in daily fractions of 1.8–2.0 Gy. (26)

Bulky disease describes tumours in the chest that are at least 13 as wide as the chest or tumours in other areas that are at least 10 centimetres (about 4 inches) across. The B-symptoms describe patients with unexplained fever, copious night sweats, or less than six months of loss of more than 10% of body weight. The staging was done according to the Ann Arbor staging system.(27)**.**

Progression-free survival (PFS) was the interval (in months) from the beginning of treatment to progression at any time or death. Disease-free survival (DFS) starts from the date of CR until the date of relapse or death. Overall survival (OS) refers to the interval (in months) from diagnosis to death or the last follow-up.

Ethical approval: The study was conducted following the ethics of the World Medical Association (Helsinki Declaration of 1975, revised in 2000) for studies involving humans.(28). The study (no. ZU-IRB#/3/2543) was approved by the Institutional Review Board (IRB) - Faculty of Medicine, Zagazig University, Egypt.

RT-PCR and qRT-PCR techniques

EBV detection:

DNA extraction

The QIAamp tissue kit (Qiagen) was used to extract viral DNA from fresh tissue nasopharyngeal biopsy samples. According to the manufacturer's instructions, viral DNA from paraffin-embedded blocks was extracted.

Nested PCR and DNA Sequencing

The presence of the EBNA-1 gene was determined in all samples using a nested PCR approach with two rounds of amplification. The EBNA-1 was targeted with two sets of primers: outer and inner primers. For amplification, the Taq PCR Master Mix Kit (Qiagen, Germany) was used. The 20-ul reaction mixture consisted of 10 ul of master mix, 0.5 uM of each outer primer, 5 ul of DNA, and the rest was water. The first round of amplification lasted 5 minutes at 94 °C, followed by 35 cycles of 30 seconds at 94 °C, 1 minute at 50 °C, 1 minute at 72 °C, and a final extension of 10 minutes at 72 ° C.10 ul of the master mix, 0.5 uM of each inner primer, and 1 ul of the PCR Products from the first round of PCR, in addition to water, were used for the second round of PCR. After 5 minutes at 94°C, the mixture was subjected to 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 50 seconds, and the final extension. As a negative control, we utilise a mix of reagents and primers without DNA. The results of nested PCRs were examined using electrophoresis on 2 percent agarose, which was then stained with ethidium bromide and observed.The amplified products were purified (using Promega USA's Wizard SV Gel and PCR cleaning equipment) and sequenced using the dideoxynucleotide chain termination method. The ABITM3130 Genetic Analyzer was used to examine the sequences (Applied Biosystems, Foster City, CA, USA). Sequencing was done with the EBNA-1 outer forward and reversed primers. Chromas 2.6.5 was used to inspect the sequencing files (Technelysium, Helensvale, Australia). The retrieved sequences were compared to the EBV prototype sequences from the GenBank database. The EBNA-1 gene was targeted with two sets of primers: outer and inner primers in all samples using the method previously designed by Ahmed et al. (29)

EBNA-1 inner	F:5$-AGATGA\mathbb{C}CAGGAG\forall GG\mathbb{C}C\forall GC-3$
	R:5$-C\forall AGGGGAGACGACTC\forall TGGTGT-3$
EBNA-1 outer	F:5$-GTAG\forall GG\mathbb{C}A\top\ \top T\mathbb{C}AC-3$
	R:5$-CT\mathbb{C}ATCGTC\forall AGCTGCA-3$

mRNA detection of the JAK / STAT and NF-kB pathways:

As described by Wook Moon et al.,(8) JAK2, STAT1, IRF-1, and PD-L1 were detected, while IFN-γ, NFkB, and Bcl-XL, as previously described by Zhang et al (30) with the following sequences of the primers:

JAK2	F:5'- CCAGATGGAAACTGTTCGCTCAG-3'
	R:5'- GAGGTTGGTACATCAGAAACACC-3'
STAT1	F:5'-ATGGCAGTCTGGCGGCTGAATT-3`
	R: 5'-CCAAACCAGGCTGGCACAATTG-3
IRF-1	F:5'-GAGGAGGTGAAAGACCAGAGCA-3`
	R: 5'-TAGCATCTCGGCTGGACTTCGA-3
PD-L1	F:5'-TGCCGACTACAAGCGAATTACTG-3’
	R:5'-CTGCTTGTCCAGATGACTTCGG-3’
IFN-γ	F:5'-GAGTGTGGAGACCATCAAGGAAG-3’
	FR5'-TGCTTTGCGTTGGACATTCAAGTC-3’
NFkB	F:5'-GCAGCACTACTTCTTGACCACC-3'
	R:5’-TCTGCTCCTGAGCATTGACGTC-3'
Bcl-xL	F:5’-GCCACTTACCTGAATGACCACC-3’
	R:5’-AACCAGCGGTTGAAGCGTTCCT-3’
COX-2	F:5’-CGGTGAAACTCTGGCTAGACAG-3’
	R:5’-GCAAACCGTAGATGCTCAGGGA-3’

The TRIzol reagent (Invitrogen Inc., Carlsbad, CA, United States) was used to isolate total RNA from cells, and then, using the Shanghai Genechem Co., Ltd., Shanghai, China, MiRNA reverse transcript kit, the RNA was reverse transcribed. At the same time, U6 functions as an internal control. Reverse transcription tests were carried out to determine mRNA using a Takara Biotechnology Ltd., Dalian, Liaoning, China) mRNA reverse transcript kit, and as an internal reference, we used GAPDH. A SYBR Green quantitative PCR analysis was carried out on a 7500 real-time fluorescence quantitative PCR. The target genes' transcription levels were then estimated using the 2MMCT technique. OriGene Technologies (Beijing, China) provided the primer sequences for STAT1 (# HP210040), JAK2 (# HP208201), IRF-1 (# HP205934), PD-L1 (# HP210654), IFN- (# HP200586), NFkB (# HP207409), Bcl-xL (# HP234144), COX-2 (# HP200900), GAPDH (# HP205798).

