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EBV and not HPV sensitizes tobacco associated head and neck cancer cell line FaDu to radiotherapy

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Abstract

Conclusion—EBV radiosensitized the p53 mutant tobacco associated head and neck cell line, FaDu.

Objectives—In the head and neck, HPV is a major risk factor associated with tonsil and base of tongue cancers, while a majority of undifferentiated nasopharyngeal cancers are positive for EBV. Clinically, head and neck tumors positive for HPV or EBV are more radiosensitive than tumors associated with tobacco and alcohol. We aimed to evaluate whether viral infections can sensitize tobacco-associated head and neck squamous cell carcinoma cell line that harbors multiple mutations, especially *TP53*, to radiotherapy.

Method—Four FaDu cell lines (vector control - FaDu-DN; FaDu expressing HPV16 E6/E7 - FaDu-HPV; FaDu infected with EBV - FaDu-EBV; and FaDu-HPV infected with EBV - FaDu-HE) were evaluated for their radiation sensitivity using clonogenic assay. Cell cycle, protein expression, apoptosis and cellular senescence were analyzed.

Results—FaDu-EBV and FaDu-HE exhibited significantly increased radiosensitivity in comparison with the control cell line. Radiation-induced cell cycle arrest was altered in all cell lines expressing viral genes. The observed distribution of cells at G1 and S phases was associated with a significant increase in expression of p21 protein along with decreased levels of pAKT/AKT and pERK/ERK ratio ($p < 0.05$) and increased cellular senescence ($p < 0.05$).

Keywords

squamous cell carcinoma; radiation; radiosensitivity; clonogenic assay; co-infection; cell cycle; senescence

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INTRODUCTION

Head and Neck cancer arise from the mucosal surfaces of the upper aerodigestive tract including the oral cavity, nasal cavity, paranasal sinuses, pharynx, and larynx. Most head and neck cancers of the upper aerodigestive tract are squamous in origin [1]. Squamous cell carcinoma of the head and neck is the sixth most common cancer worldwide, with 600,000 new cases diagnosed and 350,000 cancer deaths every year. Although tobacco usage and alcohol abuse have been considered as the major risk factors for head and neck squamous cell carcinoma (HNSCC), several studies have reported an etiological role of infectious agents such as Human Papillomavirus (HPV) and Epstein Barr Virus (EBV) specifically in the oropharynx and nasopharynx [1].

The incidence of HPV has increased over the past 20 years, therefore it has been recognized as an important risk factor for head and neck carcinogenesis [1]. HPV accounts for about 25–30% of HNSCC, with 90% of HPV positive being associated particularly with HPV type 16. The carcinogenic function of HPV is mainly due to the two major oncoproteins E6 and E7. Expression of these proteins selectively inhibits tumor suppressor proteins such as p53 and pRb, resulting in cell cycle deregulation, telomerase inactivation and cell immortalization. Similarly, EBV has been strongly associated with nasopharyngeal carcinoma (NPC), and to a lesser extent, hypopharyngeal and laryngeal tumors [2]. The tumorigenic property of EBV is established when the virus is in its latent phase controlled by latent membrane proteins (LMP1, LMP2A and LMP2B) and EBV nuclear antigens (EBNA1, EBNA2 and EBNA3). The LMPs of EBV inhibit epithelial cell differentiation to induce hyperplasia and aberrant expression of keratin [3]. Over the last few years strong association between EBV and HPV has been reported and it is presumed that EBV and high risk HPV co-infection may have a significant role in the neoplastic transformation of oral epithelial cells. Accordingly co-infection of these viruses have been reported in nasopharyngeal carcinomas (15–52.9%), other head and neck tumors (11%), oral cancers (13%) and SCC of the tonsil (25%) and base of tongue (70%) [4].

