Regulation of EBV LMP1-triggered EphA4 downregulation in EBV-associated B lymphoma and its impact on patients’ survival
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Abstract

Epstein-Barr virus (EBV), an oncogenic human virus, is associated with several lymphoproliferative disorders, including Burkitt’s lymphoma, Hodgkin’s disease, diffuse large B-cell lymphoma (DLBCL) and post-transplant lymphoproliferative disorder (PTLD). In vitro, EBV transforms primary B cells into lymphoblastoid cell lines (LCLs). Recently, several studies have shown that receptor tyrosine kinases (RTKs) play important roles in EBV-associated neoplasia. However, details of the involvement of RTKs in EBV-regulated B cell neoplasia and malignancies remain largely unclear. Here, we found that EphA4, which belongs to the largest RTK Eph family, was downregulated in primary B cells post-EBV infection at the transcriptional and translational levels. Overexpression and knockdown experiments confirmed that EBV-encoded latent membrane protein 1 (LMP1) was responsible for this EphA4 suppression. Mechanistically, LMP1 triggered the ERK pathway and promoted Sp1 to suppress EphA4 promoter activity. Functionally, overexpression of EphA4 prevented LCLs from proliferation. Pathologically, the expression of EphA4 was detected in EBV-negative tonsils but not in EBV-positive PTLD. In addition, an inverse correlation of EphA4 expression and EBV presence was verified by immunochemical staining of EBV-positive and EBV-negative DLBCL, suggesting EBV infection was associated with reduced EphA4 expression. Analysis of a public dataset showed that lower EphA4 expression was correlated with a poor survival rate of DLBCL patients. Our findings provide a novel mechanism by which EphA4 can be regulated by an oncogenic LMP1 protein and explore its possible function in B cells. The results provide new insights into the role of EphA4 in EBV-positive PTLD and DLBCL.
Key points:

1. EBV LMP1 dysregulates EphA4 expression via the ERK-Sp1 pathway.

2. Downregulation of EphA4 is demonstrated in EBV-positive DLBCL, which is significantly correlated with poor DLBCL survival.
Introduction

Epstein-Barr virus (EBV) is oncogenic and its infection is associated with multiple human diseases, especially lymphoproliferative disorders, such as infectious mononucleosis, Burkitt’s lymphoma (BL), Hodgkin’s disease (HD), NK/T cell lymphoma, post-transplant lymphoproliferative disorder (PTLD) and EBV-positive diffuse large B-cell lymphoma (DLBCL)\(^1,2\). In vitro, EBV can transform human primary B cells into lymphoblastoid cell lines (LCLs), which support its association with human malignancies. Like other human herpes viruses, EBV may be found in two states: latency and lytic replication. It is well documented that viral latent products contribute largely to EBV oncogenic activities. The main EBV latent products include six EBV nuclear antigens (EBNA1, 2, LP, 3A, 3B, 3C), three latent membrane proteins (LMP1, 2A, 2B), small RNAs (EBER 1, 2) and miRNAs (BARTs)\(^1\). According to previous studies, EBV uses various strategies to manipulate cell gene expression so that it can persist in infected B cells. These include altering the expression of protein tyrosine kinases (PTKs), cytokines, adhesion molecules and anti-apoptotic genes\(^1,3-8\). Among them, we are particularly interested in PTKs because they are important at many aspects of cell proliferation, differentiation, apoptosis, migration and tissue development. In total, 90 PTKs have been defined following sequencing of the human genome\(^9\). They can be divided into two major categories: 58 receptor tyrosine kinases (RTKs) and 32 non-receptor tyrosine kinases\(^9\). In the case of EBV-associated malignancies, several EBV genes products are involved in dysregulation of RTKs. EBV-encoded LMP1 elevates amounts of Recepteur d’Origine Nantais (RON) via NF-κB, to promote B cell proliferation and induce the migration and invasion of epithelial cells\(^3,4\). In addition, epidermal growth factor receptor (EGFR) is induced and activated by LMP1 through PKCδ and NF-κB\(^10-12\).
Also, c-Met is upregulated by LMP1 via Ets1 in epithelial cells\textsuperscript{13}. Activation of discoidin domain receptor 1 (DDR1) by LMP1 contributes to the survival of Hodgkin’s lymphoma\textsuperscript{14}. Recently, LMP1 has been reported to increase the expression of fibroblast growth factor receptor 1 (FGFR1), enhancing aerobic glycolysis and cell invasiveness\textsuperscript{15}. Furthermore, LMP1 activates the insulin-like growth factor 1 (IGF1) receptor via upregulation of its ligand, IGF1, to promote cell proliferation and colony formation\textsuperscript{16}. In addition, Zta, a lytic EBV transactivator, can upregulate the expression of Trk-related tyrosine kinase (TKT), which may enhance the metastasis of nasopharyngeal carcinoma\textsuperscript{5,6}. Human epidermal growth factor receptor 2 (HER2) and HER3 are dysregulated by EBV encoded BARF0, which not only increases the level of HER2/3 but also enhances cell anchorage independence\textsuperscript{17}. In the cases mentioned above, many EBV products are responsible for increasing the expression or enhancing the kinase activity of RTKs to deliver signaling that favors cell proliferation, migration or invasion. Herein, we wondered whether there are other mechanisms involved in EBV immortalization of B cells through other RTKs. In our previous study, a kinase display was used to investigate the differential expression of PTKs between primary B cells and LCL. RON and TKT were found to be upregulated\textsuperscript{3-6}. In this manuscript, we reveal another RTK, namely erythropoietin-producing hepatocellular receptor A4 (EphA4), which is involved in EBV lymphoproliferation.