Immunohistochemistry (IHC)

Reagents:

The primary antibodies used were rabbit monoclonal PD-L1 (clone: CAL10; Biocare Medical, Concord, USA, 1:100), rabbit monoclonal COX-2 (clone: SP21; Invetrogen, Thermo Fisher Scientific USA, 1:100) and rabbit monoclonal Bcl-xL (clone: 7D9; Invetrogen, Thermo Fisher Scientific USA, 1:200).

Technique:

The sections were rehydrated after the blocks were deparaffinized. Endogenous peroxidase activity was inhibited by 3% hydrogen peroxide.All primary antibodies were antigen recovered for 15 minutes in 10 nM citrate buffer with a pH of 6.0 in a microwave oven. The primary monoclonal antibodies were incubated on the sections for 2 hours at room temperature. At room temperature, tissues were incubated with a biotin-conjugated secondary antibody for 1 hour after being washed in PBS. When diaminobenzidine tetrahydrochloride was added, the reactions could be seen (31).

Immunohistochemical Evaluation:

PDL1 immunostaining

The following anti-PD-1 scores were assigned based on the cytoplasmic and membrane intensity of expression: 0 indicates no reaction, 1 indicates mild staining, 2 indicates moderate, and 3 indicates intense staining. Human retinopathy stem cell (HRS) tumour cells and peritumoral cells were all studied independently. When more than 5% of the tumour population was stained, the positivity of the microenvironment was realised when more than 20% of the whole cell population was stained; regardless of intensity, HRS cells were classified as positive or negative. Immunohistochemistry was used to determine whether PD-L1 was positive or negative cytoplasmic or membranous for HRS cells and the microenvironment (26, 29).

COX-2 immunostaining:

A COX-2 immunostaining evaluation was performed by dividing the number of positively stained RS cells by the total number of RS cells. The percentage was recorded in five representative fields at 400. When more than 10% of HRS cells express COX-2, it is positive. **(32).**

Bcl-xL immunostaining:

Based on cleaved caspase-3 labeling, the apoptotic index (AI) was calculated by counting the number of H/RS cells in 10 randomly selected sectors without sclerosis or fibrosis. Bcl-xL labeled as brown granular cytoplasmic staining of H/RS cells. The labeling index for H/RS cells was calculated by dividing the

fraction of labeled H/RS cells by the total number of H/RS cells detected at a magnification of 400. The cases were classified as negative when all diagnostic cells were negative or less than 5% of these cells were positive **(33).**

Results

Patient characteristics

Clinicopathological data of the patient: (Table 1)

The patient characteristics are summarised in Table 1. The study included 64 patients with HL, of which 46.9% were EBV negative and 53.1% were EBV positive, ranging in age from 23 to 71 years with a mean age of 39.9 years SD (10.4), with nodular sclerosis being the most common histological subtype by 60.9%.57.8% of the patients were in stages I-II and 42.2% were in stages III-IV. In response to the first line of treatment, 57.8% showed complete remission (CR), 23.4% showed partial remission (PR), 12.5% showed stable disease (SD), and 6.3% showed progressive disease (PD). 42.2% of patients received the 2nd line chemotherapy, where 40.6% of patients showed CR and 1.6% showed PD. By the end of the study, 39.1% of the patients had died.

Relationship between the expression levels of the mRNAs of the JAK2 / STAT1 and NF-kB pathways and the presence of EBV. Table 2

(5.2±0.3, 13.7±1.9, 48.8±3.1, 25.3±0.8, 7.6±1.9, 8.3±0.8, 14.7±1.1 and 29.7±9.5) respectively compared to
(0.9±0.2, 2.80±0.4, 11.6±1.7, 4.40±0.5, 2.7±1.1, 4.1±0.2, 5.80±0.3, and 9.2±3.4) of -ve EBV

EBV positive patients were highly significantly associated with high expression levels of all examined mRNAs (JAK2, STAT1, IRF-1, PD-L1, IFN-γ, NFkB, Bcl-xL, COX-2) of both JAK2/STAT1 and NF-kB signaling pathways. P-value <0.001

Relationship between the expression of the mRNAs from the JAK2 / STAT1 pathway and each other. Table 3

The expression of the JAK2 / STAT1 pathway mRNAs examined (JAK2, STAT1, IRF-1, PD-L1,) were highly significantly associated with each other p-value <0.001.

Relationship between the expression of NF-kB pathway mRNAs to each other. Table 4

The expression of examined JAK2/STAT1 pathway mRNAs (IFN-γ, NFkB, Bcl-xL, COX-2) was significantly associated with each other p-value <0.001.

Correlation between EBV and the expression of end-results proteins (PD-L1, COX-2, Bcl-xL) clinicopathological and outcome parameters. Table 5

EBV-positive patients had a highly significant association with B symptoms (p = 0.022), extranodal involvement (p = 0.017), advanced stage (p = 0.018), positive expression of PD-L1 expression (p 0.001), positive expression of COX-2 (p = 0.015) and positive expression of Bcl-Xl (p = 0.0067). Individuals who tested positive for EBV had a higher risk of cancer progression and relapse after therapy (p = 0.008) and poorer DFS (p = 0.013). Moreover, they were more liable to mortality (p = 0.015) and poor OS (p = 0.028).

Relationship between the expression of the PD-L1, COX-2, and Bcl-xL protein with the clinicopathological and outcome parameters. Table 6

The positive expression of PDL1, COX-2, and Bcl-xL was associated with old age, B symptoms, a bulky tumor, extranodal involvement, and advanced stages (p 0.001). (Fig. 1) (Fig. 2) (Fig.3).

Survival analysis:

Positive patients had a higher risk of cancer progression, relapse after therapy (p 0.001, p 0.0095), poor DFS (p 0.001, p 0.001, p = 0.014), a higher mortality rate (p 0.001, p 0.003, p= 0.0095), and a lower OS rate (p 0.001, p 0.001, 0.004) (Fig. 4,5).

