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Differential Signaling Pathways Are Activated in the Epstein-Barr Virus-Associated Malignancies Nasopharyngeal Carcinoma and Hodgkin Lymphoma

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ABSTRACT

EBV is associated with the epithelial cancer, nasopharyngeal carcinoma (NPC), and the lymphoid malignancy, Hodgkin lymphoma (HL). The EBV latent membrane proteins 1 and 2A are expressed in these tumors. These proteins activate the phosphatidylinositol 3′-OH kinase (PI3K)/Akt pathway, which is commonly activated inappropriately in malignancy. In this study, the status of Akt activation and its targets, glycogen synthase kinase-3β (GSK-3β) and β-catenin, was investigated in NPC and HL clinical specimens. In the majority of HL and NPC specimens, Akt was activated, indicating an important role for this kinase in the development and/or progression of these tumors. Akt phosphorylates and inactivates GSK-3β, a negative regulator of the proto-oncoprotein β-catenin that is aberrantly activated in many cancers. GSK-3β was phosphorylated and inactivated with concomitant nuclear β-catenin accumulation in the majority of NPC specimens. The malignant cells of the majority of HL cases, however, did not have inactivated GSK-3β and lacked nuclear β-catenin expression. These data indicate that this signaling arm of PI3K/Akt is universal and important in NPC pathogenesis but is apparently not affected in HL. These findings point to a divergence in pathways activated by EBV in different cellular contexts.

INTRODUCTION

EBV is a persistent and widespread pathogen, infecting >90% of the adult human population. Primary infection typically occurs during childhood via salivary exchange; however, infection during adolescence or early adulthood is common and often results in infectious mononucleosis. As with all herpesviruses, once a host is infected, the infection remains throughout his or her life. EBV infection is usually asymptomatic; however, infection with the virus is associated with development of several benign or malignant diseases such as oral hairy leukoplakia and various types of lymphomas and carcinomas (1, 2).

EBV primarily infects B lymphocytes and epithelial cells and may give rise to either lytic or latent forms of infection (1, 2). Lytic infection can occur in B cells or epithelial cells and results in viral DNA replication with the assembly and release of progeny virions. Latent infection is typically associated with infection of B cells and is characterized by limited viral gene expression. Latent EBV infection is additionally divided based on viral expression profiles into three types: types I, II, and III. In type I latency, which is typical of Burkitt's lymphoma, the only viral gene product expressed is EBV nuclear antigen (EBNA) 1 in addition to the transcription of EBV-encoded RNAs (EBERs) and the *Bam*HI-A rightward transcripts. Type II latency is found in nasopharyngeal carcinoma (NPC) and Hodgkin lymphoma (HL) and is characterized by expression of EBNA1, latent membrane proteins (LMPs) 1, 2A, and 2B, and the EBERs and *Bam*HI-A rightward transcripts. In type III latency in lymphoblastoid cell lines transformed with EBV *in vitro* or in post-transplant lymphoproliferative disease *in vivo*, the EBERs, EBNAs 1, 2, 3A, 3B, 3C, and LP, *Bam*HI-A rightward transcripts, and LMP1, 2A, and 2B are expressed.

Although many studies have been conducted investigating the effects of EBV latent proteins, the underlying pathogenesis for most EBV-related cancers has not been elucidated. LMP1 is considered to be an EBV oncoprotein and can transform rodent fibroblasts *in vitro* and induce lymphomas in transgenic mice expressing LMP1 under the control of the immunoglobulin heavy chain promoter (1, 3, 4). LMP2A has been shown to confer oncogenic capacity when expressed in the epithelial cell line HaCaT (5), although it is not required for the immortalization of B lymphocytes by the virus *in vitro*. However, LMP2A does confer a survival advantage for lymphocytes in transgenic mice (6, 7). EBNA2 is essential for B-cell immortalization and can activate transcription of several viral and cellular genes. However, EBNA2 is not expressed in malignancies with type II latency expression such as NPC and HL, implying a more prominent role for the LMPs in the genesis and/or progression of these cancers.

NPC is an epithelial cancer, the histology of which ranges from well-differentiated, keratinizing squamous cell carcinoma to undifferentiated, nonkeratinizing carcinoma (8, 9). The undifferentiated variety of NPC is invariably associated with EBV, as determined via detection of the EBERs by *in situ* hybridization. The tumor cells contain clonal viral genomes, indicating that EBV infection occurred at an early stage of tumor development, and express EBV gene products with a type II latency pattern (10). Although NPC is rare in the United States and Western Europe, it is endemic to other parts of the world, including Southern China and parts of Southeast Asia. The tumor is comprised of malignant, EBV-infected epithelial cells surrounded by reactive lymphocytes.

As with NPC, the EBV-associated lymphoma HL has a type II latency expression profile. However, unlike NPC, only an estimated 40–60% of HL cases are linked to EBV (*i.e.*, EBER-positive by *in situ* hybridization). In some developing countries where the age of primary EBV infection is younger, the percentage of HL cases associated with EBV approaches 100% (9, 11). HL is an unusual malignancy in which the malignant cells, termed Hodgkin/Reed-Sternberg (HRS) cells, comprise a minority of the total cell population of the tumor, usually <1%. These cells appear as atypical immunoblasts or polylobated giant cells with prominent nucleoli and marginated heterochromatin. The cytoplasm is abundant, and these cells are derived from germinal center B cells. The scarce HRS cells are embedded in a background of reactive lymphoid stroma, including B and T lymphocytes, plasma cells, eosinophils, and granulocytes. As with NPC, the EBV-infected malignant cells contain clonal viral genomes, implying that infection occurred early in tumor development (9, 12, 13). Classical HL is divided into three subtypes: nodular sclerosis, mixed cellularity, and lymphocyte-depleted. These variants differ in their association with EBV, histology, and prognosis. Nodular sclerosis HL has a good prognosis and comprises 60–70% of cases, and roughly 20% of these are EBV infected (11). Lymphocyte-depleted HL has the poorest prognosis but only constitutes ~5–10% of HL cases (11). Almost all cases of the lymphocyte-depleted HL subtype are EBV

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associated. Mixed cellularity HL has an intermediate prognosis and comprises ~20–30% of cases, of which, 70% are EBV-infected (11).