Of note, Eph receptor is the largest family of RTKs in humans, being composed of nine EphAs and five EphBs. Usually, EphA receptors bind to glycosyolphosphatidylinositol-linked Eph receptor interacting protein (ephrin)-A ligands and Eph B receptors bind to transmembrane ephrin-B ligands\textsuperscript{18,19}. The interactions between Eph receptors and their ligands trigger both forward Eph kinase activity in Eph receptor-expressing cells and reverse Src family kinase activity in
ephrin-expressing cells\textsuperscript{19}. These bi-directional signaling pathways make the Eph family distinct from other RTKs. Conventionally, their functions are recognized in developmental processes and tissue homeostasis\textsuperscript{20}. Recently, dysregulation of Eph receptors and ephrins has been reported in many human cancers\textsuperscript{18,19,21,22}. Of interest, various reports indicated that Eph can function in oncogenic promotion or tumor suppression in several human cancers at different stages or in different cancers\textsuperscript{18,19,21,22}. Notably, the involvement of Eph in viral infection has been explored in some human oncogenic viruses. EphA2 is the entry receptor for KSHV\textsuperscript{23} and also acts with EGFR as a co-receptor for hepatitis C virus entry\textsuperscript{24}. Collectively, Ephs not only play roles in physiological activities but also are involved in pathogenesis\textsuperscript{25}. According to our kinase display data, EphA4 is clearly downregulated in LCL, compared to uninfected B cells. Little is known about the role of EphA4 in B lymphocytes, so the expression and regulation of EphA4 in LCL and EBV-associated diseases will be explored in this paper.
Materials and Methods

B cell purification and EBV infection

Peripheral blood mononuclear cells were kindly provided by anonymous donors at Taipei Blood Center of Taiwan Blood Service Foundation and CD19-positive B cells were purified as described previously. The methods of EBV virion (B95.8 strain) production and EBV infection of primary B cells have been described previously. Experiments using human samples were approved by Institutional Review Boards (IRB) of National Taiwan University Hospital, Taipei, Taiwan.

Cell culture

Human peripheral blood CD19-positive B cells were infected by B95.8 EBV to establish LCLs in vitro. BJAB cells are EBV-negative Burkitt’s lymphoma cells. All B-cell lines were cultured in complete RPMI medium containing 10% fetal calf serum (FCS), 1 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. A nasopharyngeal carcinoma cell line, TW01, was maintained in complete DMEM containing 10% FCS, 1 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

Kinase display assay

The tyrosine kinase display assays were performed as described previously. Briefly, total RNAs were extracted and cDNAs of protein tyrosine kinases were generated and
amplified by using \([\gamma^{33}P]-\)labeled degenerate primers designed for the conserved kinase domain. The resulting 170 base pairs PCR products were gel-purified and digested separately using 16 restriction enzymes. The digested PCR products were resolved in a denaturing polyacrylamide gel. The patterns of restriction enzyme digestion were identified for each specific protein tyrosine kinase in the data bank.

**Plasmids**

The EBNA1, EBNA2, Zta and LMP1 expressing plasmids were constructed as described previously. The luciferase reporter plasmid, driven from the *EPHA4* promoter (nucleotides -1000 to +42), was inserted into the pGL3-basic vector (Promega). The LMP1 expressing lentivirus plasmid, pSIN-LMP1, and its mutant plasmids, including LMP1-deleted CTAR1 (ΔCTAR1) with the deletion of LMP1 amino acids (aa) 194 to 232, deleted CTAR2 (ΔCTAR2) with deletion of LMP1 aa 351 to 386 and LMP1-deleted CTAR1/2 (ΔCTAR1/2) plasmids were reported previously. The shLMP1, shSp1 lentivirus plasmids and its vector control shLuc were constructed in the pLKO.1 plasmids as described previously. The pLKO.1-shERK1/2 lentivirus plasmids were purchased from National RNAi Core Facility (Academia Sinica, Taipei, Taiwan) with sequences targeting human ERK1#1 (5’- CTATAACCAAGTCCATCGACAT -3’), ERK1#2 (5’-TCCCTGTCAAGCTGACTT -3’) and ERK2 (5’-
TATCCATTCAACGTATCTC -3’). The EphA4 (pCDNA3.1-EphA4) full length (WT) and mutant plasmids are kindly provided by Dr. Tang-Long Shen (Department of Plant Pathology and Microbiology, National Taiwan University, Taipei, Taiwan). The Flag-tagged EphA4 WT and mutant lentivirus plasmids were constructed into pWPI lentiviral plasmid, which is a gift from Dr. Min-Liang Kuo (Institute of Toxicology, National Taiwan University, Taipei, Taiwan).

**RNA extraction, reverse transcription and quantitative polymerase chain reaction (RT-Q-PCR)**

Total RNAs were extracted by using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RT-Q-PCR has been described previously. EphA4 mRNA was determined using forward primer, 5’- GATAGCAAGCCCTCTGGAG -3’, reverse primer, 5’- CCAATCAGTTCGTAGCCAGTT -3’ and Roche no. 20 universal probe. GAPDH mRNA were determined by using forward primer, 5’-TCCACTGGCGTCTTCACC -3’, reverse primer, 5’-GGCAGAGATGATGACCCTTTT -3’ and Roche no. 45 universal probe. MAGOH mRNA were determined by using forward primer, 5’- AAAGAGGATGATGCATTGTGGT -3’, reverse primer, 5’-TCTTCTCAATGACGATTTCA T -3’ and Roche no. 51 universal probe.