Discussion

Hodgkin's lymphoma (HL) is a malignant lymphoid tumour that accounts for less than 1% of all malignancies diagnosed worldwide each year. The presence of Reed Sternberg (RS) multinucleated giant cells in an inflammatory environment is used to identify HL (34). Despite significant therapeutic advances in lymphoma treatment, disease progression occurs in up to 40% of patients without HL and 15% of those with HL after standard chemoradiotherapy. Because treatment failure is so common, finding novel therapy modalities and engaging in clinical trials to obtain extended remission and greater survival (18).

EBV can induce its pathogenesis in different human disorders such as nasopharyngeal carcinoma, gastric carcinoma, several lymphomas, and lymphoproliferative diseases through activation of the main six pathways that result in enhanced epithelial-mesenchymal transition, angiogenesis, and inhibition of apoptosis with subsequent activation of tumour cell survival, proliferation, and metastasis.. (4)

To our knowledge, this is the first time to check these pathways together in different patients' samples, not in cell line experiments.

The JAK2 / STAT1 and NF-kB pathways played the primary role in EBV-induced tumorigenicity in different lymphomas.(4) Our results revealed high expression of all examined mRNAs (JAK2, STAT1, IRF-1, PD-L1, IFN-, NFkB, Bcl-xL, COX-2) of both the JAK2/STAT1 and NF-kB signalling pathways were significantly related to patients with EBV-HL. Then, each mRNA had a strong connection to other members of the same signalling pathway, which was in line with Moon et

al.(8), who reported that activating JAK2/STAT1/IRF-1 increased PD-L1 expression levels in EBV positive tumour cells more than in EBV negative cells. Also, Ding et al., Liu et al., Li et al., Zuo et al., (35-38) EBV-LMP1 exhibited tumorigenicity through activation of the NF-kB signalling pathway.

Apoptosis can be regulated by apoptotic proteins such as the BCL and Bax families. Increased expression of Bcl-xL, one of the potent antiapoptotic proteins of the BCL family, is a possible cause of apoptotic cascade blockade and caspase inhibition (12, 39). Furthermore, the COX-2/PG2 pathway inhibits intrinsic and extrinsic apoptosis pathways by increasing the expression of BCL proteins by activating the Ras-MAPK/ERK pathway (14).

In the current study, EBV-positive HL patients were significantly associated with positive expression of the COX-2 and Bcl-xL proteins that could inhibit the two main apoptosis pathways (12, 14, 40) rather than EBV-negative HL patients; that EBV infection explains this link's increased expression by activation of the NF-kB signalling pathways (41).

In this study, we found that EBV expression and its associated proteins PD-L1, COX-2, and Bcl-xL were correlated with poor clinic prognostic parameters and predicted poor clinical outcomes in HL patients, such as B symptoms, extranodal involvement, advanced stage, higher risk of cancer progression and relapse after therapy, as well as poor DFS. Furthermore, they were more liable to high mortality rates and poor OS. Several studies raised the same conclusion(42). Kim **et al. (26) and Shannon-Lowe et al. (27) They achieved similar results, and they stated that EBV positive HL patients were significantly associated with poorer clinical, pathological, and prognostic parameters than EBV negative HL patients. Mestre et al. and Zaky et al. proved that COX-2 expression is associated with poor clinicopathological parameters** (42, 43). In study by Ohno et al., COX-2 expression was inversely related to the apoptotic index.(40) and Kim et al.(44), who found that the apoptotic index was lower in COX-2 positive cancer cells than COX-2 negative cancer cells. Furthermore, Mestre et al.(42) reported that COX-2 expression in RS cells is a substantial independent and unfavorable prognostic factor in patients with early HL. This drew attention to the antitumor potential of non-NSAIDs through their ability to block the COX-2 pathway and reverse its effect; this was previously addressed in lymphomas caused by Kaposi sarcoma, herpesvirus, and EBV. These studies utilizing NSAIDs have shown their anticancer potential due to COX-2 and PGE2 inhibition, which is thought to have a crucial function in the etiology of numerous malignancies. (15).

Gravelle et al. (45), and Cao et al.(46) I investigated the therapeutic utility of targeting immune checkpoint proteins (PDL1) and COX-2, which may be valuable in the treatment of lymphoma, especially in cases associated with EBV, and establishing the expression of PD-1/PD-L1 cells as a significant criterion for identifying patients who are candidates for immune checkpoint blockade therapy. To date, many researchers have observed great value in the usage of anti-inflammatory drugs in the cure of different types of cancers. Kumar et al.(47) noted the additional therapeutic importance of NSAIDs and selective COX-2 inhibitors as potential chemoprotective agents against EBV-associated diseases and cancer, similarly stated by **Gravelle, et al. (45).**

Conclusion

EBV promotes the expression of proapoptotic and survival immortalization proteins through the activation of JAK/STAT and NF-kB pathways in EBV-associated HL patients. As a result, EBV-associated HL cases were associated with poor clinical and pathological parameters, a higher incidence of disease progression, relapse after therapy, and worse overall survival. Therefore, targeting NF-B, Jak2, STAT1, PD-L1, Bcl-xL or COX-2 may be valuable in the management of cases of EBV-associated Hodgkin's lymphoma.

Abbreviation

Bcl-Xl	B-cell lymphoma-extra large
COX-2	Cyclooxygenase-2
CTAR	C-Terminal Activation Region
EBV	Epstein-Barr Virus
EMT	Epithelial-mesenchymal transition
HL	Hodgkin lymphoma
IFN-γ	Interferon-gamma
IFNGR	The interferon-gamma receptor
IKKs	Ik-B kinases
IRF-1	Interferon regulatory factor-1
JAK/STAT	Janus Kinase and Signal Transducer and Activator of Transcription
LMP1	Latent membrane protein 1
NF-Kb	Nuclear factor kappa-light-chain-enhancer of activated B cells
PD-L1,	Programmed death-ligand 1
ROS	Reactive oxygen species

TGF Transforming growth factor

Declarations

- Ethics approval and consent to participate: The study (no. ZU-IRB#/3/2543) was approved by the Institutional Review Board (IRB) - Faculty of Medicine, Zagazig University, Egypt. The study was carried out following the Ethics of the World Medical Association (Helsinki Declaration of 1975, as revised in 2000) for studies involving humans, all patients or their legal representatives completed a written informed consent form for participation before being included in the study.