In the clinical setting, HPV-positive and EBV-positive head and neck patients respond to radiotherapy with a five year survival rate of 82.6%. In contrast, the response to radiotherapy in tobacco associated head and neck cancer has a five year survival rate of 20% to 40% depending on the stage and primary site [5]. Tobacco and alcohol associated cancers account for about 70% of the head and neck cancers and multiple mutations caused by tobacco carcinogens is one of the main reasons for the low survival rate. Specific molecular targets of tobacco carcinogens have not been firmly identified, which probably accounts for limitations in target-oriented therapies for head and neck cancer. In this present study we wanted to determine whether a single viral infection or the co-infection of HPV-EBV confers increased radiosensitivity to tobacco-associated HNSCC. To test our hypothesis we chose FaDu cell line isolated from a 56 year old male patient that has a TP53 mutation at codon 248 and a PTEN homozygous deletion at loci 246.

MATERIALS AND METHODS

Cell culture and generation of stable cell lines

The hypopharyngeal HNSCC cell line FaDu was obtained from American type culture collection (ATCC). FaDu cells were transfected with an HPV16 E6/E7 plasmid that has a hygromycin resistance construct. Positive colonies were selected by adding hygromycin B (0.25mg/ml), selected colonies pooled and further infected by a recombinant EBV strain resistant to neomycin. Colonies expressing both HPV and EBV proteins were selected by adding hygromycin and G418 (400µg/ml). Simultaneously empty plasmids resistant to either hygromycin or G418 were transfected to generate the control cell line. Altogether, four different combinations of HPV/EBV stable cell lines were generated *viz.*, FaDu-DN (Double negative - Control), FaDu-HPV, FaDu-EBV and FaDu-HE (Double positive HPV+/EBV+). The stable cell lines were maintained in MEM media supplemented with 10% fetal bovine serum (FBS) at 37°C in the presence of 5% CO₂, 100 IU/ml penicillin-streptomycin, 1mM Sodium pyruvate, 1X NEAA, hygromycin and G418.

Reverse Transcription PCR and Genomic PCR analysis

FaDu stable cell lines cultured in MEM media were harvested and total RNA was extracted using RNA-STAT 60 reagent (Tel Test) according to the manufacturer's instructions. The concentration and the purity of the total RNA for each sample were estimated by spectrophotometric analysis at A260 and A280. The cDNA was synthesized using 10µg of total RNA using MMLV reverse transcriptase (Invitrogen). For Real-time PCR (RT-PCR) 100ng of cDNA was used and amplification performed with Power SYBR master mix (Applied Biosystems, Carlsbad, CA). Specific primers for indicated genes were used at a concentration of 320nM described in [6, 7]. Amplification of all the genes by qRT-PCR used the following cycling parameters: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The mRNA levels were quantified using a relative standard curve analysis based on the EBV B958 cell line as a control. The housekeeping genes, HPRT or GAPDH, were used to normalize RNA input. Standard RT-PCR was performed using primers and conditions previously described [6]. The PCR products were separated in 2% agarose gels and stained with ethidium bromide. miRNA abundance was analyzed using the qScript miRNA quantification system (Quanta Biosciences, Gaithersburg, MD) following the specifications recommended by the manufacturer. In this approach, miRNAs in 1µg of total RNA were poly-A tailed and converted to cDNA using an adaptor primer provided by the manufacturer. Specific forward Primers to EBV miR-BART7 (5'GCA TCA TAG TCC AGT GTC CA3') and human miR-16 (5'CGC AGT AGC AGC ACG TA3') were designed using miRPrimer. Real time PCR amplification using Power SYBR green, 200nM primers and 10ng of cDNA was performed using the cycling parameters mentioned above. Primers consisted of miR-BART7 or miR-16 as forward primers with a universal reverse primer (Quanta Biosciences). ddCT analysis was used for calculation of miRNA abundance among the various samples. Viral DNA was quantified using viral specific primers to the BHRF1 region of the EBV genome. A relative standard curve, using the Namalwa cell line carrying 2 copies of EBV served as a reference. Samples were normalized to the cellular gene, CRP.