**Western blotting and antibodies (Abs)**
Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer and Western blotting was performed as previous study\(^7\). Abs were used as follows: EphA4 (Santa Cruz and ECM Biosciences), \(\beta\)-actin (Sigma-Aldrich), phospho-Akt S473 (Cell Signaling), Akt (Santa Cruz), phospho-I\(\kappa\)B-\(\alpha\)-S32/36 (Cell Signaling), p65 (Santa Cruz), phospho-JNK T183/Y185 (Cell Signaling), JNK (Millipore), phospho-ERK T202/Y204 (Cell Signaling), ERK (Santa Cruz), Sp1 (Santa Cruz). Abs against EBV viral products including EBNA1, EBNA2, LMP1, Zta were as reported previously\(^7,27\).

**Infection of lentivirus**

The method of production and infection with lentivirus was reported previously\(^7\). For EphA4 WT and mutant expressing lentivirus infection, \(5 \times 10^5\) LCLs were infected with lentivirus at a multiplicity of infection (MOI) of 4. For LMP1, Sp1 and ERK1/2 knockdown, LCLs were seeded at a density of \(1 \times 10^6\) cells/mL and infected with shLuc control, shLMP1, shSp1 or shERK1/2 lentivirus at MOI of 1. BJAB cells were seeded in \(1 \times 10^6\) cells/mL and infected with pSIN-LMP1 and its deletion mutant expressing lentivirus at a range of 0.5-4 MOI.

**Treatment with inhibitors**

LCLs were seeded at \(1 \times 10^6\) cells/mL and treated with 20 \(\mu\)M of PI3K inhibitor LY294002, 20 \(\mu\)M of JNK inhibitor SP600125, 20 \(\mu\)M of MEK inhibitor PD98059, 2.5 \(\mu\)M of NF-\(\kappa\)B inhibitor BAY 11-7082 for 48 hours, respectively. TW01 cells were
treated with 500 nM of Sp1 inhibitor mithramycin for 24 hours. The inhibitors mentioned above were purchased from Merck Millipore. Dimethylsulfoxide (DMSO) served as a solvent control.

**Reporter assay**

TW01 cells were seeded at a density of 1×10^5 cells/well in a 12-well plate and then cotransfected with 0.5 μg *EPHA4* promoter luciferase reporter plasmids, 0.25 μg pSG5-LMP1 and 0.05 μg green fluorescent protein (GFP) expressing plasmids (pEGFP-C1, Promega) using T-Pro Non-liposome transfection Reagent II (T-Pro NTR II, T-Pro Biotechnology) according to the manufacturer’s instructions. Cells were harvested and the luciferase activities and GFP fluorescent intensity were detected using the Bright-Glo Luciferase Assay System kit (Promega) 2 days post transfection. The relative fold induction of luciferase activity from each transfectant was first standardized with EGFP, followed by normalization to the control vector pGL3.

**Proliferation assay**

Lentivirus-infected LCLs were seeded at 1×10^4 cells per well in 96 well plates for 5 days. Before the indicated time point, cells were treated with AlamarBlue (Thermo) for 4 hours and the absorbance measured according to the manufacturer’s instructions.

**Chromatin immunoprecipitation (ChIP) assay**

BJAB vector control and LMP1-expressing cells were harvested. DNA-protein
complexes were immunoprecipitated using anti-Sp1 antibody as described previously. The DNA was extracted and analyzed by PCR with the EPHA4 promoter spanning the Sp1-binding sites. The amplification of the GAPDH promoter region was as described previously.

**Immunohistochemistry (IHC) and in situ hybridization (ISH) of EBER assays**

Tonsil, PTLD, and DLBCL biopsies were obtained from the National Taiwan University Hospital. IHC assays of EphA4 and LMP1 were performed using the Starr Trek Universal Detection system (Biocare) according to manufacturer’s protocol and ISH of EBER assays were described in our previous study.

**Kaplan-Meier plots**

The microarray datasets of DLBCL were downloaded from the National Center for Biotechnology Information (NCBI) and Gene Expression Omnibus (GEO) database (accession number GSE4475). Overall survival was analyzed by generating Kaplan-Meier plots. Patients were divided into high and low EphA4 groups, according to the median expression of EphA4.

**Statistical Analysis**

Statistical analysis was conducted using GraphPad Prism software program. Quantitative data were reported as mean ± SEM and compared using the unpaired Student’s t test. Clinical correlation was determined by Fisher’s exact test. For
Kaplan-Meier survival analysis, a log-rank test was employed to compare the
difference between two groups. Data were considered statistically significant at $p < 0.05$. 
Results