- Consent for publication: All patients or their legal representatives signed a written informed consent for the publication of their data.

- Availability of data and materials: The raw data supporting the findings of this study are available permanently at :

https://drive.google.com/drive/folders/1uTOA2C2dDmOvF0PN0x1Fq1E9gtnnUJs3?usp=sharing

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Tables

Table 1 : Clinicopathological and molecular parameters of the studied Hodgkin lymphoma patient (N= 64)

Clinicopathological and molecular feature		No.	(%)
Age group	<45 years	44	68.8
	=>45 years	20	31.3
Sex	Male	34	53.1
	Female	30	46.9
Type of Hodgkin	Lymphocytic rich	6	9.4
	Mixed cellularity	19	29.7
	Nodular sclerosis	39	60.9
EBV (EBNA-1)	Negative	30	46.9
	Positive	34	53.1
JAK2 RT-PCR	Negative	24	37.5
	Positive	40	65.6
STAT1 RT-PCR	Negative	22	34.3
	Positive	42	65.6
IRF-1 RT -PCR	Negative	35	54.6
	Positive	29	45.3
PD-L1 RT-PCR	Negative	39	61.0
	Positive	25	39.0
IFN-γ RT-PCR	Negative	26	40.6
	Positive	38	59.3
NFkB RT-PCR	Negative	23	35.9
	Positive	41	64.1
Bcl-xL RT-PCR	Negative	33	51.5
	Positive	31	48.4
COX-2 RT-PCR	Negative	35	54.6
	Positive	29	45.3
PD-L1 IHC	Negative	36	56.2
	Positive	28	43.7
COX-2 IHC	Negative	39	60.9
	Positive	25	39.1
Bcl-xLIHC	Negative	37	57.8
	Positive	27	42.2
B-symptoms	Absent	42	65.6
	Present	22	34.4
Bulky	Absent	50	78.1
	Present	14	21.9
Extra-nodal involvement	Absent	49	76.6
	Present	15	23.4
Stage	Stage I-II	37	57.8
	Stage III-IV	27	42.2
Response to 1st line chemotherapy	CR	37	57.8
	PR	15	23.4
	SD	8	12.5
	PD	4	6.3

2nd line chemotherapy	No	37	57.8
	Yes	27	42.2
Response to 2nd line chemotherapy	CR	26	40.6
	PD	1	1.6
	Not involved	37	57.8
Relapse	Absent	38	59.4
	Present	26	40.6
Mortality	Alive	39	60.9
	Died	25	39.1

Table 2: qRT-PCR expression of JAK2/STAT1 and NF-kB signaling pathway mRNAs in EBV positive and negative Hodgkin patients (N=64).

Parameters	**EBV**		p-value
	negative (N=30)	positive (N=34)	
JAK2 qRT-PCR	0.9±0.2	5.2±0.3	<0.001
STAT1 qRT-PCR	2.8±0.4	13.7±1.9	<0.001
IRF-1 qRT-PCR	11.6±1.7	48.8±3.1	<0.001
PD-L1 qRT-PCR	4.4±0.5	25.3±0.8	<0.001
IFN-γ qRT-PCR	2.7±1.1	7.6±1.9	<0.001
NFkB qRT-PCR	4.1±0.2	8.3±0.8	<0.001
Bcl-Xl qRT-PCR	5.8±0.3	14.7±1.1	<0.001
COX-2 qRT-PCR	9.2±3.4	29.7±9.5	<0.001

Table 3: RT-PCR expression of JAK2/STAT1 signaling pathway mRNAs in EBV positive and negative Hodgkin patients and its relation to each other (N=64).

	EBV		**P**	**JAK2**		**P**	**STAT1**		**P**	**IRF-1**		**P**	**PD-L1**		**I**
	-Ve (n=30)	+ve (n=34)		-ve (n=24)	+ve (n=40)		-ve (n=22)	+ve (n=42)		-ve (n=35)	+ve (n=29)		-ve (n=39)	+ve (n=25)	
EBV															
-ve (N=30)	-	-	-	22	8	<0.001	19	11	<0.001	26	4	<0.001	27	3	<
+ve(N=34)	-	-	-	2	32		3	31		9	25		12	22	
JAK2															
-ve (N=24)	22	2	<0.001	-	-	-	20	4	<0.001	19	5	0.002	22	2	<
+ve(N=40)	8	32		-	-	-	2	38		16	24		17	23	
STAT1															
-ve(N=22)	19	3	<0.001	20	2	<0.001	-	-	-	19	3	<0.001	21	1	<
+ve(N=42)	11	31		4	38		-	-	-	16	26		18	24	
IRF-1															
-ve(N=35)	26	9	<0.001	19	16	0.002	19	16	<0.001	-	-	-	30	5	<
+ve(N=29)	4	25		5	24		3	26		-	-	-	9	20	
PD-L1															
-ve(N=39)	27	12	<0.001	22	17	<0.001	21	18	<0.001	30	9	<0.001	-	-	-
+ve(N=25)	3	22		2	23		1	24		5	20		-	-	-

Table 4: RT-PCR expression of NFkB signaling pathway mRNAs in EBV positive and negative Hodgkin patients and its relation to each other (N=64).

