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The Epstein-Barr virus EBNA2 protein induces a subset of NOTCH target genes in thyroid cancer cell lines but fails to suppress proliferation

Sean Golden, BA^a, Xiao-Min Yu, MD, PhD^a, Scott Odorico^a, Vansh Jain^a, Ana Marina^a, Shidong Ma, PhD^b, Shannon Kenney, MD^b, and Herbert Chen, MD^c

^aDepartment of Surgery, University of Wisconsin School of Medicine and Public Health, Madison, WI

^bDepartment of Oncology, University of Wisconsin School of Medicine and Public Health, Madison, WI

^cDepartment of Surgery, University of Alabama at Birmingham, Birmingham, AL

Abstract

Background.—Epstein-Barr virus is associated with lymphoid and epithelial malignancies and has been reported to infect thyroid cells. The Epstein-Barr virus protein, EBNA2, regulates viral and cellular promoters by binding to RBP-jk. Similarly, NOTCH1, a tumor suppressor protein in thyroid epithelial cells, competes with EBNA2 for binding to overlapping sites on RBP-jk. EBNA2 activates a subset of NOTCH-responsive genes in lymphocytes and myocytes; however, the effect of EBNA2 expression on NOTCH targets in epithelial cells is unknown. Here we have explored whether EBNA2 activates NOTCH1 targets in thyroid cancer lines and examined its effect on cellular proliferation.

Methods.—Two human thyroid cancer lines, follicular FTC-236 and anaplastic HTh7, were transfected with EBNA2, NOTCH1, or control vectors. Notch targets were measured using quantitative reverse transcriptase polymerase chain reaction. Cellular proliferation was measured by MTT analysis.

Results.—EBNA2 activated only a subset of NOTCH1 targets. Expression of HES1 and HEY1 were increased 10-fold in FTC-236 and HTh7 cells, respectively, but the majority of NOTCH1 targets examined were not affected. In contrast to NOTCH1, EBNA2 did not suppress proliferation.

Conclusion.—EBNA2 does not activate most Notch1-responsive genes or suppress proliferation in human thyroid cancer cells. Instead, EBNA2 may compete with NOTCH1 for limiting amounts of RBP-jk in epithelial cells and inhibit certain aspects of NOTCH1 signaling.

EPSTEIN-BARR VIRUS (EBV) is a gamma herpesvirus that infects >90% of adults worldwide. EBV is the causative agent of infectious mononucleosis and is associated with the

development of epithelial and lymphoid malignancies, including nasopharyngeal carcinoma, gastric cancer, Burkitt's lymphoma, and Hodgkin lymphoma. Although EBV infects primarily B lymphocytes, it has also been shown to infect T lymphocytes, myocytes, and epithelial cells of the oropharynx and stomach.¹ Whether EBV infects the thyroid gland is not known. In recent years, a handful of studies have found evidence of EBV infection of thyroid tissue using PCR, in situ hybridization, and immunohistochemistry,²⁻⁵ but to the best of our knowledge, no study has examined the effect of EBV on cellular signaling or proliferation in thyroid cancer cell lines.

EBV encodes a transcription factor called EBV nuclear antigen 2 (EBNA2) that is essential to B-cell immortalization.⁶ Interestingly, EBNA2 can be considered a functional homologue of an activated NOTCH receptor. NOTCH genes encode a family of transmembrane signaling proteins that function as tumor suppressors in thyroid cells.⁷ Both EBNA2 and activated (cleaved) NOTCH receptors bind to a cellular protein called RBP-j κ (also known as CBF1, RBP-J, KBF2, and CSL) to modulate expression of other genes. Through this common interaction with RBP-j κ , EBNA2 and NOTCH activate a set of promoters that is overlapping, yet not identical, in lymphocytes.⁶

Previous studies in EBV-infected B cells have demonstrated that EBNA2 activates the NOTCH-responsive *HES1* (hairly/enhancer of split) promoter.⁸ Similarly, EBNA2 mimics the effect of an activated NOTCH receptor on proliferation in a myogenic cell line, most likely through induction of *HES1*.⁶ EBNA2 is expressed during EBV infection of normal tongue epithelial cells as well as in a subset of nasopharyngeal carcinomas^{9,10}; however, the effect of EBNA2 on NOTCH-responsive genes in epithelial cells is unknown.