EphA4 is downregulated post-EBV infection

Because RTKs play a critical role in EBV infected cells, the RTK expression profile was analyzed by kinase display assay using degenerate primers⁵. According to the BstNI, HhaI, and MnlI restriction enzyme digestion profile, downregulation of EphA4 was found in three LCLs compared to control primary B cells (Figure 1A). To confirm this result, mRNA and protein expression levels of EphA4 were detected in cells infected with EBV for 3, 7, 14, 21 or 28 days (defined as LCL), compared to uninfected primary B cells. Clearly, mRNA and protein expression levels of EphA4 were reduced to approximately 50% to 10% post-EBV infection, compared to primary B cells (Figure 1B-C). Expression of EBNA1 and LMP1 indicated that the primary B cell was successfully transformed by EBV (Figure 1C). In addition to EBV infection, B cells can be activated by various treatments including anti-CD40 antibody plus IL-4, lipopolysaccharide (LPS) and poly I:C to mimic T-cell dependent or independent activation²⁹. To clarify whether B-cell activation can decrease EphA4 expression, EphA4 transcripts were measured after 3 days of incubation. As shown in Figure 1D, EphA4 was downregulated not only by EBV infection but also by anti-CD40 antibody plus IL-4 treatment. This fit previous findings that EBV LMP1 functionally mimics constitutive active CD40 receptor associated with TRAFs³⁰,³¹. Meanwhile, treatments
of LPS or poly I:C which triggered TLR4 or TLR3 signal pathway did not alter EphA4 expression.

To determine whether this effect is common in B cells infected by EBV, expression of EphA4 mRNA was detected by RT-Q-PCR in another eight LCL cell lines established in our lab. Compared to primary B cells, EphA4 transcripts were also detected at 10% to 50% normal levels in LCLs (Figure 1E). To verify these observations, total RNA and protein were collected from two paired primary B cells and their corresponding LCLs. Consistent with the data above, EphA4 mRNA and protein expression were reduced in LCLs, suggesting that EBV infection influenced the expression of EphA4 at the transcriptional and translational levels (Figure 1F-G).

**LMP1 is responsible for EphA4 suppression**

Many EB viral products are able to regulate gene expression. To determine which EB viral gene was involved in EphA4 suppression, selected viral genes were expressed ectopically in the EBV-negative nasopharyngeal carcinoma cells TW01 through transfection. Based on the results of Figure 2A-H, we demonstrated that LMP1 was the candidate protein that inhibits EphA4 expression. Other EBV gene products, including Zta, EBNA2, EBER1, EBER2 and BARF0, had no effect on the EphA4 expression level (Figure 2C-H). This LMP1-mediated EphA4 downregulation was also observed in a dose dependent manner in EBV-negative Burkitt’s lymphoma cells.
BJAB transduced with LMP1 lentivirus, at mRNA and protein levels (Figure 2I-J). Of note, upregulation of EphA4 protein and mRNA expression were seen in LCLs using an shLMP1 knockdown approach (Figure 2K-L). Taken together, all the evidence indicated that LMP1 was the viral protein responsible for EphA4 downregulation.

**LMP1 suppresses EphA4 through ERK pathways**

Structurally, LMP1 resembles a CD40 receptor but its activity is ligand-independent. LMP1 delivers signaling through its long C-terminal activation regions (CTARs). To determine which CTAR domains were involved in EphA4 downregulation, lentiviruses containing LMP1 full length (WT), CTAR1 deleted mutant (ΔCTAR1), CTAR2 deleted mutant (ΔCTAR2), and both CTAR1 and CTAR2 deleted mutant (ΔCTAR1/2) were transduced into BJAB cells. It seems that both CTAR1 and 2 of LMP1 are involved in this repression (Figure 3A). Usually, these CTARs associate with TRAFs and activate downstream PI3K/Akt, NF-kB and MAPK, including the JNK, ERK and p38 pathways. To dissect which signaling pathway was necessary for LMP1-mediated EphA4 downregulation, LCLs were treated with MEK inhibitor PD98059, JNK inhibitor SP600125, PI3K inhibitor LY294002 or NF-kB inhibitor Bay11-7082. Expression of EphA4 was increased in LCLs treated with PD98059 but not other inhibitors (Figure 3B). Furthermore, LMP1-triggered EphA4 downregulation through ERK pathway was confirmed by silencing both ERK1/2 by lentiviral
transduction of LCLs (Figure 3C).

**LMP1 represses EphA4 promoter activity through Sp1**

LMP1 downregulated the expression of EphA4 at the transcriptional and translational levels (Figure 2) through the ERK pathway (Figure 3B-C). ERK has been reported to phosphorylate Sp1 at T453 and T739 to regulate the promoter activity of targeted genes and we speculated that LMP1 may inhibit EphA4 promoter activity through Sp1. To test this hypothesis, EphA4 protein was detected in Sp1-knockdown LCLs and its amount was augmented (Figure 4A). Meanwhile, EphA4 protein amounts were restored in LMP1-overexpressing BJAB cells, when cells were simultaneously knocked down of Sp1 (Figure 4B). These data suggested that Sp1 was involved in LMP1-mediated EphA4 downregulation. The effect of Sp1 on EphA4 promoter activity was investigated further. The *EPHA4* promoter (-1000 to +42) sequence was analyzed and four Sp1 binding sites were predicted. Clearly, EphA4 promoter activity was upregulated in Sp1-silenced LCLs and in Sp1-knockdown, LMP1-expressing TW01 cells (Figure 4C and 4D). Also, addition of the Sp1 inhibitor mithramycin, which interferes with Sp1 binding to the GC rich sequence, to the LMP1 transfectants increased the EphA4 promoter activity (Figure 4E). Furthermore, to determine whether LMP1 promotes Sp1 binding to the EphA4 promoter, a ChIP assay was performed in BJAB cells expressing LMP1. We showed that Sp1 bound to the EphA4
promoter at the region of -51 to -60 nt in BJAB cells, following LMP1 expression (Figure 4F).