	EBV		P	IFN-γ		P	NFkB		P	Bcl-xL		P	COX-2		I
	-Ve (n=30)	+ve (n=34)		-ve (n=26)	+ve (n=38)		-ve (n=23)	+ve (n=41)		-ve (n=33)	+ve (n=31)		-ve (n=35)	+ve (n=29)	
EBV															
-ve (N=30)	-	-	-	25	5	<0.001	19	11	<0.001	24	6	<0.001	21	9	(
+ve(N=34)	-	-	-	1	33		4	30		9	25		14	20	
IFN-γ															
-ve (N=26)	25	1	<0.001	-	-	-	20	4	<0.001	18	8	0.019	23	3	<
+ve(N=38)	5	33		-	-	-	3	37		15	23		12	26	
NFkB															
-ve(N=23)	19	4	<0.001	20	3	<0.001	-	-	-	21	2	<0.001	21	2	<
+ve(N=41)	11	30		4	37		-	-	-	12	29		14	27	
Bcl-xL															
-ve(N=33)	24	9	<0.001	18	15	0.019	21	12	<0.001	-	-	-	23	10	(
+ve(N=31)	6	25		8	23		2	29		-	-	-	12	19	
COX-2															
-ve(N=35)	21	14	0.02	23	12	<0.001	21	14	<0.001	23	12	0.0128	-	-	-
+ve(N=29)	9	20		3	26		2	27		10	19		-	-	-

Table 5: Relation between clinicopathological and outcome parameters with EBV positive and negative Hodgkin patients (N=64).					
Parameters	EBV				p-value
	negative (N=30)		positive (N=34)		
	No.	(%)	No.	(%)	
Age	-	-	-	-	
<45 years (N=42)	19	(43.2%)	25	(56.8%)	0.380
=>45 years (N=22)	11	(55%)	9	(45%)	
Sex					
Male (N=34)	16	(47.1%)	18(	52.9%	0.975
Female (N=30)	14	(46.7%)	16(	53.3%)	
Type of hodgkin	-	-	-	-	
Lymphocytic rich (N=06)	1	(16.7%)	5(	83.3%)	
Mixed cellularity (N=19)	9	(47.4%)	10(	52.6%)	0.286
Nodular scelrosis (N=39)	20	5(1.3%)	19(	48.7%%)	
B-symptoms	-	-	-	-	
Absent (N=42)	24	(57.1%)	18(	42.9%)	0.022
Present (N=22)	6	(27.3%)	16(	72.7%)	
Bulky	-	-	-	-	
Absent (N=40)	25	(50.0%)	25(	50.0%)	0.344
Present (N=14)	5	(35.7%)	9(	64.3%)	
Extra-nodal envolv.	-	-	-	-	
Absent (N=49)	27	(55.1%)	22(	44.8%)	0.017
Present (N=15)	3	20%)	12(	80%)	
Stage	-	-	-	-	
Early (I-II) (N=37)	22	(59.5%)	15(	40.5%)	0.018
Advanced (III-IV) (N=27)	8	(29.6%)	19(	70.4%)	
PDL-1	-	-	-	-	
Negative (N=36)	26	(72.2%)	10(	27.7%)	<0.001
Positive (N=28)	4	(14.2%)	24(	87.5%)	
Cox-2	-	-	-	-	
Negative (N=39)	23	(58.9%)	16(	41.0%)	0.015
Positive (N=25)	7	(28.0%)	18(	85.7%)	
Bcl-xL	-	-	-	-	
Negative (N=37)	12	(32.4%)	25 (	66.7%)	0.006
Positive (N=27)	18	(67.6%)	9(	33.3%)	
Response to 1st line chemotherapy					
CR (N=37)	22	(60.0%)	15	(55.9%)	0.018
PR (N=15)	6	(26.7%)	9	(20.6%)	
SD (N=9)	1	(6.7%)	8	(17.6%)	
PD (N=3)	1	(6.7%)	2	(5.9%)	
Response to 2nd line chemotherapy		-	-	-	
CR (N=26)	16	(100.0%)	-	10	(93.3%)	0.87
PD (N=1)	0	(0.0%)	-	1	(6.7%)	

Relapse		-	-	-	-	-	
Absent	(N=38)	23	(76.7%)		15(	44.1%)	0.008
Present	(N=26)	7	(23.3%)		19(	55.9%)	
Mortality		-	-	-	-	-	
Alive	(N=39)	23	(58.9%)		16	(41.1.7%)	0.015
Died	(N=25)	7	(28%)		18	(72%)	
DFS		-	-	-	-	-	
Mean (months)(95%CI)			52.1			43.4	0.013
Median DFS			NR			43.0	
5-year DFS			76.7%			44.1%	
OS		-	-	-	-	-	
Mean (months)(95%CI)			56.1			53.7	0.028
Median OS			NR			57	
5-year OS			76.7%			47.1%	

Categorical variables were expressed as number (percentage); continuous variables were expressed as mean (95%CI); 95%CI: 95% confidence interval; § Chi-square test; † Log rank test; p< 0.05 is significant

Table 6 : Relation between clinicopathological, outcome parameters ,and PDL-1, Cox-2, BcL-xL expression in the studied patients (N=64).