In epithelial thyroid cells, the NOTCH1 isomer is believed to function as a tumor suppressor. NOTCH1 signaling is absent or decreased in thyroid cancer cell lines, and the relative quiescence of NOTCH1 signaling correlates with tumor severity.⁷ Likewise, overexpression of NOTCH1 decreases cellular proliferation and promotes differentiation in in vitro and murine models.⁷ While NOTCH requires activation through binding of extracellular ligands before engaging RBP-j κ , EBNA2 is constitutively active.

Given the ability of both NOTCH and EBNA2 to interact with limiting amounts of RBP-j κ , we hypothesized that EBNA2 could potentially either mimic or inhibit transcription of NOTCH1 targets in thyroid cancer cell lines. Here we show that only a subset of NOTCH1 targets are activated by EBNA2 in thyroid cancer cells.

MATERIALS AND METHODS

Cell culture.

The human FTC cell line, FTC236, was purchased from European Collection of Cell Cultures (ECACC) through Sigma-Aldrich (St. Louis, MO) and maintained in Dulbecco's Modified Eagle/Ham's F-12 medium (1:1; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 0.01 IU/mL thyroid-stimulating hormone, 40 μ g/mL insulin (Sigma-Aldrich), and 50 μ g/mL penicillin/streptomycin (Invitrogen).

HTh7, a human-derived anaplastic thyroid cancer (ATC) cell line authenticated by DNA profiling, was provided kindly by Dr Rebecca Schweppe, (University of Colorado, Denver, CO) and maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 50 $\mu\text{g}/\text{mL}$ penicillin/streptomycin (Invitrogen). Both cell lines were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C.

Transfection.

The pSG5 Vector (Stratagene, La Jolla, CA) is a eukaryotic expression vector plasmid containing an SV40 early promoter. The pSG5 Vector and a plasmid containing *EBNA2* in pSG5, were provided kindly by Dr Eric Johannsen (University of Wisconsin, Madison, WI). TAN1, an LNCX1 vector plasmid containing a constitutive *NOTCH1* intercellular domain (NICD), was provided kindly by Dr Douglas W. Ball (Johns Hopkins University, Baltimore, MD). FTC-236 and HTh7 cells were transiently transfected with *EBNA2*, *NOTCH1*, or an appropriate vector control (pSG5 or LNCX1) using Lipofectamine 3000 (Invitrogen) as described previously.¹¹

Western blot analysis.

FTC-236 and HTh7 cells were transfected transiently with *EBNA2*, *NOTCH1*, or an empty vector control (pSG5 or LNCX1). After 48 h, cells were washed with PBS and then treated with lysis buffer. The protein lysates were prepared as described previously.¹¹ Protein concentration was quantified using the BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Denatured protein extracts were resolved by electrophoresis on 4% to 15% Criterion TGX precast gels (Bio-Rad Laboratories, Hercules, CA), transferred onto nitrocellulose membranes (Bio-Rad Laboratories), then blocked and incubated with the following primary antibodies overnight at 4°C: β -actin (1:2,000; Cell Signaling Technology, Danvers, MA), Notch1 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000; Trevigen, Gaithersburg, MD), and anti-EBNA2 PE2 (1:250; Abcam, Cambridge, United Kingdom).

After washing, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated, antirabbit/mouse secondary antibodies. The immunoreactive bands were visualized using Immunstair (Bio-rad Laboratories), Supersignal West Pico, or SuperSignal West Femto (Pierce Biotechnology, Rockford, IL) reagents. Expression levels of β -actin or GAPDH were used as the loading control.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Total RNA was isolated from cultured cells 48 h after transient transfection with *EBNA2*, *NOTCH1*, or control expression vector (pSG5 or LNCX1) using an RNeasy Plus Mini kit (Qiagen, Valencia, CA). After isolation, RNA samples were quantified by NanoDrop (Thermo Scientific) to ensure purity and integrity of RNA. Next, 2 μg of the isolated RNA was reverse transcribed into complementary DNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Sequences for each pair of PCR primers were published previously.¹² The quantitative, real-time PCR reactions were performed on a MyiQ Thermal Cycler (Bio-Rad Laboratories). Target gene expression was normalized to the housekeeping gene

s27 from the same sample measured concurrently. The comparative cycle threshold (Ct) method was used to calculate relative expression levels of target genes.

Cell proliferation assay.