**The Eph JM domain, but not kinase domain, is involved in preventing LCL proliferation**

Like other EphA family members, EphA4 contains an extracellular domain, transmembrane (TM) and juxtamembrane (JM) domains, and an intracellular kinase domain\(^\text{19}\). To understand the biological function of EphA4 in B cells, EphA4 full length (WT) and mutants, including EphA4 with a kinase-dead mutant with V653M (KD) and EphA4 with mutations of the tyrosine auto-phosphorylation site Y569F and Y602F of the JM region (2M), as illustrated in Figure 5A, were delivered to LCLs by lentivirus infection. The transduced LCLs expressed WT or mutated EphA4 at physiologic level, compared to primary B lymphocytes (Figure 5B-C, right panel).

Cell proliferation was inhibited significantly in the LCLs expressing EphA4 WT and KD but not in the LCLs expressing EphA4 2M (Figure 5B-C). These data showed that EphA4 repressed LCL proliferation through its JM region, suggesting that the kinase domain is not important for EphA4 repression of LCL proliferation.

**EphA4 can be detected in tonsil biopsies but not in PTLD**

EBV-positive PTLD exhibits similar biological features to LCLs. Thus, to address whether EphA4 was downregulated in PTLD biopsies, we examined EphA4
expression levels in 14 EBER-positive PTLD biopsies and 5 EBER-negative tonsil biopsies by IHC assay. The results of the IHC assay showed that all cases of tonsil biopsies were positive for EphA4 staining. In addition, EphA4 was predominantly expressed in the cytoplasm and cell membranes in tonsil biopsies. On the other hand, 13 of 14 cases (92.9%) of PTLD were negative for EphA4 expression (Figure 6 and Table 1). In addition, LMP1 was responsible for downregulation of EphA4 in our studies (Figure 2-4). Therefore, the expression levels of LMP1 were measured in EBER-positive PTLD biopsies by IHC assay and 12 of 14 cases (85.7%) of PTLD were positive for LMP1 staining (Figure 6 and Table 1).

A reverse correlation between EphA4 and the survival rate of EBV-positive DLBCL is revealed

According to 2016 WHO classification, EBV-positive DLBCL of the elderly, which is equivalent to EBV-positive DLBCL, not otherwise specified (NOS), displays a similar EBV viral protein expression as LCL. To confirm that EphA4 expression is correlated with EBV infection and LMP1 expression, 16 EBER-positive DLBCL and 11 EBER-negative DLBCL biopsies from patients without HIV infection or any iatrogenic immunosuppression were examined. The IHC assay indicated that all cases of EBV-negative DLBCL biopsies were positive for EphA4 staining (Figure 7A and Table 2). In contrast, 10 of 16 cases (62.5%) of EBV-positive DLBCL were negative
for EphA4 expression, three showed very weak, positive signals and three were
stained positively (Table 7A and Table 2). That the presence of EBV was associated
with reduced EphA4 expression was statistically significant (Table 2, \( p=0.0011 \),
Fisher’s extract test). On the other hand, 12 of 15 cases (80%) of EBV-positive
DLBCL were positive for LMP1 staining (Table 7A and Table 1). LMP1 expression
was also inversely correlated with EphA4 expression (Table 2, \( p=0.0375 \), Fisher’s
extract test). Next, we asked whether there was correlation between EphA4
expression and patients’ survival. So, we analyzed the survival data of patients with
DLBCL from GEO datasets (accession number GSE4475). The profile of overall
survival in patients was used to estimate whether patients with low EphA4 expression
have worse prognosis. Based on the median expression value of EphA4, 123 patients
were grouped into two clusters; 60 patients had high EphA4 and 63 patients had low
EphA4. Patients with low EphA4 had worse overall survival (Figure 7B, \( p=0.0414 \)).
Our results indicated that low EphA4 expression may potentially contribute to a poor
prognosis for these DLBCL patients.
Discussion

Physiologically, Eph receptors and their ligands are critical for many developmental processes. Pathologically, the Eph family is dysregulated in a variety of human diseases, especially cancers. During cancer formation, Eph can function in tumor suppression or tumor promotion, depending on the type of cancer, the interacting ligands and cross-talk with other RTKs. Among them, EphA2 is a good example. Limited expression of EphA2 is required for mammary gland development but its overexpression may enhance the malignancy of breast cancer\(^35\). In brain tumors, the presence of ephrin-A1 determines the direction of a reciprocal loop between EphA2 and Akt, which results in inhibition or promotion of the tumor grade\(^36\). In addition to neuronal development, EphA4 has been found to be involved in tumor progression\(^18,19,21,22,37,38\). For example, EphA4 is upregulated in colon cancer with liver metastasis\(^22\). In invasive cervical carcinoma, EphA4 expression is decreased due to chromosomal deletions, with loss of heterozygosity\(^21\). EphA4 mRNA is downregulated or lost in metastatic melanoma\(^37\). The regulatory mechanisms of EphA4 mRNA expression have been explored in terms of transcription. In the cervical cancer cell line HeLa and U373 glioma cells, EphA4 is reduced due to mRNA instability through regulation of its 3’untranslated regions by HuR\(^39\). In hepatocellular carcinoma, microRNA-10a targets EphA4 mRNA and overexpression of EphA4
inhibits cell migration and invasion\textsuperscript{40}. Moreover, a Pax3/FKHR oncogenic fusion protein has been shown to bind directly to the promoter region of EphA4, thus increasing EphA4 transcription in SaOS-2 cells\textsuperscript{41}. Furthermore, DNA methylation of EphA4 is observed in acute lymphoblastic leukemia\textsuperscript{42}. Similar with the transcriptional regulation mentioned above, our results showed that EBV LMP1 inhibits EphA4 promoter activity through Sp1 (Figure 4).