Parameters	PDL1				p-value	Cox-2				p-value	Bcl-xL			
	Negative (N=36)		positive (N=28)			negative (N=39)		positive (N=25)			negative (N=37)		positive (N=27)	
	No.	(%)	No.	(%)		No.	(%)	No.	(%)		No.	(%)	No.	(%)
Age														
<45 years (N=42)	32	(76.1%)	10	(23.8%)	<0.001	33	7(5.0%)	11	(25.0%)	0.001	23	(52.3%)	21	(47.7%)
=>45 years (N=22)	4	(18.1%)	18	(81.8%)		6	(30.0%)	14	(70.0%)		4	(20.0%)	16	(80.0%)
Sex														
Male (N=34)	11	(32.3%)	13(	38.2%)	0.193	20	(58.8%)	14	(41.2%)	0.712	19	(55.9%)	15	(44.1%)
Female (N=30)	25	(83.8%)	15(	50.0%)		19	(63.3%)	11	(36.7%)		8	(26.7%)	22	(73.3%)
Type of hodgkin														
Lymphocytic rich(N=06)	1	(16.6%)	5(	83.3%)	0.117	2	(33.3%)	4	(66.7%)	0.346	2	(33.3%)	4	(66.7%)
Mixed cellularity(N=19)	11	(57.8%)	8(	42.1%)		12	(63.2%)	7	(36.8%)		7	(36.8%)	12	(63.2%)
Nodular scelrosis(N=39)	24	(61.5%)	15(	38.4%)		25	(64.1%)	14	(35.9%)		18	(46.2%)	21	(53.8%)
EBV														
Negative (N=30)	26	(86.6%)	4(	13.3%)	<0.001	23	(76.6%)	7	(23.3%)	0.015	18	(60.0%)	12	(40.0%)
Positive (N=34)	10	(29.4%)	24(	70.5%)		16	(47.0%)	18	(52.9%)		9	(26.5%)	25	(73.5%)
B-symptoms														
Absent (N=42)	34	(80.9%)	8(	19.0%)	<0.001	38	(90.5%)	4	(9.5%)	<0.001	25	(59.5%)	17	(40.5%)
Present (N=22)	2	(9.1%)	20(	90.9%)		1	(4.5%)	21	(95.5%)		2	(9.1%)	20	(90.9%)
Bulky														
Absent (N=50)	35	(70.0%)	15(	30.0%)	<0.001	38	(76.0%)	12	(24.0%)	<0.001	26	(52.0%)	24	(48.0%)
Present (N=14)	1	(7.1%)	13(	92.8%)		1	(7.1%)	13	(92.9%)		1	(7.1%)	13	(92.9%)
Extra-nodal envolement														
Absent (N=49)	35	(71.4%)	14(	10.2%)	<0.001	36	(73.5%)	13	(26.5%)	<0.001	26	(52.0%)	23	(46.9%)
Present (N=15)	1	(6.6%)	14(	93.3%)		3	(20.0%)	12	(80.0%)		1	(7.1%)	14	(93.3%)
Stage														
Early (I-II) (N=37)	37	(100.0%)	0(	0.0%)	<0.001	35	(94.6%)	2(	5.4%)	<0.001	24	(64.9%)	13	(35.1%)
Advanced (III-IV) (N=27)	11	(40.7%)	16(	59.3%)		4	(14.8%)	23(	85.2%)		3	(11.1%)	24	(88.9%)
Response to 1st line chemotherapy														
CR (N=37)	27	(72.9%)	10	(27.0%)	0.001	29	(74.4%)	8	(32.0%)	0.001	22	(81.5%)	15	(40.5%)
PR (N=15)	8	(53.3%)	7	(46.6%)		8	(20.5%)	7	(28.0%)		5	(18.5%)	10	(27.0%)
SD	1	(12.5%)	7	(87.5%)		2	(5.1%)	6	(24.0%)		0	(0.0%)	8	(21.6%)

(N=8)														
PD (N=4)	0	(0.0%)	4	(100.0%)		0	(0.0%)	4	(16.0%)		0	(0.0%)	4	(10.8%)
Response to 2nd line chemotherapy														
CR (N=26)	14	(100.0%)	12	(92.3%)	0.481	10	(100.0%)	16	(94.1%)	1.000	21	(95.5%)	5	(100.0%)
PD (N=1)	0	(0.0%)	1	7.7%		0	(0.0%)	1	(5.9%)		0	(0.0%)	1	(4.5%)
Relapse														
Absent (N=38)	29	(76.3%)	9(	23.6%)	<0.001	30	(78.9%)	8	(21.05%)	<0.003	27	(71.05%)	11	(28.9%)
Present (N=26)	7	(26.9%)	19(	73.1%)		9(	34.6%)	17	(65.3%)		10	(38.4%)	16	(61.5%)
Mortality														
Alive (N=39)	29	(74.3%)	10	(25.6%)	<0.001	30	(76.9%)	9	(23.1.0%)	0.001	28	(71.7%)	11	(28.2%)
Died (N=25)	7	(28.0%)	18	(72.0%)		9	(36%)	16	(64.0%)		9	(36%)	16	(64%)
DFS														
Mean (months) (95%CI)		53.7		38.3	<0.001		54.02		37.36	<0.001		51.1		42.5
Median DFS		NR		39.00			NR		30.0			NR		40
5-year DFS		81.6%		26.9%			76.9%		32%			73.0%		40.7%
OS														
Mean (months) (95%CI)		56.9		51.8	<0.001		57.8		50.2	<0.001		56.5		52.5
Median OS		NR		54.00			NR		55			NR		55
5-year OS		81.6%		30.8%			76.9%		36.0%			75.7%		40.7%

Categorical variables were expressed as number (percentage); continuous variables were expressed as mean (95%CI); 95%CI: 95% confidence interval; § Chi-square test; † Log rank test; p< 0.05 is significant NR Not Reached Yet

Figures

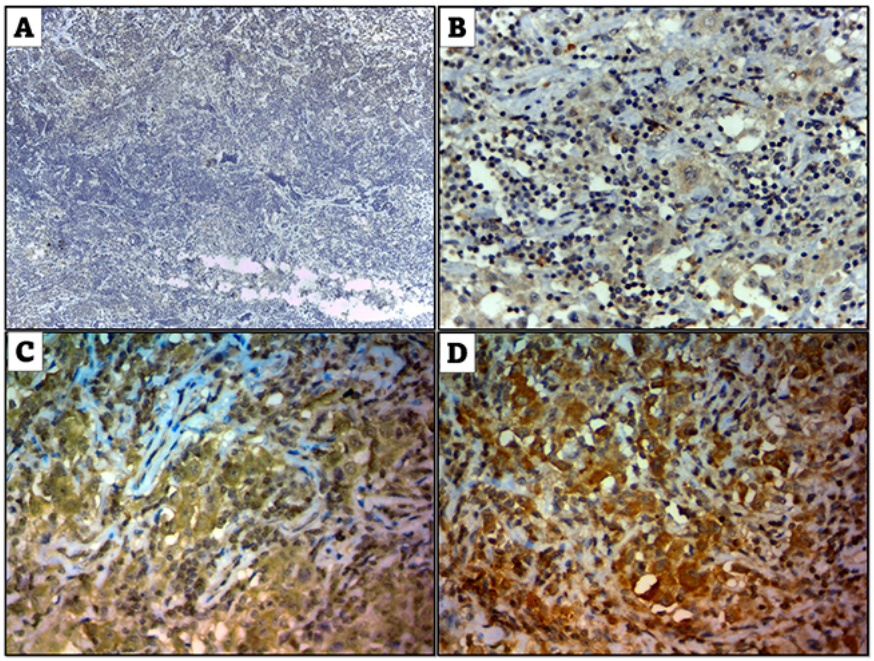

Figure 1

Immunohistochemical expression of PD-L1 in tumor cells (RS), and peritumoral microenvironment indicated by brown cytoplasmic and/or membranous staining, (A): Showing negative expression of PD-L1 with less than 5% of positive cells (100X). (B): Showing weak PD-L1 expression (400X). (C): Showing moderate PD-L1 expression (400X). (D): Showing strong expression of PD-L1 (400X).