Radiation treatment and Clonogenic assay

A clonogenic assay was performed to determine the survival percent of cells following radiation treatment. In brief, the cells were seeded on gelatin coated 60 mm culture dishes to obtain ~50 surviving colonies per dish post-irradiation. After the cells attached they were subjected to radiation (2, 4 and 6 Gy) at room temperature using a ^{137}Cs γ -ray source (J.L. Shepard and Associates, San Fernando, CA) and allowed to grow for 15 days. Seeding was performed in triplicate; the colonies formed were stained with 1% gentian violet and counted. Groups of 50 or greater cells were counted as a colony and the reduction in the ability to form colonies was the measure of radiosensitivity. Colony counts were averaged from three plates and the surviving fraction was calculated as the ratio of the plating efficiency of irradiated cells to the plating efficiency of the control cells. The whole set of experiments was repeated three times before being analyzed statistically. Radiation treatment of 4 Gy was used to analyze effects on EBV reactivation and viral gene expression.

Cell cycle analysis

To determine the effect of radiation on the stable cell lines in the distribution of cells at different phases, we performed cell cycle analysis. The cells were washed with PBS and harvested using 0.25% trypsin, followed by a second wash with PBS, cells counted, and adjusted to 1×10^6 cells/ml. The cells were fixed in 100% ethanol, treated with 100mg/l RNase at 37°C for 30 min and then stained with 50mg/l propidium iodide (Sigma, St. Louis, MO, USA) for 30 min. The cells were analyzed by flow cytometry (BD FACS Calibur flow cytometer, San Jose, CA, USA).

Annexin-V binding assay

Phosphatidylserine externalization (PS) was assessed by measuring annexin V-FITC binding according to the manufactures protocol (BD PharmaginTM, San Diego, CA, USA). Briefly, approximately 1×10^5 cells were collected and washed twice in 1 ml of cold PBS (pH 7.4). The cells were then resuspended in 500 μl of binding buffer (pH 7.4) and added to 5 μl annexin V-FITC and 5 μl PI. After 15 min of incubation in the dark at room temperature, the samples were analyzed at 1 hr using a flow cytometer. In all flow cytometric determinations, cell debris and cell clumps were excluded from the analysis by suitable gating (Becton-Dickinson, San Jose, CA, USA). For each experiment, 25,000 events were collected and analyzed. Each experiment was repeated three times.

Senescence-Associated β -Galactosidase Staining Assay

Staining for senescence-associated β -galactosidase (SABG), senescence markers, was performed using the Senescence β -Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA). In brief, 10,000 cells were counted and plated in small 35mm dishes and allowed to attach for 24h. Upon attachment the cells were irradiated at 4 Gy followed by senescence staining at indicated time points as per manufacturer's protocol. The SABG positive (blue-stained) cells were counted at $\times 200$ magnification.

Western blot analysis

Protein lysates were prepared from stable cells at 24h post irradiation at 4 Gy. The cells were subjected to sonication (Tekmar Sonic disruptor, TM-600, Rolla biotech, Anaheim, CA, USA) using cell lysis buffer (Cell signaling technology, Boston, MA, USA), containing protease cocktail inhibitor (Roche, Indianapolis, IN, USA), operated at 3sec for three cycles and repeated twice. Protein concentration was determined using the BCA protein assay reagent (Thermo Scientific, Rockford, IL, USA). Total protein (30ug) was subjected to electrophoresis in a polyacrylamide gel and transferred to 0.45µm PVDF membrane (Immobilon, Millipore, Billerica, MA, USA) by electroblotting. The blots were incubated overnight at 4°C with rabbit anti-AKT, pAKT, p21, pERK, Bcl2, Bax, or LMP1 (S12) antibodies (Cell Signaling Technology, Danvers, MA). Signals were visualized with a Chemiluminescent Detection Kit according to the manufacturer's protocol (Thermo Scientific, Rockford, IL, USA). The membranes were reprobed with anti-actin antibody or tubulin and all the signals were analyzed by densitometric scanning (Epson perfection V-500 photo, Long beach, CA, USA) and Imagequant analysis (GE health care Biosciences, Pittsburgh, PA, USA).