Sp1, which is expressed ubiquitously, can serve as a transactivator or repressor to regulate the promoter activities of target genes via phosphorylation\textsuperscript{43}. For example, FGF-2 stimulation enhances Sp1 binding to repress the promoter of platelet-derived growth factor receptor-alpha (PDGFR-\(\alpha\)) in smooth muscle cells through ERK mediated phosphorylation of Sp1 at T453 and T739\textsuperscript{44}. HER2 also has been reported to suppress the RECK promoter by ERK-mediated Sp1 phosphorylation\textsuperscript{45}. In this study, LMP1 activated the ERK pathway to inhibit the EphA4 promoter through Sp1 binding (Figure 4). We provide this new insight into how a viral oncoprotein can regulate Eph expression.

The function of EphA4 associated with cancer progression is controversial. As a tumor suppressor, EphA4 downregulates ERK phosphorylation to inhibit migration and invasion in non-small cell lung cancer cells\textsuperscript{38}. However, EphA4 can act as tumor promoter; for example, EphA4 interacts directly with FGFR1 through its cytoplasmic
domains, phosphorylates the docking protein, FRS2α, and enhances cell migration and proliferation\(^{46,47}\). In our case, EphA4 prevents LCL from proliferation (Figure 5), suggesting that it probably plays an inhibitory role in EBV-positive B lymphoproliferative disorders. This is confirmed by the IHC results of PTLD and DLBCL (Figure 6 and 7A). In this study, expression of EphA4 was undetectable in the cells of EBER-positive PTLD biopsies (Figure 6 and Table 1). Because the incidence of EBV in PTLD cases is over 90%\(^2\), these data imply that EphA4 is involved in the pathogenesis of EBV-positive PTLD.

In addition to PTLD, our results indicated that cells in EBV-positive DLBCL biopsies exhibited lower expression levels of EphA4 than those in EBV-negative biopsies (Figure 7A and Table 2), verifying the inverse correlation of EphA4 and EBV infection in DLBCL patients. It has been reported that patients with EBV-positive DLBCL have worse overall survival and progression-free survival than their EBV-negative counterparts\(^{34,48}\), yet the underlying mechanism has not been fully understood. Here we provided observation to show that low EphA4 correlated with poor survival outcome for DLBCL (Figure 7B). Also, some EphA4 ligands, such as ephrin-A1, A2, A3, A4 and B1 were downregulated post-EBV infection (data not shown). The results of overexpression of EphA4 in LCL indicated that EphA4 prevention of B cell proliferation may be ligand-independent.
Taken together, LMP1-mediated EphA4 repression accelerates B cell proliferation post-EBV infection. Our clinical results suggest that downregulation of EphA4 in patients with PTLD and DLBCL may provide new insights into pathogenesis and a poor prognostic marker for EBV-associated B cell malignancies. EphA4 may be considered as a potential therapy target for PTLD and DLBCL.
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Authorship Contributions

Y.-C.H designed experiments, performed experiments, analyzed the data and co-wrote the manuscript; S.-J.L designed experiments and co-wrote the manuscript; K.-M.L performed experiments; Y.-C.C performed experiments and analyzed the data; C.-W.L provided materials; S.-C.Y provided materials and analyzed the data; C.-L.C provided materials and analyzed the data; T.-L.S provided materials; J.L performed experiments; C.-K.C provided materials and analyzed the data; M.-R.C provided materials and C.-H.T designed experiments and co-wrote the manuscript.

Conflict of Interest Disclosures

The authors declare no competing financial interests.
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### Table 1
EBV products and EphA4 in tissue biopsies

| Case   | EBER | | | LMP1* | | | EphA4 | | | **p**-value as determined by Fisher’s exact test. **Statistically significant.** |
|--------|------|---|---|------|---|---|------|---|---|---|---|---|---|---|
|        | Case | Negative | Positive | Negative | Positive | Negative | Positive | **p**-value as determined by Fisher’s exact test. **Statistically significant.** |
|        |      | 5/5      | 0/5      | 5/5      | 0/5      | 0/5      | 5/5      | **p**-value as determined by Fisher’s exact test. **Statistically significant.** |
| Tonsil | 5    | 5/5      | 0/5      | 5/5      | 0/5      | 0/5      | 5/5      | **p**-value as determined by Fisher’s exact test. **Statistically significant.** |
| PTLD   | 14   | 0/14     | 14/14    | 2/14     | 12/14    | 13/14    | 1/14     | **p**-value as determined by Fisher’s exact test. **Statistically significant.** |
| DLBCL  | 27   | 11/27    | 16/27    | 14/26    | 12/26    | 10/27    | 17/27    | **p**-value as determined by Fisher’s exact test. **Statistically significant.** |

*One case of LMP1 status was not determined in EBER-positive DLBCL.

### Table 2
Relationship between EphA4 and EBV in DLBCL

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<th>Negative</th>
<th><strong>p</strong>-value as determined by Fisher’s exact test. <strong>Statistically significant.</strong></th>
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*One case of LMP1 status was not determined in EBER-positive DLBCL.
Figure Legends

Figure 1. EphA4 expression is decreased post-EBV infection.