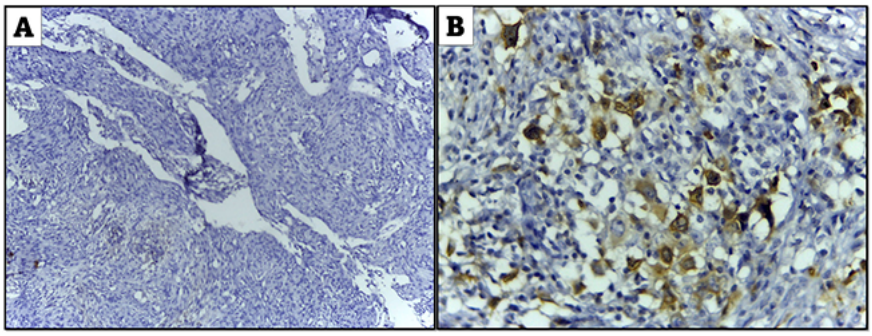

Figure 2

Immunohistochemical expression COX-2 in tumor cells (RS), indicated by brown cytoplasmic/ membranous staining, (A): Showing negative expression of COX-2, less than 10% of RS positive cells (100X). (B): Showing positive expression of COX-2, more than 10% of RS positive cells (400X).

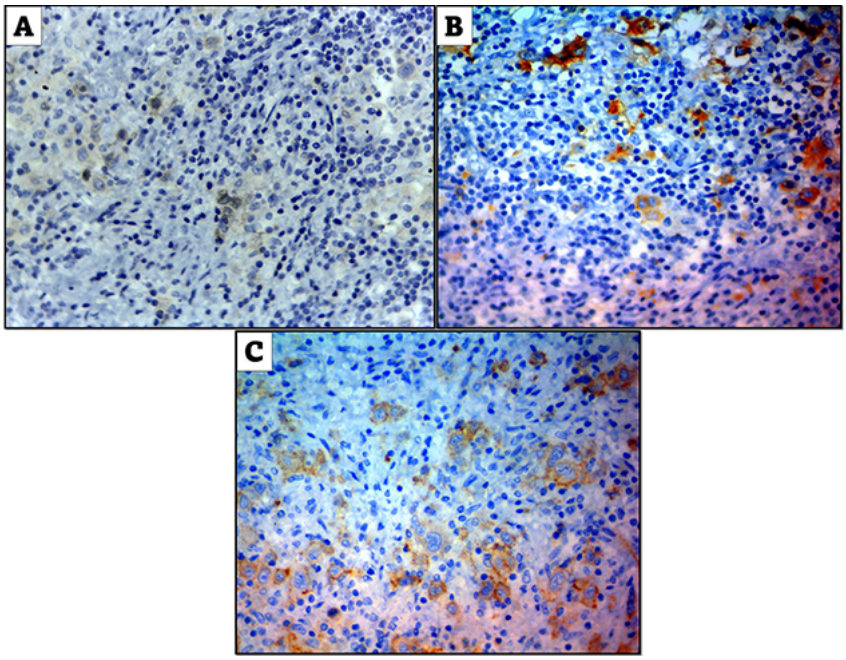

Figure 3

Immunohistochemical expression of Bcl-xL in the tumor cells (RS) of HL, expression of Bcl-xL is indicated by granular brown cytoplasmic staining of RS cells, (A) Negative expression of Bcl-xL in a patient with HL showing less than 5% Bcl-xL positive RS cells. (B&C) Positive expression of Bcl-xL showing more than 5% Bcl-xL positive RS cells (400X).