Virologic studies of NPC and HL suggest a critical role for EBV infection in the development of these malignancies. It is important to determine the effects of expression of viral LMP1 and LMP2A in these diseases. The signaling pathways activated by LMP1 and LMP2A are likely important for the contribution of EBV to disease pathogenesis. Both of these viral proteins have properties that could contribute to the formation and/or progression of malignancies. Both LMP1 and LMP2A can activate phosphatidylinositol 3′-OH kinase (PI3K) and its target Akt in epithelial cells. LMP2A has been shown to activate PI3K and Akt in B lymphocytes as well (5, 14, 15). PI3K is a phospholipid kinase that phosphorylates phosphoinositides at the 3′-position. These 3′-phosphoinositides recruit signaling molecules to the plasma membrane where they ultimately become activated. One of the effectors activated as a result of PI3K signaling is the serine/threonine kinase Akt, which is activated after phosphorylation on Ser^{473} and Thr^{308} (16, 17). Activated Akt has many targets, including glycogen synthase kinase-3β (GSK-3β), Bad, and the Forkhead family of transcription factors. Through their various signaling effectors, activated PI3K and Akt affect cell cycle progression and cellular proliferation and inhibit apoptosis, thereby contributing to dysregulated cell growth control when inappropriately activated. The PI3K/Akt signaling pathway is activated in many types of cancers, including prostate and breast carcinomas, melanoma, and leukemia (17). Therefore, LMP1- and LMP2A-mediated activation of this pathway could provide a growth advantage and contribute to abnormal cellular proliferation in the context of an EBV-infected, transformed cell.

Recently, we have shown that LMP2A activates β-catenin signaling in epithelial cells (18), and activation of β-catenin has been detected in EBV-infected lymphoid cells with type III latency expression (19). Aberrant activation of Wnt/β-catenin signaling is central to many different cancers, including colon cancer in which this pathway is inappropriately activated in 90% of cases, hepatocellular carcinoma, medulloblastomas, prostate cancer, and skin cancer (20, 21). In canonical Wnt signaling, a Wnt ligand binds its receptor, leading to a series of events in the cytoplasm that ultimately leads to inactivation of GSK-3β and the β-catenin destruction complex. In an unstimulated cell, GSK-3β, in conjunction with adenomatous polyposis coli, axin, and casein kinase I, phosphorylates the proto-oncoprotein β-catenin and targets it for proteasomal degradation (22). This destruction complex functions constitutively to limit β-catenin accumulation and signaling in the cell. Inactivation of GSK-3β downstream of Wnt or other signals leads to β-catenin stabilization, cytoplasmic accumulation, and nuclear translocation. In the nucleus, β-catenin acts in concert with members of the T-cell factor/lymphoid enhancer factor transcription factor family to activate gene expression of targets such as c-*myc* and *CCND1* (cyclin D1; Refs. 23–25). Therefore, nuclear β-catenin expression is a hallmark of cancers with aberrant activation of this pathway.

The PI3K/Akt and Wnt/β-catenin pathways are activated in many types of cancers; therefore, activation of these pathways by EBV proteins could contribute to transformation and tumor development in the appropriate cellular context. In this study, the activation of these pathways in NPC and HL was determined via immunohistochemistry for activated Akt, inactivated GSK-3β, and β-catenin in primary NPC and HL tissues, as well as in two NPC xenografts passaged in nude mice. Akt was activated in the malignant cells of the majority of both NPC and HL specimens examined; however, GSK-3β inactivation and β-catenin expression and signaling were significant only in the NPC tissues. These data implicate the PI3K/Akt/GSK-3β/β-catenin pathway in the initiation and/or progression of NPC. It is likely that a distinct effector of PI3K/Akt is critical in HL carcinogenesis. This study identifies key differences in signaling and pathway activation in the pathogenesis of two different EBV-associated cancers with a similar viral expression profile. In disparate cellular contexts (*i.e.*, lymphoid *versus* epithelial), EBV proteins might signal differently and have distinct consequences.

MATERIALS AND METHODS

Tumor and Normal Tissue Specimens. The C15 and C17 human NPC xenografts were passaged in mice as described previously (26). The C15 xenograft was derived from a primary NPC and expresses the EBV oncoprotein LMP1. The C17 xenograft was derived from a NPC metastasis in a patient who had received chemotherapy and radiotherapy, and it does not express LMP1 and is deleted for *TP53* (27). After passaging in nude mice, the C15 and C17 tumors were embedded in paraffin or snap-frozen in liquid nitrogen. A specimen of normal nasopharynx was obtained from the Southern Division of the Cooperative Human Tissue Network. Paraffin-embedded sections of 13 primary and metastatic NPC specimens of 11 patients were obtained from the archives of the Subang Jaya Medical Centre in Malaysia. Paraffin-embedded sections of 47 HL specimens from 47 different patients were obtained from the archives at University of North Carolina Hospitals and the University of Texas Health Science Center at San Antonio.

Immunohistochemistry. Paraffin-embedded sections were deparaffinized in two 10-min treatments with Histoclear (National Diagnostics, Atlanta, GA) and rehydrated through 5-min washes in 100, 95, and 70% ethanol followed by Tris-buffered saline with 0.1% Tween 20. Antigen retrieval was performed by proteinase K (DAKO, Glostrup, Denmark) treatment for 3–6 min (phospho-Akt stains) or by steaming in 1× citrate buffer for 20 min (phospho-GSK-3β and β-catenin stains; Zymed Laboratories, San Francisco, CA). After blocking in 5% normal goat serum (Vector Laboratories, Burlingame, CA) in Tris-buffered saline with 0.1% Tween 20, the sections were incubated with primary antibodies diluted in 1–3% normal goat serum/Tris-buffered saline/0.1% Tween 20. A 1:100 dilution of phospho-Akt antibody (Cell Signaling, Beverly, MA) for 2–3 h at room temperature or a 1:50 dilution of phospho-GSK-3β (Cell Signaling) or β-catenin antibody (BD Biosciences, Mississauga, Ontario, Canada) was applied overnight at 4°C. After three washes, the sections were incubated with the EnVision polymer (DAKO) conjugated to alkaline phosphatase and antirabbit and antimouse antibodies for 30 min at room temperature. Negative controls to ensure staining specificity were incubated with rabbit immunoglobulin or mouse IgG_1 at the corresponding dilution. Sections were washed three times in Tris-buffered saline/0.1% Tween 20 and treated with 5-bromo-4-chloro-3-indoxyl phosphate/nitro blue tetrazolium substrate with levamisole (DAKO) for 30 min. Cells were counterstained with nuclear fast red (DAKO) for 1–3 min, dehydrated through 5-min washes in 70, 95, and 100% ethanol and Histoclear, mounted using Permount (Fisher Scientific, Pittsburgh, PA), and visualized via brightfield microscopy on a Zeiss Axioskop.

The C15 tumor served as a positive control for the HL sections because its expression and activation profiles were confirmed by immunoblot analysis. Positive and negative controls were included in parallel each time immunohistochemistry was performed. For scoring of the phospho-Akt and phospho-GSK-3β NPC stains, ++ indicates strong positive staining in the cytoplasm and/or nucleus; +, positive staining in cytoplasm and/or nucleus; +/−, weak positive staining; and −, no appreciable staining. For the β-catenin NPC immunohistochemistry, ++ indicates strong positive staining in the cytoplasm and the majority of nuclei; +, positive staining in the cytoplasm and many tumor nuclei; +/−, weak positive staining in cytoplasm and nuclei; and −, no appreciable nuclear staining. For scoring of the HL cases for phospho-Akt and phospho-GSK-3β, ++ indicates HRS cells stain strongly positive in cytoplasm and/or nucleus; +, most HRS cells positive; +/−, occasional positive HRS cell; and −, very few to no positive HRS cells. For the β-catenin HL immunohistochemistry, ++ indicates strong positive staining in the nucleus (plus or minus the cytoplasm) of most HRS cells; +, positive staining in nucleus (plus or minus the cytoplasm) of some HRS cells; +/−, occasional HRS cells with positive nuclear (with or without cytoplasmic) staining; and −, very few to no nuclear-positive HRS cells.