(A) Total RNAs were harvested from uninfected primary B cells, from peripheral blood, and EBV-immortalized LCLs from three different donors. cDNAs were generated using \[^{33}P\]-labeled degenerate primers for PTKs. EphA4-specific cDNAs were digested by three restriction enzymes, BstNI, HhaI and MnlI. Relative fold EphA4 expression was compared to primary B cells. (B-C) Peripheral CD19-positive B cells were seeded at 1×10^6 cells/mL and infected with EBV strain B95.8. Total RNAs and protein were harvested from primary B cells at the days indicated post-EBV infection. (B) Expression levels of EphA4 mRNA were measured by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control MAGOH and standardized with uninfected primary B cells. (C) EphA4, EBNA1, LMP1 and β-actin proteins were measured by Western blotting. EphA4 protein relative folds were normalized to internal control β-actin and compared with uninfected primary B cells. (D) Total RNAs were extracted from primary B cells, EBV infection or B-cell stimulations including anti-CD40 antibody plus IL-4, LPS or poly I:C for 3 days. EphA4 transcripts were measured by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control MAGOH and standardized with uninfected primary B cells. (E) Total RNAs were extracted from primary B cells and
eight LCL lines. Expression levels of EphA4 mRNA were measured by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control MAGOH and standardized with uninfected primary B cells. (F-G) Total RNAs and protein were harvested from paired uninfected B cells and LCLs generated from the peripheral blood mononuclear cells of two healthy donors. (F) Expression levels of EphA4 mRNA were measured by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control MAGOH and standardized with uninfected primary B cells. (G) EphA4, EBNA1, LMP1 and β-actin proteins were measured by Western blotting. EphA4 protein relative folds were normalized to β-actin and compared with uninfected primary B cells.

**Figure 2. EphA4 is downregulated by LMP1.**

(A-H) EBV-negative TW01 cells were transfected with plasmids harboring the EBV viral genes LMP1, Zta, EBNA2, EBER1, EBER2 and BARF0. Total RNAs and protein lysates were obtained from each transfectant at day 3 post transfection. (A, C, E, G) EphA4 transcripts were detected by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control GAPDH and then standardized with vector controls. (upper panels of B, D, F, H) Total proteins were harvested from the vector control and each transfectant. EphA4 protein relative folds were normalized to internal control β-actin and standardized with vector controls. Expression levels of LMP1, Zta,
EBNA2 and β-actin protein were estimated by Western blotting. (lower panels of F and H) The *EBER1, EBER2* *BARF0* and β-actin transcripts were analyzed by RT-PCR. (I and J) EBV-negative BJAB cells were infected with LMP1 expressing lentivirus at MOI 0.5 or 1. The expression levels of EphA4 mRNA were determined by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control GAPDH and then standardized with the pSIN vector control. (J) EphA4, LMP1 and β-actin were detected by Western blotting. EphA4 protein relative folds were normalized to internal control β-actin and then standardized with pSIN vector control. (K and L) Knockdown of LMP1 in LCLs was performed by lentiviral transduction at the MOI of 1 for 5 days and infected cells were selected with 2 μg/mL puromycin for 2 days. (K) Expression levels of EphA4 mRNA were measured by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control GAPDH and then standardized with vector control shLuc. (L) EphA4, LMP1 and β-actin were determined by Western blotting. EphA4 protein relative folds were normalized to β-actin and then standardized with vector control shLuc. (*, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001, Student’s *t* test).

**Figure 3.** The ERK pathway is critical for LMP1-mediation of EphA4 downregulation.

(A) BJAB cells were infected with pSIN, LMP1, ΔCTAR1, ΔCTAR2 or ΔCTAR1/2 expressing lentiviruses for 5 days. EphA4, LMP1 and β-actin were detected by
Western blotting. EphA4 protein relative folds were normalized to β-actin and standardized with pSIN vector control. (B) LCLs were treated with 20 μM of PD98059, SP600125, LY294002 or 2.5 μM of Bay11-7082 for 48 hours. EphA4, phosphorylated (p-) and total (t-) proteins of ERK, JNK, Akt and IkBα were determined by Western blotting. β-actin served as an internal control. EphA4 protein relative folds were normalized to β-actin and standardized with DMSO solvent control. (C) Knockdown of ERK1/2 in LCLs achieved using a lentivirus expressing shERK1 plus shERK2 for 3 days. Infected cells were selected with 2 μg/mL puromycin for 2 days. EphA4, phospho-ERK, total ERK proteins were then determined by Western blotting. EphA4 protein relative folds were normalized to β-actin and standardized with vector control shLuc.

Figure 4. Sp1 is the key suppressor of LMP1-hampered EphA4 promoter activity.