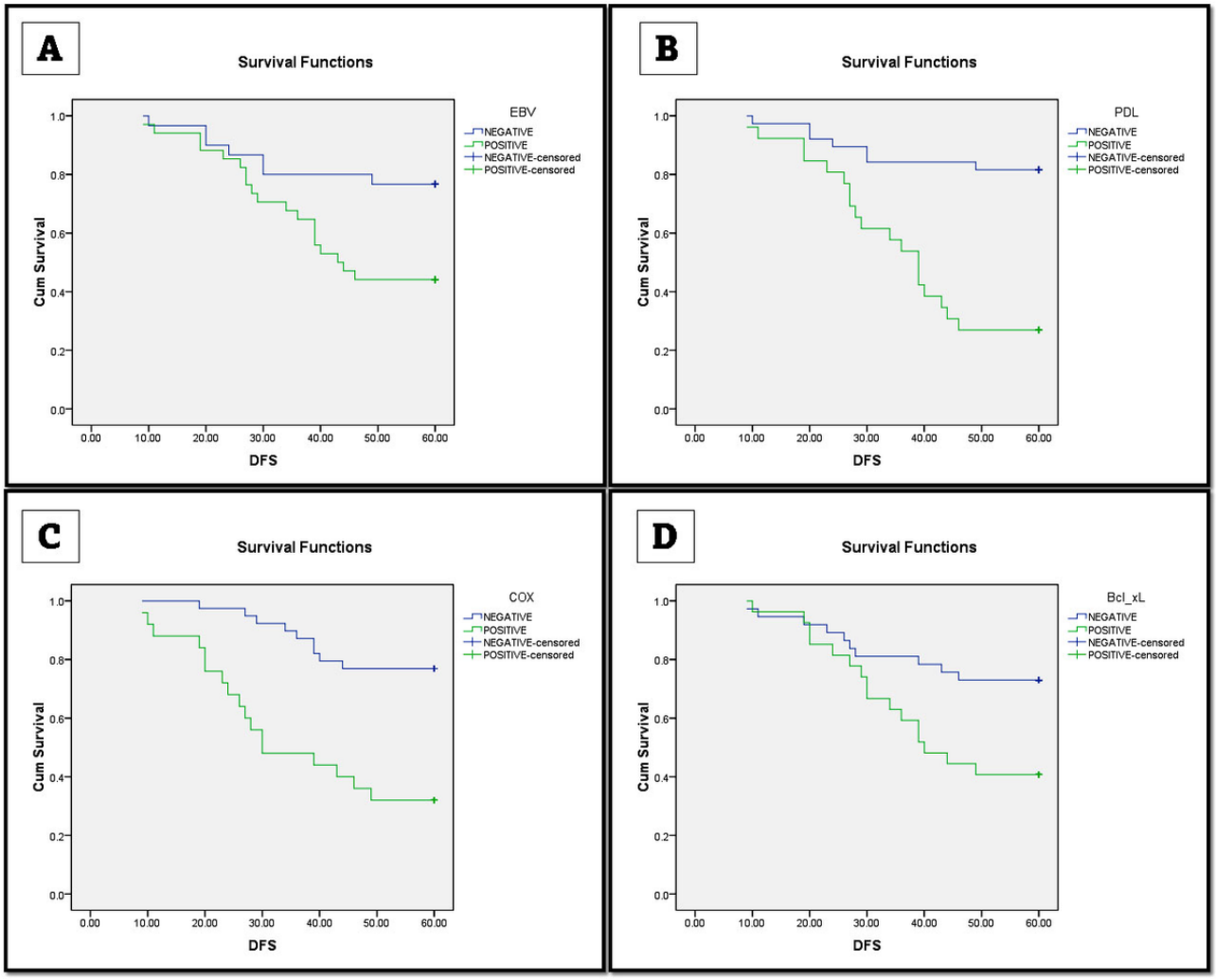

Figure 4

Kaplan-Meier plot of disease-free survival (DFS): (A) all patients (N=64) stratified by EBV; (B) stratified by PDL-1; (C) stratified by COX-2; (D) stratified by Bcl-xL

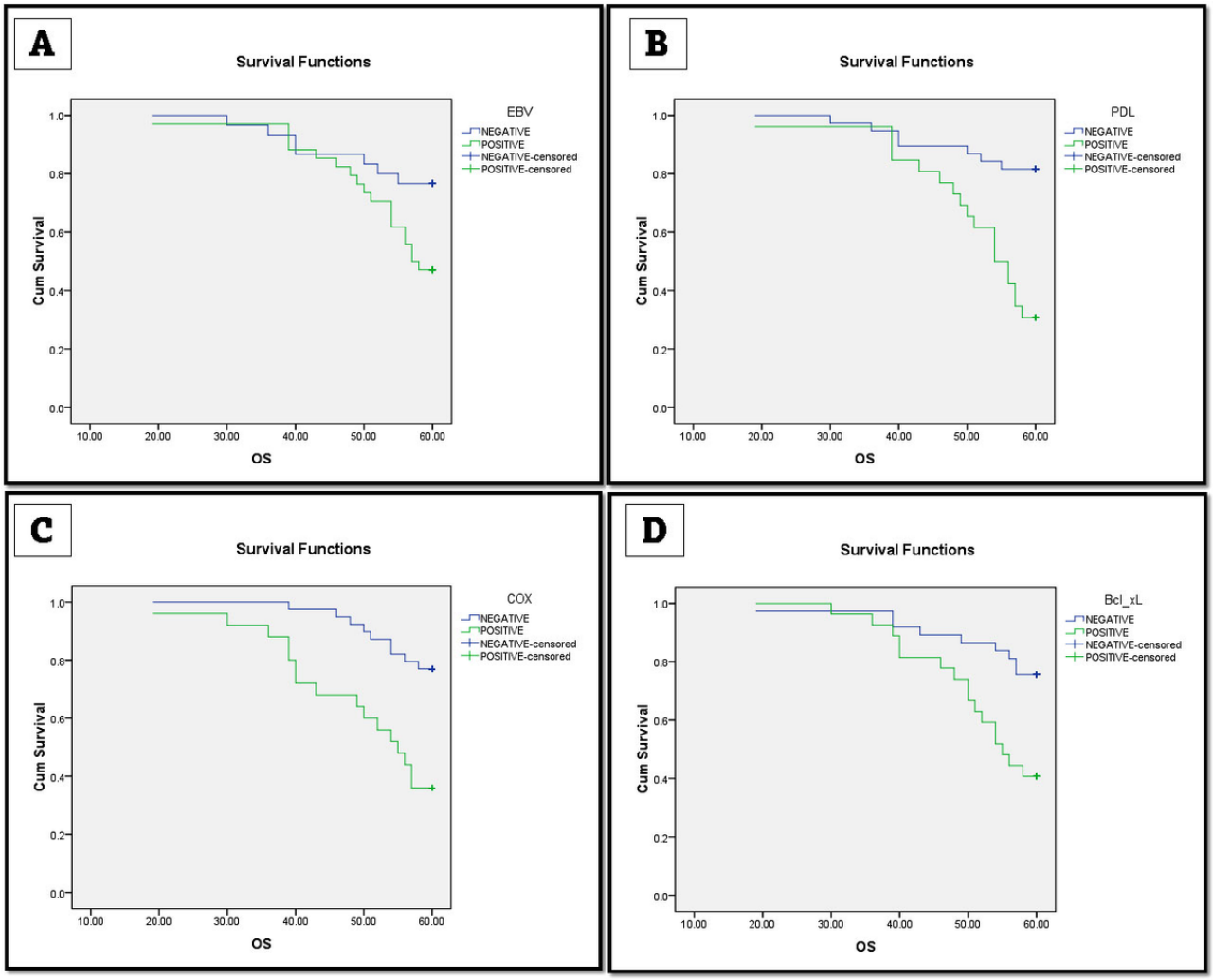

Figure 5

Kaplan-Meier graph of overall survival (OS): (A) all patients (N=64) stratified by EBV; (B) stratified by PDL-1; (C) stratified by COX-2; (D) stratified by Bcl-xL

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GA.png