Cellular proliferation was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) rapid colorimetric assay (Sigma-Aldrich) as described previously.¹¹ Briefly, cells were plated in quadruplicate on 24-well plates at a density of 15,000 cells per well. Cells were allowed to adhere overnight, then were transfected transiently with 1 or 2 concentrations of *EBNA2*, *NOTCH1*, or an empty vector control (pSG5 or LNCX1). Cell proliferation was measured immediately after transfection as well as 24 h, 48 h, 96 h, or 144 h after transfection. On the day of measurement, the medium in each well was replaced with 250 μ L of serum-free medium containing 0.5 mg/mL MTT and incubated for 4 hours at 37°C. Next, 750 μ L MTT DMSO (Fischer Scientific, Pittsburg, PA) was added to each well and mixed to dissolve the MTT formazan crystals. Absorbance was measured at 540 nm using a μ Quant spectrophotometer (Bio-Tek Instruments, Winooski, VT).

RESULTS

EBNA2 activated only a subset of NOTCH1 targets in FTC-236 and HTh7 cell lines.

To determine whether EBNA2 activates NOTCH-responsive promoters in thyroid cancer cell lines, protein and mRNA expression of selected genes were measured after transfection of an *EBNA2* expression vector or empty vector control (pSG5). First, Western blot analysis was utilized to confirm that EBNA2 was expressed selectively in *EBNA2* transfected cells (Fig 1, A). As expected, EBNA2 was expressed strongly in *EBNA2* transfected cells in both FTC-236 and HTh7 cell lines but not in cells transfected with the empty vector control.

Though EBNA2 is not known to activate the NOTCH promoter, the Western blot was also stained using a NOTCH1 antibody (NICD) to confirm that expression of EBNA2 did not modulate expression of the NOTCH1 protein. As expected, NOTCH1 protein expression did not change after transfection of *EBNA2* in either cell line. β -actin was used as a loading control. The Western blot indicated that *EBNA2* was transfected successfully in both cell lines and that EBNA2 did not alter protein expression of NOTCH1. In order to compare EBNA2 expression to NOTCH1 activation, FTC-236 and HTh7 cell lines were also transfected with a *NOTCH1* expression vector or empty vector control (LNCX1) (Fig 1, B and C).

Next, qRT-PCR was utilized to assess mRNA expression of NOTCH1, as well as NOTCH1 target genes of the Hairy/Enhancer of Split (HES) family, *HES1*, *HES5*, and *HES6*, and Hairy/Enhancer Split (HEY) *HEY1* (Fig 2). HES and HEY are transcriptional repressors that are activated by the transcriptional activation complex formed by the NOTCH intracellular domain, RBP-j κ , the coactivator Mastermindlike, and other proteins. *EBNA2* transfection of thyroid cancer cells resulted in marked induction of only a subset of NOTCH1 downstream targets, and the effect differed among the cell lines.

In FTC-236 cells, expression of HES1 was increased roughly 10-fold; in contrast, 3 other NOTCH1 targets were not affected by EBNA2 expression. Importantly, NOTCH1 mRNA expression was unchanged. In HTh7 cells, expression of HEY1 was increased 10-fold, while 3 other NOTCH targets were not activated; NOTCH1 mRNA expression was unchanged. Interestingly, the levels of EBNA2-induced activation of HES1 and HEY1 were similar to those observed with *NOTCH1* transfection in previous experiments.^{13–16}

EBNA2 failed to suppress thyroid tumor growth in vitro.

After determining that EBNA2 activates expression of only a subset of downstream NOTCH1 targets in thyroid cancer cells, we investigated whether EBNA2 mimics NOTCH1 in its capacity to suppress proliferation of the thyroid tumor cell lines in vitro using MTT assays. Cell viability was measured 1 and 2 days after transfection of 2 concentrations of *EBNA2* or an empty vector control (pSG5) in FTC-236 and HTh7 cell lines (Fig 3, *A* and *B*). The concentrations used in the MTT assays were comparable or greater than the concentrations used in the Western blot and qRT-PCR experiments.

In both cell lines, *EBNA2* transfected cells did not demonstrate a decrease in cellular growth at either time point or concentration when compared to the control group. While both the control and *EBNA2* transfected cells demonstrated a significant decrease in cell viability between the 0- and 1-day measurements, this was likely due to toxicity associated with the transfection reagents, because a similar decrease in cell viability occurred in the control and *EBNA2* vectors.

Conversely, in FTC-236 and HTh7 cells transfected with a *NOTCH1* expression vector, cellular proliferation was decreased approximately 25% compared to cells transfected with an empty vector control (LNCX1), although this decrease was not seen until Day 6 for HTh7 cells (Fig 3, *C* and *D*). The MTT results suggest that EBNA2 does not decrease tumor proliferation in vitro at concentrations sufficient to activate expression of NOTCH1 targets.