(A) LCLs were infected with an shSp1 expressing lentivirus for 5 days and the infected cells were selected with 2 μg/mL puromycin for 2 days. EphA4, Sp1 and β-actin expression levels were detected by Western blotting. EphA4 protein relative folds were normalized to β-actin and standardized with vector control shLuc. (B) BJAB cells were infected simultaneously with LMP1 and shSp1 expressing lentiviruses for 3 days and infected cells were selected with 2 μg/mL puromycin for 2
days. EphA4, Sp1, LMP1 and β-actin were determined by Western blotting. EphA4 protein relative folds were normalized to β-actin and standardized with pSIN plus shLuc controls. (C) LCLs were co-infected with shSp1 and GFP-tagged \textit{EPHA4} promoter (-1000~+42)-expressing lentiviruses for 3 days and then the infected cells were selected with 2 µg/mL puromycin for 2 days. EphA4 relative luciferase activity was first normalized to GFP, followed by standardization with the vector pCDHGL3 (**, \( p < 0.001 \), Student’s \( t \) test). Sp1, LMP1 and internal control β-actin were analyzed by Western blotting (lower panel). (D) TW01 cells were infected with an shSp1-expressing lentivirus for 3 days and the infected cells were selected with 2 µg/mL puromycin for 2 days. The Sp1-knockdown TW01 cells were co-transfected with LMP1 plasmid or its vector control pSG5, combined with reporter plasmids \textit{EPHA4} promoter (-1000~+42) or vector control pGL3 and internal control pEGFPC1 plasmids for 2 days. EphA4 relative luciferase activity was first normalized to GFP, followed by standardization with the control vector pGL3 (**, \( p < 0.01 \), Student’s \( t \) test). Expression of Sp1, LMP1 and β-actin proteins was analyzed by Western blotting (lower panel). (E) TW01 cells were co-transfected with LMP1 or pSG5 plasmids, reporter plasmids of pGL3 vector control or \textit{EPHA4} promoter (-1000~+42) and internal control pEGFPC1 plasmids. 24 hours post-transfection, 500 nM mithramycin was added to the cells for another 24 hours. EphA4 relative luciferase activity was
determined as described above (*, \( p < 0.05 \), Student’s \( t \) test). Expression levels of LMP1 and β-actin were measured by Western blotting (lower panel). (F) BJAB cells were transduced with pSIN or LMP1 expressing lentiviruses for 5 days and a ChIP assay was performed as described previously. DNA-protein complexes were immunoprecipitated using anti-Sp1 Ab or isotype control rabbit IgG. \( EP HA 4 \) promoter and control \( GAP DH \) promoter DNA were detected in the immunoprecipitates by PCR. Total DNA was harvested from BJAB cells and used as the input control.

**Figure 5. The EphA4 JM domain is required for preventing LCL proliferation.**

(A) Flag-tagged EphA4 WT, KD (kinase dead with V653M) and 2M (JM region mutant with two tyrosine auto-phosphorylation sites Y569F and Y602F) expression plasmids for lentivirus packaging were illustrated. (B-C) LCLs from two donors were infected with EphA4 WT, mutants or vector control expressing lentiviruses for 2 days and then reseeded at 1×10^4 cells per well in 96 well plates for 5 days. Cell proliferation assays were measured by AlamarBlue reduction. Relative folds of proliferation were standardized with vector controls (*, \( p < 0.05 \); **, \( p < 0.01 \), Student’s \( t \) test). Total proteins were obtained from primary B cells and LCLs expressing WT and mutant forms of EphA4 at day 5 post reseeding. EphA4, LMP1 and β-actin expression levels were detected by Western blotting (right panel). β-actin
served as an internal control.

**Figure 6. Expression of EphA4 in PTLD biopsies.**

Paraffin-embedded PTLD and tonsils sections were subjected to IHC assays and hematoxylin was used for the nuclear counterstaining. Positive signals of EphA4 were indicated as a brown color in tonsil biopsies but not in PTLD biopsies. LMP1 expression was also detected as a brown color by IHC assay in PTLD biopsies. The nuclei of the cells are colored blue. Magnification x200 (Scale bar 50 μm).

**Figure 7. Detection of EphA4 expression in DLBCL biopsies.**

(A) Paraffin-embedded DLBCL sections were stained for EphA4. Positive signals of EphA4 could be seen as a brown color in EBV-negative DLBCL, but not in EBV-positive DLBCL biopsies, by IHC. LMP1 expression was also detected in EBV-positive DLBCL biopsies. The nuclei were observed as a blue color and hematoxylin was used for the nuclear counterstain. Magnification x200 (Scale bar 50 μm). (B) This survival curve of DLBCL was obtained from the GEO datasets. Patients were divided into high (n=60) and low EphA4 (n=63) groups, according to the median expression level of EphA4. A Kaplan-Meier plot showed that patients with low EphA4 had worse overall survival ($p =0.0414$, Log-rank test).
Figure 4

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Figure 5

A

EphA4 (WT)

1

JM (570-620)

TM

Kinase domain

Flag

EphA4 (KD)

1

KD

Y635M

Kinase domain

Flag

EphA4 (2M)

1

Y596F

Y602F

Kinase domain

Flag

B

Relative fold of reduced AlamarBlue

0 1 2 3 4 5 Day

LCL-48

Vector

WT

KD

2M

C

Relative fold of reduced AlamarBlue

0 1 2 3 4 5 Day

LCL-49

Vector

WT

KD

2M

primary B cells

EphA4

LMP1

β-actin

primary B cells

EphA4

LMP1

β-actin
Figure 6

Case 1

Tonsil

EphA4

Case 2

PTLD

EphA4

LMP1
Figure 7

A

Case 1

EBV- DLBCL

EphA4

Case 2

EBV+ DLBCL

LMP1

B

GSE4475

Overall Survival (%)

100
50
0
0 50 100 150 200 250 Months

Log-rank test

EphA4 high (n=60)

EphA4 low (n=63)

ρ = 0.0414
Regulation of EBV LMP1-triggered EphA4 downregulation in EBV-associated B lymphoma and its impact on patients’ survival

Ya-Chi Huang, Sue-Jane Lin, Kai-Min Lin, Ya-Ching Chou, Chung-Wu Lin, Shan-Chi Yu, Chi-Long Chen, Tang-Long Shen, Chi-Kuan Chen, Jean Lu, Mei-Ru Chen and Ching-Hwa Tsai