Statistical analysis

For continuous variables, data are represented as mean $\pm$ SD from 3 independent experiments and analyzed using ANOVA with SAS 9.4 (SASInc., Cary, NC, USA), the surviving fraction of all the data versus dose was fitted by polynomial regression. The student *t*-test was used to compare α and β values of the HPV and EBV expressing cell lines. The survival data for the cell lines were also compared by calculating the area under the curve for each cell line by using the Trapezoidal Rule, followed by the student *t*-test to compare the values. All *p* values < 0.05 of all analyses were considered statistically significant.

RESULTS

HPV16-E6 and EBV oncogene expression in FaDu stable cell lines

To validate the generated model in the FaDu cell line, the transcriptional levels of candidate HPV and EBV genes were assessed for expression by RT-PCR. The signature genes were amplified with gene specific primers. HPV positive head and neck cell line UM-SCC47 for HPV16-E6, a EBV positive B-cell lymphoblastic cell line B958 for EBER1 and LMP1, and a lung adenocarcinoma cell line A549 for GAPDH were used as positive controls. (Figure 1). The results indicated equal HPV-E6 expression in FaDu-HPV and FaDu-HE cell lines. EBV EBER1 levels were similar in FaDu-EBV and FaDu-HE. Next, we examined the EBV latent gene expression program of the EBV-positive FaDu cell lines. The FaDu-EBV and FaDu-HE were previously shown to express equal levels of LMP2 mRNA [4]. Both FaDu-EBV and FaDu-HE were positive for LMP1 mRNA, but levels differed between the two EBV-positive cell lines (Figure 1). EBNA2 mRNA was not detected (data not shown). The expression of LMP1 and 2 in the absence of EBNA2 indicated a type II latency pattern typical of EBV infection in carcinoma.

Expression of HPV16-E6/E7 and EBV genes radiosensitize FaDu cells

The survival graph (Figure 2) showed that FaDu-EBV was the most radiosensitive followed by FaDu-HE. Using the linear quadratic equation model we analyzed changes in the alpha/beta ratio to determine the cell killing effect for various doses of radiation tested (Table 1). Among the viral positive cell lines only FaDu-EBV showed a significant difference compared to FaDu-DN using the α/β ratio analysis ($p<0.05$). However analysis of the area under the curve showed a significant sensitization effect for both FaDu-EBV and FaDu-HE cell lines compared to FaDu-DN ($p<0.05$). FaDu-HPV did not attain significance in radiosensitivity in comparison with the control cell line either by α/β ratio or by area under the curve.

Radiation-induced cell cycle arrest is altered in the cell lines expressing viral genes

In HPV- and EBV-infected HNSCC, it has been reported that a distinct large set of specific cell cycle genes are upregulated, leading to the deregulation of cell cycle distribution [8, 9]. In the first 12 hours after radiation treatment all the cell lines showed a similar pattern of cell cycle changes – reduction of G1 phase and an increase of S and G2 phases (Figure 3A). The percentage of cells in G1 phase returned to baseline levels at 24h in the viral positive clones, but not in the FaDu-DN control cell line. With respect to S phase the percentage of the cells was reduced in the viral positive clones at 24h. FaDu-DN cells displayed the same pattern with restored G1 and reduced number of cells in S phase at 48h. We observed an increase in p21 expression 24h after radiation treatment (Figure 3B). This was significantly higher in the viral cell lines as compared to FaDu-DN cell line. These results suggest that the presence of viral genes promoted increased G1 accumulation in viral positive FaDu stable cells by modulating the expression of cell cycle regulatory proteins.