Preparation of Cell Lysates and Western Blot Analyses. Frozen normal tissue and tumor specimens were pulverized in a Braun Mikro-Dismembrator.

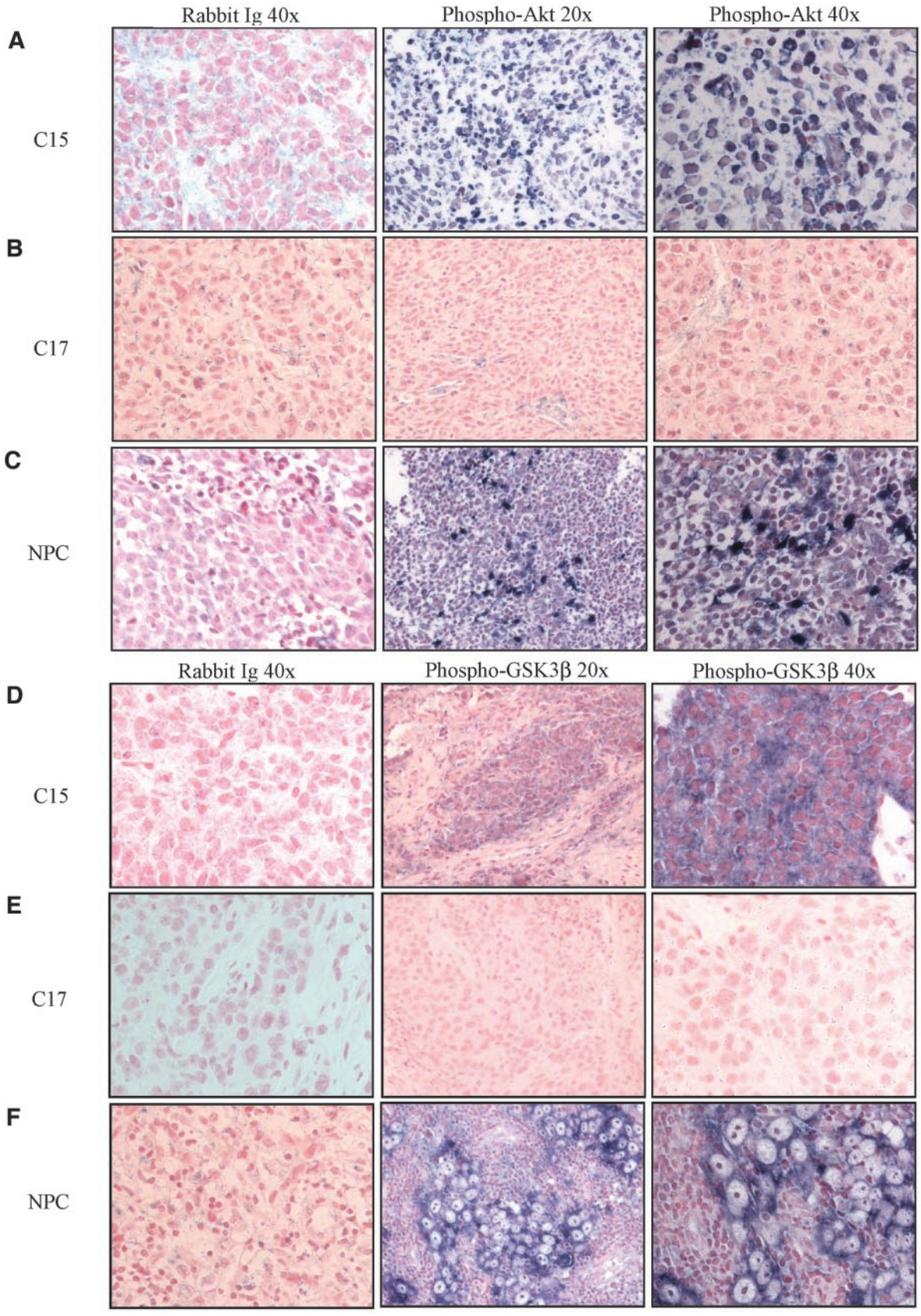

Fig. 1. The Akt signaling pathway is activated in nasopharyngeal carcinoma (NPC). Immunohistochemistry was performed on paraffin-embedded primary NPC tissues and NPC xenografts to detect phosphorylated Akt (*A–C*) and phosphorylated glycogen synthase kinase-3β (GSK-3β; *D–F*). A polyclonal rabbit antibody specific for the phosphorylated form of Akt was used to detect activated Akt in the C15 (*A*) and C17 (*B*) xenografts. Phospho-Akt staining of a representative primary NPC specimen is also shown (*C*). The specimens are shown at $\times$20 and $\times$40 magnifications, and positive staining is indicated by blue/purple coloration. Negative control staining was performed on each section using rabbit immunoglobulin (*left panels*) and is shown at $\times$40 magnification. A polyclonal rabbit antibody specific for the phosphorylated form of GSK-3β was used to detect inactivated GSK-3β in C15 (*D*), C17 (*E*), and a representative NPC specimen (*F*).

After washing in PBS (Invitrogen-Life Technologies, Inc., Carlsbad, CA), cells were lysed in NP40 lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP40, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and protease and phosphatase mixtures at 1:100 (Sigma-Aldrich, St. Louis, MO). Protein concentrations were determined with the Bio-Rad DC Protein Assay system (Bio-Rad, Hercules, CA), using a modification of the Lowry method. Lysates were mixed with SDS sample buffer, boiled for 5 min, and electrophoresed on a SDS-7.5% polyacrylamide gel (SDS-PAGE). Proteins were transferred to an Optitran nitrocellulose membrane (Schleicher & Schuell, Keene, NH) in a Bio-Rad transfer unit, and Western blot analysis was performed. Antibodies included anti-phospho-Akt Ser^{473} (1:500), anti-Akt (1:500), and anti-phospho-GSK-3β Ser^{9} (1:1000) from Cell Signaling, anti-β-catenin (1:500) from BD Biosciences, and anti-GRP78 (1:500) and anti-actin (1:200) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-tagged secondary antibodies against rabbit, mouse (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom), and goat (DAKO) antibodies were used at a 1:1000 dilution for 1 h, and antibody-bound proteins were detected using the Pierce SuperSignal West Pico System (Pierce, Rockford, IL). Staining of nitrocellulose membranes with Ponceau S (Sigma) was performed to assess levels of protein loading.