DISCUSSION

Growing evidence suggests that NOTCH1 plays an important role as a tumor suppressor in thyroid cancers as well as some other epithelial neoplasms of the head and neck.¹⁷ NOTCH1-activating compounds, from synthetic histone deacetylase inhibitors to natural flavonoids, are currently under investigation for their anticancer properties. In other tumor types, such as T-cell and B-cell lymphomas, however, NOTCH functions as an oncogene.¹⁸

EBV promotes both B-cell and epithelial cell malignancies, and the ability of the EBV EBNA2 protein to hijack the Notch pathway in B cells is required for EBV transformation of B cells. EBNA2 does not bind DNA directly but instead interacts with RBP-j κ (the crucial cofactor for NOTCH) to induce transcription of all other viral proteins as well as modulate the expression of cellular genes.⁶

EBNA2 is expressed in some EBV-infected nasopharyngeal carcinomas, as well as in EBV-infected normal tongue epithelium, but the effect of EBNA2 on NOTCH target genes in epithelial cells has not been examined. In this study, we explore whether EBNA2 mimics

NOTCH signaling in a follicular and an ATC cell line. Our results show that EBNA2 activates only a subset of NOTCH1 target genes in thyroid cancer cells and does not share the tumor suppressor properties of NOTCH1 in epithelial cells.

Consistent with the ability of EBNA2 to activate promoters that have RBP-j κ motifs, we found that EBNA2 activates strongly the NOTCH1 responsive targets, HES1 and HEY1, in FTC-236 and HTh7 cells, respectively. The HES and HEY families are important transcriptional repressors activated by the NOTCH/RBP-j κ activation complex and play a central role in proliferation and differentiation of thyrocytes.

Our findings are in line with earlier experiments⁸ conducted in B lymphocytes that found that EBNA2 could activate the *HES1* promoter. The relative upregulation of HES1 and HEY1 in FTC-236 and HTh7 cells, respectively, was robust and reproducible, and most importantly, comparable to the upregulation of these targets after NOTCH1 activation seen in previous experiments.^{13–16} Furthermore, the effects of EBNA2 were demonstrated by Western blot analysis and qRT-PCR to be independent of activation of the *NOTCH1* promoter.

Importantly, however, EBNA2 did not activate all NOTCH1 targets, and its effects differed between the 2 cell lines. This observation suggests that while EBNA2 mimics NOTCH1 in its ability to transcriptionally activate certain gene targets, this overlap is limited, perhaps due to differences in the physical interaction between the 2 transactivators and RBP-j κ . While EBNA2 and NOTCH are believed to contact the same hydrophobic pocket within the RBP-j κ protein, they appear to also bind to distinct amino acids in the vicinity of this pocket.^{19,20} Furthermore, EBNA2 was shown recently to drive formation of new chromosome binding sites and target genes for RBP-j κ , which may differ from the binding sites regulated by NOTCH1.²¹

Of note, EBNA2 failed to suppress proliferation of thyroid cancer cell lines in MTT assays, a key difference in phenotype between EBNA2 and NOTCH1. The mechanisms underlying this incongruence are not yet clear but may derive from the same variations in binding to RBP-j κ outlined earlier. Alternatively, this finding may highlight the irreplaceable role of NOTCH1 signaling in suppressing thyroid malignancies. Conversely, because the binding of EBNA2 and Notch to RBP-j κ is mutually exclusive, EBNA2 may disrupt essential NOTCH1 functions by blocking its interaction with RBP-j κ .²² In this case, EBNA2 expression in epithelial cell types in which NOTCH1 functions as a tumor suppressor may help to promote malignancy.

Because a recent study examining almost 500 papillary thyroid carcinomas did not find evidence of EBV infection in fully formed neoplasms,²³ the possible contribution of EBV in the development of these neoplasms may occur at an earlier time point. In addition to the potential role of EBNA2 for inhibiting the tumor suppressor function of NOTCH signaling, 2 other EBV latency proteins (LMP1 and LMP2A) promote EBV-associated epithelial cell neoplasms by inducing cytosine methylation of tumor suppressor genes.²⁴ Indeed, a number of the same tumor suppressor genes that are methylated commonly in EBV-positive

nasopharyngeal carcinomas or gastric cancers (including PTEN, RASSF1A, and DAPK) are also hypermethylated commonly in thyroid cancer.^{25–27}

Given the immunogenicity of EBV proteins, the EBV genome may be lost during later stages of thyroid cancer progression once cellular transformation is complete and no longer requires the presence of EBV-encoded proteins. Similarly, the EBV genome is lost commonly in EBV-positive nasopharyngeal carcinoma and gastric cancer cell lines grown in cell culture, because cellular mutations and methylation of tumor suppressor genes can substitute for EBV genes at late stages of tumor progression.