Effect of HPV16 E6/E7 and EBV on radiation induced apoptosis and senescence

The percentage of apoptosis induced by radiation treatment in the FaDu stable cell lines was assessed by flow cytometric analysis at 24–72h time points. Annexin V-FITC and PI staining were used to count the total number of apoptotic cells in samples subjected to radiation. The percentage of Annexin V-FITC/PI positive fraction of cells compared to FaDu-DN cells was not significantly different between the virally-positive FaDu cell lines (Figure 4A). FaDu-EBV alone showed a slight increase in apoptosis over FaDu-DN at all the time points analyzed, but was not statistically significant. Western blotting analysis was performed to check the expression of two counteracting mitochondrial proteins, Bax and Bcl2. We observed a slight increase in the Bax/Bcl2 ratio in the viral clones as compared to control following radiation treatment (Figure 4B), but the increased levels did not reach statistical significance. FaDu-EBV and FaDu-HE cell lines displayed a significant decrease in pro-survival signaling (reduced pAKT/AKT and pERK/ERK ratios) after radiation compared to FaDu-DN ($p<0.05$) (Figure 4C). Although FaDu-EBV and FaDu-HE cells have reduced pro-survival signaling and elevated p21 level after radiation this was not associated with a statistically significant increase in apoptosis, suggesting an alternate mode of death may be responsible for enhanced radiosensitivity of FaDu-EBV and FaDu-HE cells.

Senescence was assessed by counting cells showing β -galactosidase activity at pH 6.0 (SABG). At the baseline level and at 24h post irradiation FaDu-HE cells ($p<0.01$) showed a

significant amount of senescent cells compared to FaDu-DN cells. After irradiation, at 48h and 72h FaDu-HPV ($p<0.01$), FaDu-EBV ($p<0.05$) and FaDu-HE ($p<0.001$) cell lines had significantly higher percentages of senescent cells in comparison with respective FaDu-DN cell line at indicated time points (Figure 5). Our samples displayed reduced AKT and ERK activities (Figure 4 C and D) accompanied by elevated p21 after radiation in agreement with upregulated senescence level in FaDu HPV/EBV cell lines.

Expression of EBV miR-BART7 associated with radiosensitization

To explain the radiosensitization of EBV-infected FaDu-EBV and FaDu-HE cell lines, we examined if radiation induced EBV reactivation. We examined the mRNA expression of the lytic switch genes BZLF1 and BRLF1, which convert the latency to productive infection, using qRT-PCR. Both cell lines had background levels of BZLF1 and BRLF1 mRNA levels and these levels did not change upon radiation (data not shown). As viral reactivation results in the amplification of the viral genome, we measured DNA levels by qPCR in the FaDu-EBV and FaDu-HE cell lines following radiation treatment. DNA was harvested in 24 intervals between 48 and 96 hours following irradiation. Viral DNA levels were normalized to the viral copy number in untreated cells. As shown in Figure 6A, EBV DNA levels did not increase upon radiation treatment suggesting that other factors other than induction of viral reactivation contributed to the radiosensitivity of the EBV-positive FaDu cell lines.