Cytosolic and Nuclear Fractionation of Cells. Pulverized normal and tumor tissue was washed in PBS, and fractionations were performed using OptiPrep (Sigma-Aldrich, St. Louis, MO), as adapted from the manufacturer's protocol. Briefly, cells were resuspended in Buffer A containing 20 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and protease and phosphatase inhibitor mixtures at 1:100 (Sigma-Aldrich) with 1% NP40. Crude nuclei were pelleted at low speed, and the cytosolic fractions were extracted. Nuclei were purified over an OptiPrep gradient and lysed with hypotonic NE buffer (20 mM Tris-HCl, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and protease and phosphatase inhibitor mixtures at 1:100). Protein concentrations of cytosolic and nuclear extracts were measured, and the extracts were subjected to SDS-PAGE and Western blotting as described above.

RESULTS

Akt and β-Catenin Signaling Are Activated in NPC. Because the EBV proteins LMP1 and LMP2A have been shown to activate Akt and LMP2A leads to inactivation of GSK-3β and subsequent β-catenin stabilization and nuclear translocation in epithelial cells (5, 14, 15, 18), it was of interest to investigate whether activation of these pathways occurs in the presence of these viral products in the EBV-associated epithelial malignancy NPC. Paraffin-embedded sections of the NPC xenografts C15 and C17, as well as primary NPC specimens from 11 Malaysian patients, were obtained. Immunohistochemistry was performed to detect activated Akt, inactivated GSK-3β, and β-catenin. There are phospho-specific antibodies readily available to detect the activated form of Akt that is phosphorylated at Thr^{308} or Ser^{473} and the inactivated form of GSK-3β, phosphorylated at Ser^{9}, in tissues by immunohistochemistry or immunoblot analysis. These reagents are specific to the phosphorylated forms of these proteins and do not react with the unphosphorylated forms of these kinases, which are located in the cytoplasm under normal, unstimulated conditions. Negative control staining with isotype controls was performed in parallel to ensure staining specificity, and the nuclei of negative cells were visualized by counterstaining and appeared pink (Fig. 1*A–C*, *left panels*).

Akt was phosphorylated and activated in the C15 NPC tumor (Fig. 1*A*, *middle and right panels*), with positive staining indicated in blue/purple. This was expected as the C15 tumor expresses both LMP1 and LMP2A, which are each capable of activating Akt (5, 14, 15, 18). The activated Akt was detected in the cytoplasm and nucleus of the tumor cells, and these findings are consistent with observations that activated Akt is located in the cytoplasm and also in the nucleus in many contexts (28). Furthermore, GSK-3β, a target of activated Akt, was phosphorylated and inactivated in the cytoplasm of C15 tumor cells (Fig. 1*D*). Unlike the C15 tumor, however, the C17 NPC xenograft lacked phosphorylation of Akt and GSK-3β (Fig. 1, *B* and *E*, respectively). In contrast to the C15 tumor derived from a primary NPC, the C17 tumor is derived from a NPC metastasis postradiation treatment and polychemotherapy, contains only three copies of the EBV genome/cell (compared with 30 for C15), and does not express LMP1 (26). Therefore, the C17 tumor is atypical in comparison to the EBV-associated primary NPC prototype C15.

To investigate the status of the Akt pathway in primary, EBV-associated NPC, similar immunohistochemical analyses were performed on 11 primary and 2 metastatic NPC specimens from Malaysian patients. Akt was activated in the cytoplasm and many nuclei of the tumor cells of a majority of NPC cases, and a representative specimen is shown (Fig. 1*C*). In accordance with Akt activation, its target GSK-3β was also phosphorylated and inactivated in the cytoplasm of a majority of the NPC cells (representative shown in Fig. 1*F*). It is noteworthy that pockets of infiltrating lymphocytes did not stain for phospho-Akt or phospho-GSK-3β (Fig. 1, *C* and *F*), indicating that activation of the Akt pathway was limited to the NPC tumor cells. The data from these immunohistochemical studies are summarized in Table 1. Of the 13 sections examined, 85% were positive for activated Akt, and 83% were positive for inactivated GSK-3β.

Table 1 *Results of immunohistochemical staining of primary NPC*[a]

NPC sample	Diagnosis	Phospho-Akt[b]	Phospho-GSK-3β[b]	β-Catenin[c]
NPC1	No histology available	+	++	++
NPC2	Metastatic undifferentiated carcinoma from NP	++	+	++
NPC3	Undifferentiated carcinoma of NP (WHO III)	+	++	+
NPC4	Nonkeratinizing carcinoma of NP (WHO II)	++	++	++
NPC5	Nonkeratinizing carcinoma of NP (WHO II)	+	++	++
NPC6	Nonkeratinizing carcinoma of NP (WHO II)	++	++	−
NPC7	Undifferentiated carcinoma of NP (WHO III)	+/−	−	++
NPC8	Undifferentiated carcinoma of NP (WHO III)	++	++	++
NPC9	Undifferentiated carcinoma of NP (WHO III)	−	+/−	+
NPC10	Nonkeratinizing carcinoma of NP (WHO III)	++	++	++
NPC11	Recurrent undifferentiated carcinoma (WHO III)	+/−	−	++
NPC12	Metastatic nonkeratinizing carcinoma from NP	++	+	++
NPC13	Nonkeratinizing carcinoma of NP	−	ND	++
C15 NPC	Xenograft of primary NPC; poorly differentiated	++	++	++
C17 NPC	Xenograft of metastatic NPC; poorly differentiated	−	−	−

[a] NPC, nasopharyngeal carcinoma; GSK-3β, glycogen synthase kinase-3β; NP, nasopharynx; ND, not determined.

[b] For phospho-Akt and phospho-GSK-3β staining, ++ indicates strong positive staining in cytoplasm and/or nucleus; +, positive staining in cytoplasm and/or nucleus; +/−, weak positive staining; and −, no staining.

[c] For β-catenin staining, ++ indicates strong positive staining in the cytoplasm and the majority of nuclei; +, positive staining in cytoplasm and many nuclei; +/−, weak positive staining in cytoplasm and nuclei; and −, no appreciable nuclear staining.