In conclusion, our findings may support a potential role for EBV infection in the development of thyroid cancers, but further investigations of the prevalence of EBV infection of the thyroid gland and its effects on NOTCH functions in this cell type are required to examine this hypothesis.

DISCUSSION

Dr Kepal N. Patel (New York, NY): Two quick questions. One is—and I think you briefly touched on it at the end—have you looked at expression of EBNA2 in human thyroid tissues, looking at its expression in human cells? The second question is, have you looked at the relationship of this and other EBV-related tumors such as nasopharyngeal carcinoma? Is there a role of these proteins in Notch 1 and those cancers as well?

Dr Sean Golden: I will answer the second question first. If I understand it correctly, there certainly is a role for EBNA2 in nasopharyngeal carcinomas as far as I can tell, and that is already an active line of investigation in EBV fields. As far as looking for EBV infection in actual thyroid tissue samples and genomic sets that are already out there, we have not yet done that analysis, but it is certainly on our radar for the next set of experiments as we pursue this further.

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Fig 1.

Successful transfection of thyroid cancer cell lines with *EBNA2* or *NOTCH1*. (A) Detection of NOTCH intercellular domain (NICD) and EBNA2 in FTC-236 and HTh7 cells transfected with an *EBNA2* expression vector ("*EBNA2*"), pSG5 vehicle control ("*Vector*"), or no DNA control ("*Empty*"). Equal loading was confirmed with β-actin. (B) Detection of NICD in FTC-236 cells transfected with a *NOTCH1* expression vector ("*NOTCH1*") or LNCX1 vehicle control. Equal loading was confirmed with GAPDH. (C) Detection of NICD in HTh7 cells transfected with a *NOTCH1* expression vector or LNCX1 vehicle control. Equal loading was confirmed with β-actin.

Fig 2.

EBNA2 activates only a subset of NOTCH1 targets in both FTC-236 and HTh7 cell lines.

(A) Transfection of EBNA2 resulted in significant activation of NOTCH1 target HES1 but not other NOTCH1 targets in FTC-236 cells. mRNA levels of HES, HEY, and NOTCH1 were measured using qRT-PCR in FTC-236 cells transfected with EBNA2 and empty pSG5 control vectors. Target gene expression was normalized to the housekeeping gene s27 and plotted relative to cells transfected with the empty vector control. All values were expressed as mean $\pm$ SD ($n = 3$). (B) Transfection of EBNA2 resulted in significant activation ($P < .1$) of only the NOTCH1 target HEY1 in HTh7 cells.

Fig 3.

EBNA2 failed to suppress thyroid tumor growth in vitro. (A) No decrease in cellular proliferation was detected using an MTT assay in FTC-236 cells transfected with 2 concentrations (1.3 $\mu\text{g/mL}$ or 2.6 $\mu\text{g/mL}$) of an EBNA2 expression vector or empty pSG5 vector control. Cellular proliferation was measured immediately after transfection and at 1 day or 2 days after transfection. (B) No decrease in cellular proliferation was detected using an MTT assay in HTh7 cells transfected with 2 concentrations (2.6 $\mu\text{g/mL}$ or 4.0 $\mu\text{g/mL}$) of an EBNA2 expression vector or empty pSG5 vector control. Cellular proliferation was measured immediately after transfection and at 1 day or 2 days after transfection. (C) A 30% reduction in cellular proliferation was detected using an MTT assay in FTC-236 cells transfected with a NOTCH1 expression vector compared to cells transfected with an empty LNCX1 vector control. Cellular proliferation was measured immediately and at 2 days, 4 days, or 6 days after transfection. (D) A 25% reduction in cellular proliferation was detected at Day 6 using an MTT assay in HTh7 cells transfected with a NOTCH1 expression vector compared to cells transfected with an empty LNCX1 vector control. Cellular proliferation was measured immediately and at 2 days, 4 days, or 6 days after transfection.