Our observations for a decreased expression of pERK and pAkt in the EBV-positive FaDu cell lines implicated LMP1, a ligand independent receptor known to activate ERK and Akt signaling [10] in radiosensitization of the EBV-positive FaDu cell lines. LMP1 has been reported to be radioprotective; such that loss of LMP1 sensitizes cells to radiation [11]. Using qRT-PCR and unirradiated samples the FaDu-EBV cell line had 240x more LMP1 transcripts compared to the FaDu-HE cell lines. Therefore the level of LMP1 did not correlate with the radiosensitivity of the cell lines as FaDu-EBV is more sensitive than FaDu-HE. Radiation treatment also did not reduce LMP1 mRNA in either the FaDu-EBV or FaDu-HE cell line (Figure 6B). Similarly, LMP1 protein levels in the FaDu-EBV cell line remained unchanged following radiation (Figure 6C). Guided by a report that the EBV microRNA (miR) BART7 can increase the radiosensitivity of nasopharyngeal cell lines [12] we next investigated if miR-BART7 was expressed in our EBV-infected FaDu cell lines. In nasopharyngeal carcinoma (NPC), the EBV miRNAs are generated from the abundantly expressed noncoding EBV BamHI A Rightward transcripts (BART). The EBV BART miRNAs have been estimated to constitute up to 20% of the total cellular miRNA content in NPC. Both the FaDu-EBV and FaDu-HE showed similar expression of primary BART transcript (exon 1) as an indicator for expression of the EBV miRNAs (Figure 1). The BART transcript did not increase with radiation treatment (data not shown). Using a miRNA qRT-PCR assay, expression of the EBV miR-BART7 was detected in the FaDu-EBV and FaDu-HE. However, The FaDu-EBV showed an 11-fold increase in the abundance of miR-BART7 compared to FaDu-HE. Such increased levels of miR-BART7 were consistent with the enhanced radiosensitivity observed for the FaDu-EBV over the FaDu-HE. MiR-BART7 levels did not statistically change with radiation treatment in either the FaDu-EBV or FaDu-HE (Figure 6D).

DISCUSSION

HPV-positive head and neck tumors are very responsive to radiation or chemoradiation therapy as is undifferentiated carcinoma of the nasopharynx associated with EBV. Co-infection of HPV and EBV has been reported in nasopharyngeal, hypopharyngeal and oral cancers. Recently, we have shown that co-infection of these viruses in lymphoid rich sites (tonsil and BOT) of the head and neck is significantly associated with cancer status [4]. Head and neck tobacco associated cancers are recalcitrant to treatment because of multiple mutations that limit the identification of specific molecular targets. Here, we hypothesized that either HPV or EBV infection or the co-infection of these viruses may sensitize tobacco-associated HNSCCs to radiotherapy. To explore the mechanism we developed a double transgenic stable cell line model in FaDu cells as explained in the methods section.

After confirming the expression of HPV and EBV genes in the generated stable cell lines, we performed a clonogenic assay to assess the cytotoxicity. We determined that FaDu-EBV cell line alone is significantly radiosensitive compared to the FaDu-DN cell line. By using the area under the curve analysis we observed that FaDu-EBV and FaDu-HE cell lines were radiosensitive. Surprisingly, we observed only modest increase in radiosensitivity of FaDu cells expressing HPV oncogenes although the FaDu-HE was radiosensitive. This could be explained by EBV expression in the FaDu-HE being responsible for the radiosensitization effect. We presume that superimposed infection of HPV in tobacco-associated HNSCC behave differently from HPV positive tumors because sustained inactivation of p53 pathway by HPV-E6 protein is required for maintenance of the proliferative phenotype of the HPV positive carcinoma cells [13] which does not occur in the smoking associated cancers where p53 is mutated. The smoking-associated tumors have likely established proliferation due to a number of mutations. Further, some studies have shown that HPV-positive head and neck cell lines were not intrinsically radiosensitive and it is believed that the radiotherapy induces an immune response to antigenic cancer and this may be responsible for the radiosensitization effect in HPV-associated malignancies [14]. P53 mutations are also rare in EBV-associated nasopharyngeal carcinomas. EBV has several viral factors that are known to interfere with p53 (EBNA1, LMP1, EBNA3C, and BZLF1) [15–17]. However, EBV impairment of p53 is typically indirect mechanism, which differs from the direct HPV E6 degradation of p53. Thus, EBV is able to carry out its viral functions regardless of P53 status.