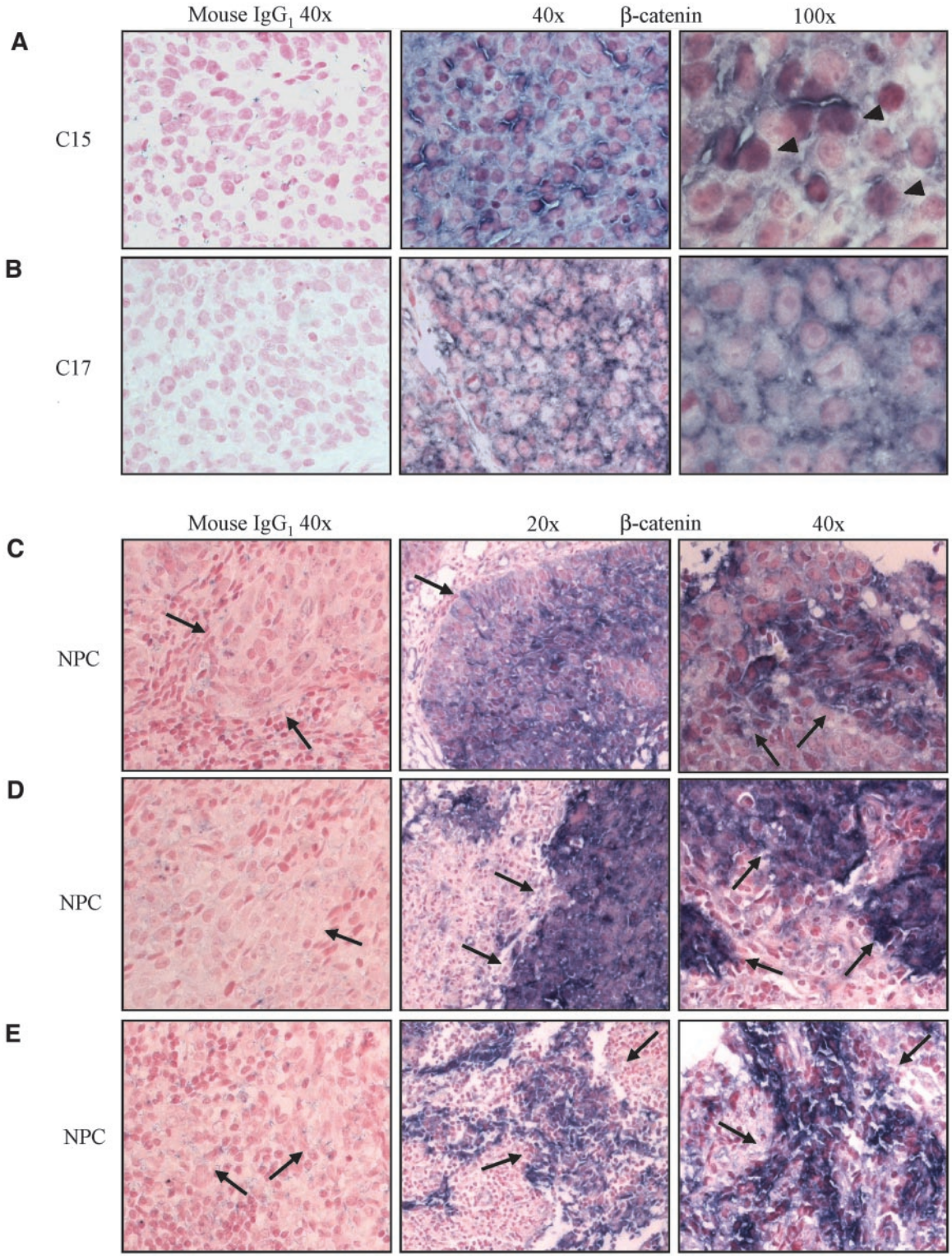

Fig. 2. The β-catenin signaling pathway is activated in primary nasopharyngeal carcinoma (NPC). Immunohistochemistry was performed on paraffin-embedded NPC xenografts and clinical NPC specimens to detect β-catenin expression. A mouse monoclonal antibody that recognizes β-catenin was used to detect β-catenin in the C15 and C17 xenografts (*A* and *B*, respectively). Negative control stains with mouse IgG1 are shown at ×40 magnification in the *left panels* (*A* and *B*). Positive staining is indicated by blue/purple coloration. The *arrowheads* depict tumor cells with nuclear β-catenin accumulation (*A*, *right panel*). Stains of representative primary NPC specimens are also shown (*C*, *D*, and *E*, respectively) at ×20 and ×40 magnification. *Arrows* indicate areas of tumor, which are embedded in a background of reactive lymphocytes.

In certain contexts upon inactivation of GSK-3β, β-catenin is stabilized, accumulates in the cytoplasm, and translocates into the nucleus to modulate transcription of its target genes. Activation of Akt and β-catenin by LMP2A has been demonstrated in normal human keratinocytes (18). Therefore, the status of β-catenin in NPC was investigated. In addition to phosphorylated Akt and GSK-3β, immunohistochemical staining of the C15 NPC tumor revealed abundant β-catenin (Fig. 2*A*). Nuclear β-catenin staining was detected in many

of the C15 cells, indicating that the β-catenin signaling pathway was activated (Fig. 2*A*, *right panel arrowheads*). Although β-catenin was detected heavily at the plasma membrane of C17 tumor cells, the nuclei were devoid of β-catenin (Fig. 2*B*), indicating that the β-catenin signaling pathway was not activated. Plasma membrane staining for β-catenin is typical for cells of epithelial origin because it is a normal component of adherens junctions. Tumor cells of the primary NPC specimens also contained abundant nuclear β-catenin (Fig. 2*C–E*). All sections except for one (92%) stained positively for nuclear β-catenin, with the vast majority of these staining very strongly (Table 1). The high prevalence for activation of this pathway in NPC suggests that this pathway contributes to the genesis and/or progression of this EBV-associated tumor.

To confirm the data from the C15 and C17 xenografts, whole cell, cytosolic, and nuclear extracts were prepared from frozen tumor tissue and subjected to immunoblot analyses. Activated Akt was readily detectable in the C15 tumor but not in the C17 tumor, although actin and total Akt levels were comparable (Fig. 3*A*). Likewise, C15 had a higher level of phosphorylated, inactivated GSK-3β in the cytosolic compartment compared with C17 and a normal tissue control (Fig. 3*B*). Both cytosolic and nuclear extracts of the tumors and a normal control were examined for β-catenin expression, and the C15 tumor contained much higher levels of both cytosolic and nuclear β-catenin compared with C17 and the normal control (Fig. 3*C*). GRP78 is an endoplasmic reticulum marker that was included in the analysis to ensure purity of the nuclear fractions. On a longer exposure, a band corresponding to GRP78 appeared in the cytosolic fraction of the normal nasopharynx control (data not shown). The lanes were equally loaded as assessed by staining of the nitrocellulose membrane with Ponceau S to detect protein levels (data not shown). These data confirm the immunohistochemistry results of the C15 and C17 tumors and reveal that Akt is activated with phosphorylation and inactivation of its target GSK-3β in the C15 tumor but not in C17. Furthermore, the C15 tumor but not the C17 tumor has β-catenin nuclear accumulation in the tumor cells, indicating that β-catenin signaling is activated.