EBV latency-associated gene expression is dependent upon host cell cycle progression, and radiation treatment disrupts the cell cycle [18]. Moreover cell cycle pathways are often interrupted by viral proteins to take control of cellular growth and proliferation. Also, viruses rely on the components of the host replicative factory, which accumulate during the S phase of the cell cycle. Since the FaDu cells expressing LMP-1 and co-expressing LMP-1 and E6 were proliferative despite DNA damage caused by radiation treatment, we speculate that these viral proteins may suppress DNA damage response (DDR) and the viral oncogenes orchestrate DDR proteins to augment senescence [19]. Our results are in agreement and exhibited significant increase in the percentage of senescence cells in the viral positive cells over time after being subjected to radiation. Previous studies in EBV associated epithelial cells have reported radiation induced transactivation of p21 mediated

G1 cell cycle arrest, and triggering of the EBV lytic switch gene, BZLF1 [20]. Although radiation induced p21 levels in our EBV positive clones, we did not observe an increase in the lytic genes BZLF1, BRLF1 or induction of EBV DNA replication. Having excluded viral reactivation as a cause for radiosensitivity of the EBV-positive cell lines, we next focused on LMP1. LMP1 is known to confer radioprotection, activating in a ligand independent manner the radioprotective signaling pathways (PI3K, STAT3, and ERK) [10]. Silencing of LMP1 has been shown to enhance the radiosensitivity of nasopharyngeal carcinoma cells [11]. Consistent with a potential loss of LMP1, pERK and pAKT protein levels were reduced following radiation in the EBV-positive FaDu cell lines. However, no change in LMP1 mRNA or protein levels were observed (Figure 6B and C), suggesting other viral players in the EBV-mediated radiosensitization of the FaDu cell line.

A recent study showed that the EBV microRNA BART7 ectopic expression enhanced the radiosensitivity of a nasopharyngeal carcinoma cell line [12]. Indeed, expression of miR-BART7 was detected in the cell lines FaDu-EBV and FaDu-HE. A higher abundance of miR-BART7 was noted in the FaDu-EBV that also displayed the greatest radiosensitivity. Intriguingly, the FaDu-EBV also displayed abundant LMP1 expression, suggesting that miR-BART7 radiosensitization may be dominant over the LMP1 radioprotection effects. In contrast, the FaDu-HE cells, with reduced radiosensitivity compared to the FaDu-EBV, exhibited low levels of both LMP1 and the miR-BART7, consistent with notion for miR-BART7 being a candidate for the EBV-mediated radiosensitization of the FaDu cell line. p53 is one of the most important tumor suppressor genes and its mutations are the most common genetic alterations found not only in tobacco-associated HNSCC, but also in many other types of cancer. Despite being the most appealing target for molecular targeted anticancer therapy, p53 is also known as a challenging target for drug discovery. MicroRNA-based therapeutics, although still in early stage of clinical development, are a promising approach to cancer treatment. Targeted delivery of miR-BART7 to p53-mutated radiation-resistant head and neck carcinoma cells may enhance sensitivity of these cells to radiotherapy and result in improved patient outcomes. As this study was not exhaustive, it is likely that other EBV factors in addition to miR-BART7 and LMP1 could influence the cellular outcome following radiation.

In conclusion, we have shown that EBV infection induces radiosensitivity of tobacco associated FaDu cell line. Similar results were not noted in a clone expressing HPV16 E6/E7, which is not surprising given patients who are smokers and have a superimposed HPV infection have a worse prognosis compared to HPV associated tumors in non-smokers. Our study presents important evidence for EBV as a radiosensitizer in a tobacco induced cancer cell line. Identification of viral factors, such as miR-BART7, will guide future studies to understand the mechanisms of increased radiosensitivity and its association with viral infection. Ectopic expression of such radiosensitizing viral factors could be employed to enhance the effectiveness of radiotherapy in the management of traditional head and neck cancer.

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Figure 1. Validation of HPV transfection or EBV infection

The expression of HPV-E6 and EBV - EBER1, LMP1, and BART transcripts in the generated FaDu stable cell line model was verified by RT-PCR based amplification. GAPDH were shown as controls for loading. RT-negative (RT-) samples were analyzed on the same gel. Images were cropped to remove extra lanes.