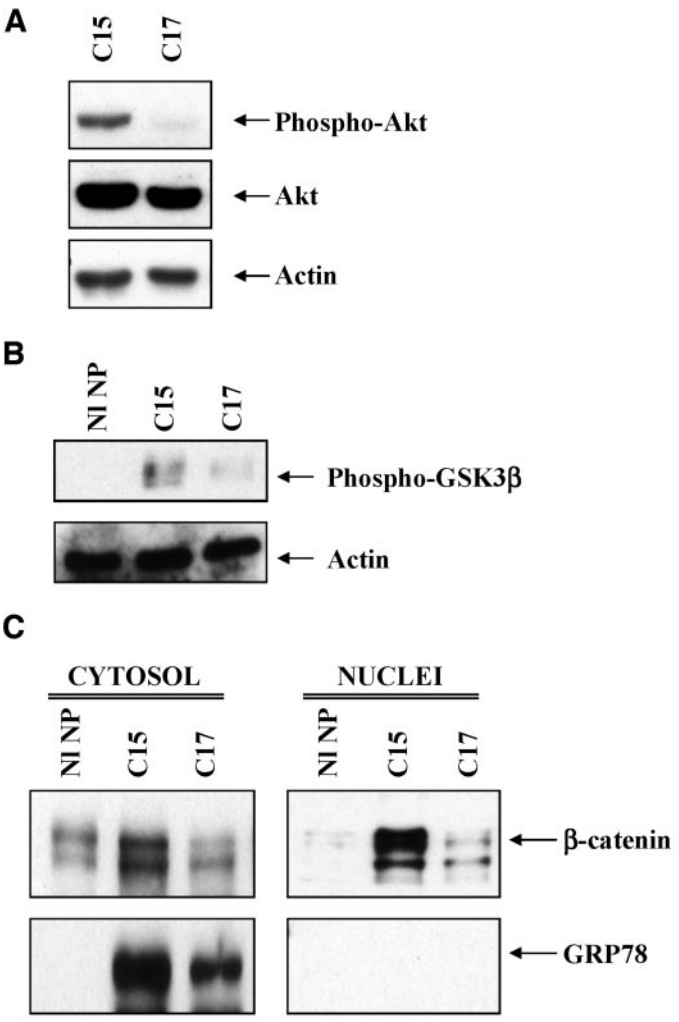

Fig. 3. The nasopharyngeal carcinoma (NPC) xenograft C15 has increased phosphorylation of Akt and glycogen synthase kinase-3β (GSK-3β) and enhanced cytosolic and nuclear β-catenin expression. *A*, immunoblot analysis was performed on whole cell lysates of the NPC xenografts C15 and C17 to detect activated phospho-Akt (*top panel*). The phospho-Akt blot was reprobed with an antibody against total Akt (*middle panel*), and an immunoblot to detect actin is included as a loading control (*bottom panel*). *B*, immunoblot analysis on cytosolic extracts of normal nasopharynx (Nl NP), C15, and C17 were performed to detect phosphorylated, inactivated GSK-3β (*top panel*). An immunoblot for actin is included as a loading control (*bottom panel*). *C*, cytosolic and nuclear extracts of normal nasopharynx, C15, and C17 were subjected to immunoblot analysis with an antibody against β-catenin (*top panels*). Immunoblot analysis with an antibody against the endoplasmic reticulum marker GRP78 was included to attest to the purity of the nuclear isolations (*bottom panel*).

Akt Is Activated in HL in the Absence of β-Catenin Signaling. Because of the abundance of Akt and β-catenin activation in NPC, it was of interest to study another EBV-associated malignancy, HL. EBV-associated HL, as with NPC, is characterized by a type II latency pattern expressing both LMP1 and LMP2A. Unlike NPC, however, the malignant cells of HL, the HRS cells, are of lymphoid origin. As is the case with epithelial cells, LMP2A has been shown to activate Akt in lymphoid cells (15), although the effects on GSK-3β were not investigated. A recent study reported activation of β-catenin signaling in a type III latency context and showed that there were elevated β-catenin levels with activation of a T-cell factor-responsive reporter in EBV-immortalized lymphoblastoid cell lines (19). To investigate the activation status of Akt, GSK-3β, and β-catenin in HL, paraffin-embedded tissues representing EBV-positive (as determined by EBER *in situ* hybridization; data not shown) and EBV-negative HL were examined by immunohistochemistry to detect phospho-Akt, phospho-GSK-3β, and β-catenin. Negative control staining for specificity was performed with rabbit and mouse immunoglobulins (Fig. 4*A*).

Phosphorylated, activated Akt was readily detectable in the cytoplasm and some nuclei of HRS cells in the majority of HL specimens. Staining was limited to the HRS cells, indicating that the reactive lymphocytes surrounding the rare HRS cells did not have activated Akt. The HRS cells can be distinguished from the reactive lymphocytes by their abundant cytoplasm and large, often polylobated nuclei with prominent nucleoli and marginated heterochromatin. Representative sections of EBER-positive HL of mixed cellularity, nodular sclerosis, and lymphocyte-depleted subtypes are shown (Fig. 4*B*). Although the majority of HL samples was positive for activated Akt in the HRS cells, not all cases were positive. One case of EBER-negative nodular sclerosis HL is shown in which the HRS tumor cells lacked Akt activation (Fig. *B, bottom right panel*). In total, 93% of EBER-positive HL cases had HRS cells containing activated, phosphorylated Akt (Table 2). The majority of EBER-negative HL cases were also positive for Akt signaling, although the proportion was somewhat lower at 67% (Table 2).

To determine whether the Akt target GSK-3β was also affected in HL, the phosphorylation status of this protein was investigated. Surprisingly, the vast majority of HL specimens were negative for phosphorylation of GSK-3β, indicating that GSK-3β had not been inactivated (Fig. 4*C*). The lack of GSK-3β phosphorylation was universal, regardless of EBV status, with 97% of EBER-positive cases and 94% of EBER-negative cases lacking GSK-3β phosphorylation (Table 2). These data reveal that, although Akt is activated in the HRS cells of

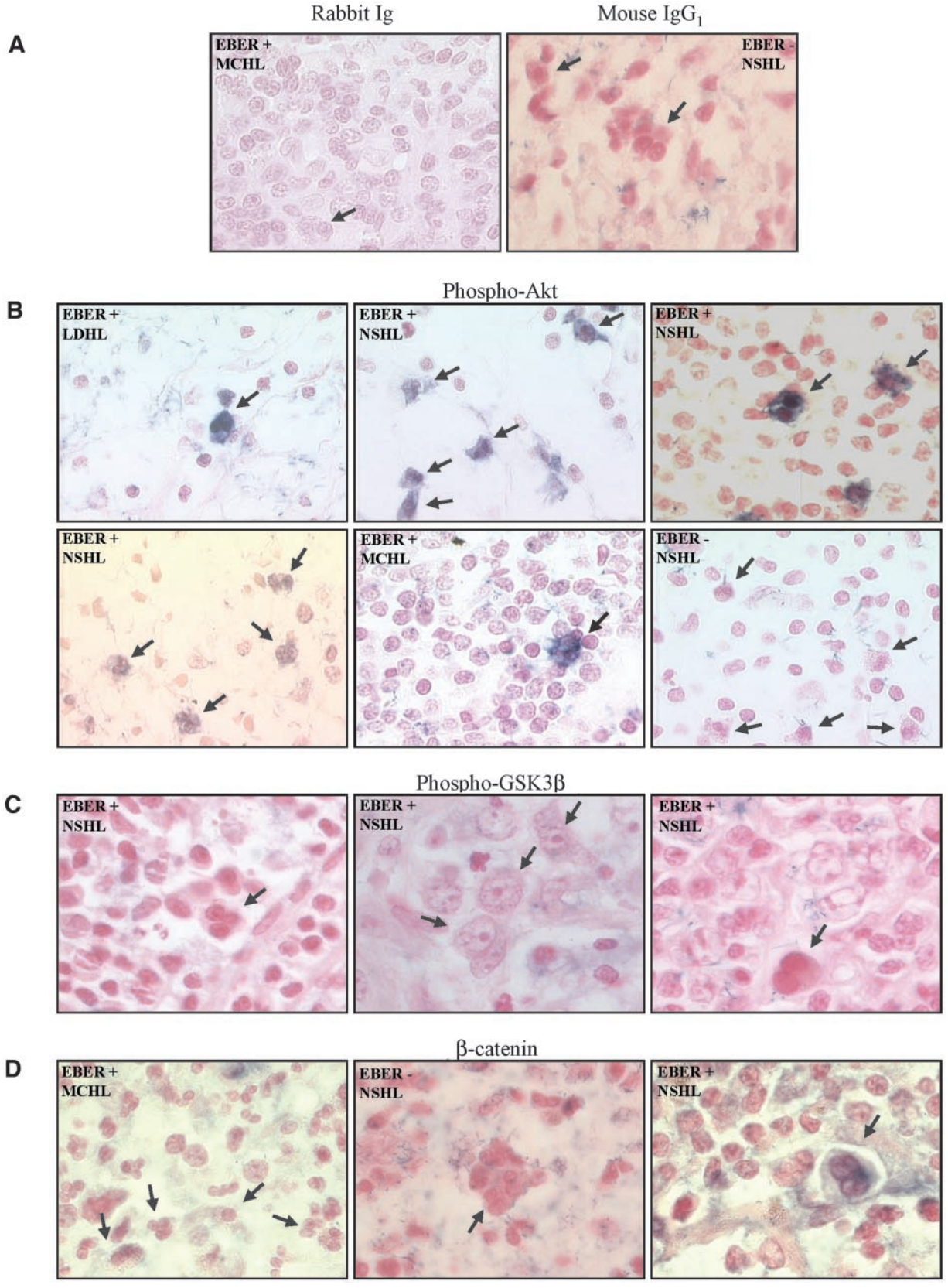

Fig. 4. Akt is activated in the Hodgkin/Reed-Sternberg cells of primary Hodgkin lymphoma (HL) in the absence of β-catenin pathway activation. Immunohistochemistry was performed on paraffin-embedded sections of primary HL tumors to detect activated phospho-Akt (*B*), inactivated phospho-glycogen, synthase kinase-3β (GSK-3β; *C*), and β-catenin expression (*D*). *A*, negative control staining was performed on sections of primary HL using rabbit immunoglobulin and mouse IgG_1. Primary HL sections were stained with a rabbit polyclonal antibody that recognizes phospho-Akt (*B*), a rabbit polyclonal antibody against phospho-GSK-3β (*C*), and a mouse monoclonal antibody that recognizes β-catenin (*D*). *Arrows* specify Hodgkin/Reed-Sternberg cells, and all panels are at ×100 magnification. Both EBV-encoded RNA (EBER)-positive and EBER-negative HL specimens as well as the classical subtypes mixed cellularity HL (MCHL), nodular sclerosis HL (NSHL), and lymphocyte-depleted HL (LDHL) are represented, and the sections are labeled to indicate EBER status and HL subtype.

Table 2 *Summary of immunohistochemical staining of primary HL sections*[a]

HL sample	Diagnosis	EBER status	Phospho-Akt[b]	Phospho-GSK-3β[b]	β-Catenin[c]
HL1	NSHL	−	++	−	−
HL2	NSHL	−	−	+/−	−
HL3	NSHL	−	−	−	−
HL4	MCHL	−	−	−	ND
HL5	NSHL	−	+/−	−	−
HL6	NSHL	−	−	−	−
HL7	NSHL	−	−	−	−
HL8	MCHL	−	+/−	−	ND
HL9	NSHL	−	+/−	−	−
HL10	NSHL	−	+/−	−	+/−
HL11	NSHL-lymphocyte rich	−	+/−	−	−
HL12	MCHL	+	+/−	−	+
HL13	LDHL-diffuse fibrosis	+	++	−	−
HL14	LDHL-diffuse fibrosis	+	+	−	−
HL15	NSHL	+	++	−	−
HL16	HL-classical NOS	+	++	−	−
HL17	LDHL-diffuse fibrosis	+	++	−	−
HL18	NSHL-interfollicular	+	++	−	−
HL19	HL- NOS	+	++	−	+/−
HL20	MCHL	+	+	−	−
HL21	NSHL	+	+	−	−
HL22	LDHL-diffuse fibrosis	+	++	−	ND
HL23	MCHL	+	++	−	−
HL24	NSHL	+	++	−	−
HL25	MCHL	+	++	−	ND
HL26	NSHL-lymphocyte depleted	+	−	−	−
HL27	HL-NOS	+	+	−	−
HL28	NSHL-fibroblastic	+	++	−	−
HL29	NSHL	+	++	−	−
HL30	NSHL-cellular phase	+	++	−	−
HL31	MCHL	+	++	−	−
HL32	LDHL	+	+/−	−	+/−
HL33	MCHL	+	+/−	−	−
HL34	MCHL	+	++	−	−
HL35	LDHL-diffuse fibrosis *versus* NSHL-fibroblastic	+	+	−	−
HL36	MCHL	+	+/−	−	−
HL37	HL-interfollicular	+	++	−	−
HL38	MCHL	+	++	+/−	ND
HL39	MCHL	+	−	−	+
HL40	MCHL	+	++	−	−
HL41	NSHL	−	++	−	ND
HL42	NSHL	−	++	−	−
HL43	NSHL-cellular phase	−	++	−	−
HL44	NSHL	−	−	ND	ND
HL45	NSHL-cellular phase	−	++	−	−
HL46	NSHL	−	++	−	−
HL47	NSHL-lymphocyte depleted	−	+	−	−

[a] HL, Hodgkin lymphoma; EBER, EBV-encoded RNA; GSK-3β, glycogen synthase kinase-3β; NSHL, nodular sclerosis Hodgkin lymphoma; MCHL, mixed cellularity Hodgkin lymphoma; ND, not determined; LDHL, lymphocyte-depleted Hodgkin lymphoma; NOS, nodular sclerosis.

[b] For phospho-Akt and phospho-GSK-3β staining, ++ indicates strong positive staining in cytoplasm or nucleus and/or many positive Hodgkin/Reed-Sternberg cells; +, positive staining in Hodgkin/Reed-Sternberg cells; +/−, occasional positive Hodgkin/Reed-Sternberg cells; and −, very few or absence of positive HRS cells.