Figure 2. Radiosensitivity of FaDu-DN and cell lines expressing HPV and EBV genes
Clonogenic survival assay following treatment with increasing doses of radiation. Survival fraction after radiation treatment was normalized to the plating efficiency of the corresponding non-irradiated sample. The mean of three independent experiments $\pm$ SD is shown (* $p < 0.05$).

Figure 3. Kinetics of cell cycle distribution
A. Cells were exposed to radiation at 4Gy, fixed in ethanol at indicated time points and stained with PI before analyzing with flow cytometry. **B.** Expression of p21 in FaDu clones before and after radiation at 4Gy. The mean of three independent experiments $\pm$ SD is shown (* $p < 0.05$).

Figure 4. Annexin-V staining

A. Phosphatidylserine internalization was determined by Annexin V-FITC binding assay in the unirradiated control and after radiation at 4Gy at indicated time points. **B.** Expression of mitochondrial proteins Bcl2 and Bax in FaDu clones with and without radiation. The mean of three independent experiments $\pm$ SE is shown. **C, D.** Change in expression pattern of AKT and ERK activity in FaDu clones with respect to radiation treatment. The mean of three independent experiments $\pm$ SD is shown (* $p < 0.05$).

Figure 5. Radiation treatment induces senescence-associated β -galactosidase (SABG) activity in FaDu cells

Increase in the percent of SABG positive cells at 24–72h after radiation. The mean of three independent experiments $\pm$ SD is shown, a- $p<0.05$, b- $p<0.01$, c- $p<0.001$.

Figure 6. Lack of viral reactivation following radiation treatment

A. DNA from FaDu-EBV and FaDu-HE cell lines was isolated at 24 hour intervals following 48 to 96 hours post-irradiation (4 Gy). Viral DNA was quantified using EBV-specific primers to the BHRF1 gene and normalized to the cellular DNA levels using primers to c-reactive protein (CRP). The fold change was determined by comparing the BHRF1/CRP ratio in irradiated samples to that of untreated controls. The average and standard deviation of the mean from 3 biological experiments is shown. **B.** LMP1 mRNAs following radiation treatment. Total RNA was extracted from cells 48 post-irradiation treatment (4Gy). QRT-PCR was performed to quantify LMP1 levels. Data was normalized to the housekeeping gene HPRT, and the fold change calculated relative to untreated cells. The average and standard deviation of the mean of 3 independent biological experiments is shown. **C.** LMP1 protein levels following radiation treatment. **D.** Abundance of miR-BART7

in EBV-infected FaDu cell lines following radiation. Total RNA was extracted from cells 48 post-irradiation treatment (4Gy). miR-BART7 RNA levels were measured by qRT-PCR following a using polyA tailing cDNA conversion approach. Relative levels were normalized to the cellular miR-16 and quantified using the delta-delta Ct method. Shown are the average of 5 independent experiments and the standard error of the mean.

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Table 1**Survival data analysis**

Cell line	Linear quadratic analysis $\pm$ SD		Area under the curve $\pm$ SD
	Alpha	Beta	
FaDu-DN	0.1897 ± 0.070	-0.0896 ± 0.018	4.0661 ± 0.059
FaDu-HPV	0.02613 ± 0.171	-0.0797 ± 0.0216	3.2404 ± 0.539
FaDu-EBV	$-0.2497^* \pm 0.172$	-0.0590 ± 0.023	$2.2285^* \pm 0.329$
FaDu-HE	-0.1394 ± 0.155	-0.0602 ± 0.018	$2.7130^* \pm 0.465$

The survival fraction data were analyzed using linear quadratic model and area under curve to determine statistically significant differences in the viral genes expressing cell lines FaDu-HPV, FaDu-EBV and FaDu-HE compared to FaDu-DN cell line. Data are represented as mean $\pm$ SD.

* Indicates statistical significance at $p < 0.05$ compared to FaDu-DN.