[c] For β-catenin stains, ++ indicates strong nuclear staining of most HRS cells; +, positive nuclear staining in some HRS cells; +/−, occasional HRS cell with some nuclear staining; and −, very few or absence of positive HRS cells.

most HL cases, activated Akt does not target and inactivate GSK-3β in this context.

Consistent with a lack of GSK-3β inactivation, nuclear β-catenin was also absent in a majority of HL specimens (Fig. 4*D*). Of note, however, the EBER-positive HL cases had approximately twice the β-catenin positivity rate at 15% than the EBER-negative cases had at 7% (Table 2). A representative β-catenin-positive HRS cell, with some cytoplasmic and nuclear staining, is shown in an EBER-positive nodular sclerosis HL (Fig. 4*D*, *right panel*). These data indicate that β-catenin signaling may be active in the HRS cells of a small percentage of HL cases, but this pathway is unlikely to be critical in HL genesis or progression as the vast majority of HL cases investigated in this study were negative for β-catenin. Consistent with the low or absent expression level of β-catenin for mature lymphocytes (29), β-catenin was not detected in most of the lymphocytes in the stroma surrounding the HRS cells.

DISCUSSION

This study reveals differential pathway activation in two EBV-associated malignancies with a similar viral gene expression pattern. The Akt/GSK-3β/β-catenin pathway was activated in the NPC specimens investigated, whereas the HRS cells of most HL cases contained activated Akt in the absence of GSK-3β phosphorylation and nuclear β-catenin expression. These data imply that, in distinct cellular contexts such as lymphoid or epithelial origins, EBV infection may impact diverse signaling pathways and result in different effects. Such impacts are relevant to consider when trying to elucidate the mechanisms of pathogenesis of these EBV-related cancers.

Akt was activated in the malignant cells of the majority of HL and NPC cases, and this activation excluded the reactive lymphoid cells surrounding or infiltrating the tumors. The observed Akt activation is consistent with the expression of LMP1 and LMP2A in these types of tumors and the ability of these viral proteins to activate PI3K/Akt signaling. Interestingly, Akt was also activated in many EBER-negative HL, indicating that Akt activation might be critical in HL carcinogenesis and, therefore, may be activated through various mechanisms, viral or otherwise. The Akt target GSK-3β was also phosphorylated and inactivated in the majority of NPC specimens investigated. Consistent with the role of GSK-3β in β-catenin degradation, the majority of NPC sections also contained abundant nuclear β-catenin in the tumor cells because β-catenin is stabilized upon the inactivation of GSK-3β. These data implicate the PI3K/Akt and β-catenin pathways in the initiation and/or progression of NPC. Conversely, the HRS cells of HL lacked phosphorylation of GSK-3β and nuclear β-catenin expression in the majority of cases, indicating that this signaling pathway activated by Akt is not operative in these tumor cells and, therefore, is unlikely to play an important role in HL carcinogenesis.

This is the first analysis of β-catenin signaling in EBV-associated HL or NPC. A previous study identified loss of E-cadherin/β-catenin complexes at the cell junctions of tumor cells in primary and metastatic NPC with an incidental finding of increased nuclear β-catenin (30). Activation of β-catenin signaling in herpesvirus infections has been described. EBV LMP2A activates β-catenin signaling in epithelial cells (18), and β-catenin is up-regulated in some EBV-infected B cell lines with a type III latency expression profile possibly through effects with deubiquitinating enzymes (19). The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus also activates β-catenin signaling in primary effusion lymphoma cell lines by sequestering and inhibiting GSK-3β (31). In the current study, the abundant activation of β-catenin signaling found in the NPC specimens implicates this pathway in the genesis and/or progression of NPC. Although extensive β-catenin activation in HL specimens was not found in our study, other pathway effectors of LMP1, LMP2A, and PI3K/Akt are likely to be operative and contribute to the development of HL. In fact, microarray analyses conducted by Portis *et al.* (32) revealed that some LMP2A-induced gene expression alterations in mouse and human B cells are similar to those of HRS cells and suggests that LMP2A expression in B cells promotes an activated, proliferative environment in which HL might develop.

The finding that Akt is activated in HL is consistent with the reported importance of PI3K/Akt signaling in several lymphoid malignancies. Akt is activated in many cases of multiple myeloma, which is a malignancy derived from plasma cells, and also in a subset of anaplastic large-cell lymphomas (33–35). Furthermore, the TCL1 oncoprotein, which is overexpressed in several types of lymphomas,

including AIDS-related non-HL and follicular lymphomas, and B- and T-cell leukemias, binds to Akt and augments its activity (36–38). Importantly, this interaction is required for the survival and proliferation of the tumor cells. In addition, there have been studies reporting expression of β-catenin in leukemia and, to a lesser extent, some lymphomas (29, 39). In fact, a small number of T/natural killer lymphomas have been identified with β-catenin mutations (40, 41). However, activation of β-catenin signaling in HL has not been investigated, and our results reveal a lack of β-catenin activation in HL.

The finding of activation of PI3K/Akt and β-catenin signaling in EBV-associated cancers confers importance to the growing ranks of pathways affected by the EBV latent proteins. Another pathway that is activated in both HL and NPC is the nuclear factor-κB (NF-κB) pathway. NF-κB activation is required by HRS cells to proliferate and inhibit apoptosis (42). Furthermore, activation of NF-κB is essential for B-cell immortalization by EBV and LMP1-mediated transformation of fibroblasts (1, 43). As is the case with Akt activation, EBER-negative HL also has NF-κB activation, indicating that in the absence of LMP1, other mechanisms exist to activate NF-κB (42, 44). For example, defects in inhibitor of nuclear factor-κB α (IκBα) and IκBε expression have been noted in HL, as well as constitutive activation of IκB kinases, which inactivate IκB and thereby activate NF-κB (45–47). Activation of NF-κB is different between EBV-associated HL and NPC as different forms of NF-κB are activated in HL and NPC. In HL, p50/p65 heterodimers are the main complexes found in the HRS cells, whereas p50/p50 homodimers in conjunction with the IκB family member Bcl-3 are nuclear and active in NPC (48, 49). LMP1 has also been shown to activate the mitogen-activated protein kinase pathways and Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway in epithelial cells, as well as in B cells (50). Furthermore, nuclear expression of STATs in NPC implies that LMP1 activation of the JAK/STAT pathway may play a role in NPC development or progression (50). Activation of the PI3K/Akt pathway could complement or synergize with these or other pathways in NPC or HL to enhance cell survival, proliferation, and motility.

In summary, this article details the important finding of differential cellular pathway activation in two different EBV-associated malignancies with similar viral expression profiles. PI3K/Akt activation with subsequent phosphorylation and inactivation of GSK-3β and nuclear β-catenin accumulation were characteristic of primary NPC specimens. The universality of these findings implicates the PI3K/Akt and β-catenin pathways in the development or progression of NPC. Alternatively, only Akt was activated in the HRS cells of the majority of HL specimens. These data imply a prominent role for PI3K/Akt signaling in HL but suggests that the GSK-3β/β-catenin effectors are unlikely to contribute to HL pathogenesis.

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