Development of a Novel Mouse Model of Spontaneous High-Risk HPVE6/E7-Expressing Carcinoma in the Cervicovaginal Tract

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ABSTRACT

Current preclinical models for cervical cancer lack important clinical and pathologic features. To improve upon these models, we aimed to develop a novel, spontaneous HPV16-expressing carcinoma model that captures major aspects of HPV-associated cancer in the female genital tract. This novel preclinical model features (i) expression of HPV oncogenes E6 and E7 in the tumors in female reproductive tract of mice, (ii) spontaneous progression through high-grade squamous intraepithelial lesion (HSIL) to carcinoma, and (iii) flexibility to model cancers from different high-risk HPV genotypes. This was accomplished by injecting plasmids expressing HPV16 E6/E7-luciferase, AKT, c-myc, and Sleeping Beauty transposase into the cervicovaginal tract of C57BL/6 mice followed by electroporation. Cell lines derived from these tumors expressed HPV16 E6/E7 oncogenes, formed tumors in immunocompetent mice, and displayed carcinoma morphology. In all, this novel HPV-associated cervicogenital carcinoma model and HPV16 E6/E7-expressing tumor cell line improves upon current HPV16-E6/E7-expressing tumor models. These tumor models may serve as important preclinical models for the development of therapeutic HPV vaccines or novel therapeutic interventions against HPV E6/E7-expressing tumors.

Introduction

Human papillomavirus (HPV) is the etiologic factor for cervical cancer (1), with high-risk types such as HPV16 and HPV18 causing the majority of cases (2). Currently, cervical cancer remains the fourth most common type of cancer affecting women worldwide (1). Although the prophylactic vaccines Gardasil and Cervarix have been developed and widely administered, they cannot cure previously established HPV infections. HPV is currently the most common venereal disease. Although usually self-limited, after HPV infection a small fraction of infections will progress to precancerous high-grade squamous intraepithelial lesions (HSIL), of which, a subsequent fraction will progress to HPV+ cancer. The HPV oncogenes E6 and E7 play a critical role in inducing tumorigenesis (3, 4), and HPV DNA encoding HPV E6 and E7 is found integrated and expressed in the vast majority of cervical cancer cells (5).

Cervical cancer progresses through HSIL to squamous cell carcinoma (SCC) in a stepwise process. There is currently substantial interest in developing improved treatments for both HSIL and cervical cancer. Currently, efficacy of HSIL treatments remains 75%–85% (6, 7), and the treatments are accompanied by various adverse effects, including increased risk for preterm birth (8). After HSIL progression to cancer, the 5-year survival rate for patients with cervical cancer is 66%–79% (9). Novel immunotherapies that target HPV proteins E6 and E7 are particularly promising strategies to improve prognoses of patients with HPV-associated cancers (9). Adequate preclinical animal models are necessary to test such therapies, yet the existing preclinical models for cervical cancer have drawbacks that limit their utility in accurately evaluating novel therapies. For instance, transgenic mice that express HPV16E6E7 under the K14 epithelial cell promoter develop gynecologic malignancies after 6 months when treated chronically with high-dose estrogen (10). However, (i) the lengthy time for tumor outgrowth, (ii) the asynchronous outgrowth of tumors and HSIL, and (iii) the development of central tolerance to HPV16 E6E7 mitigate the usefulness of this model for the evaluation of novel therapies for cervical cancer (11).

Alternatively, HPV-associated malignancies can be studied through patient-derived xenograft (PDX) models, which involve challenging immunocompromised (SCID) mice (12) with cell lines from patient-derived cervical cancers. However, PDX models (i) are incapable of evaluating novel cancer treatments that require an intact immune system, (ii) grow implanted tumors at irrelevant anatomical sites, and (iii) do not model clinical progression through precancerous lesions.

Furthermore, there are multiple immortalized cell lines that express HPV16 E6E7 and induce tumor outgrowth in syngeneic mice (13–17). These existing cervical cancer cell lines exhibit multiple drawbacks including that (i) they are not derived from anatomically relevant tissue, (ii) they do not model clinical progression through precancerous lesions, (iii) and perhaps most notably, they have failed to predict clinical outcomes of novel treatments in past studies (16).
The limitations of the currently existing preclinical models for cervical cancer impede accurate evaluation of novel cancer therapies and necessitate the development of an improved preclinical model for cervical cancer that more faithfully mirrors the clinical scenario. We reasoned such a model would (i) locally express HPV E6E7 in the reproductive tract of mice, (ii) have monitorable tumor progression through HSIL to SCC, (iii) be accompanied by a mutation in the PI3K/AKT pathway, as mutations in the PI3K/AKT pathway are the most common somatic mutations found in cervical cancers (18), (iv) be amenable for the evaluation of novel treatments for HSIL and cervical cancer, including immunotherapies, (v) and display flexibility in which HPV genotype is used for tumorigenesis as there are multiple high-risk HPV types beyond HPV16 (1).

In this study, we developed a novel strategy to induce HPV16-E6E7−expressing (HPV+) cancer in the murine reproductive tract. Because HPV E6E7 is insufficient to induce cervical cancer alone, we hypothesized that if we integrated clinically relevant oncogenes along with HPV E6E7 oncogenes in mouse cervicovaginal tract, we may be able to locally induce HPV+ cancer. Such a tumor model would likely represent an improved preclinical model for cervical cancer. To accomplish this, we utilized the Sleeping Beauty transposase (SB100) to induce local HPV+ tumor outgrowth in the reproductive tract of mice. SB100 integrates DNA flanked by specific inverted repeat sequences into AT dinucleotides in genomic DNA (19, 20), reflecting the random integration of HPV DNA seen in cervical cancer. We integrated plasmids that encoded (i) constitutively active AKT (myrAKT) as it is an oncogene in the PI3K/AKT pathway, (ii) c-myc, because the upregulation of c-myc has been noted in HPV E6E7−transfected cells as well as patients with cancer, where it has been found to be a negative prognostic factor for survival (21–27), and (iii) HPV16-E6E7 with a luciferase reporter gene, abbreviated as AMES-16 plasmids. Using this methodology, we transfected AMES-16 oncogenes into the reproductive tract of C57BL/6 mice, which induced HPV+ tumor outgrowth that progressed through HSIL to invasive SCC. In addition, we isolated and characterized a HPV16+ cancer cell line, Tal3, from an intraperitoneal metastasis of a tumor induced by the AMES-16 model. The AMES-16 model and Tal3 cell line have potentially important implications in the future of preclinical evaluations of treatments for HSIL and cervical cancer.

**Materials and Methods**

**Mice and animal care**

Six- to 8-week-old female C57BL/6NTac mice were purchased from Taconic. All mice were maintained at the Johns Hopkins University School of Medicine Animal Facility (Baltimore, MD) under specific pathogen-free conditions. All procedures were performed according to the protocols approved by the Johns Hopkins Institutional Animal Care and Use Committee and in accordance with recommendations for the proper use and care of laboratory animals.

**Plasmid vectors**

The construction of the Pkt2-Luc-T2a-E7-T2a-E6 plasmid has been described previously (28). To generate Pkt2-LucHPV18E7E6, HPV18E7E6 was synthesized by GenScript, and the sequence was cloned into the Xhol and BstXI sites of the Pkt2-LucHPV16E7E6, as described previously (28). The construction of the pCMV(CAT)T7-SB100 plasmid has been described previously (29). The plasmid was purchased from Addgene (plasmid #34879). The construction of the pT2C-Luc//pPGK-SB13 plasmid has been described previously (30). The plasmid was purchased from Addgene (plasmid #20207). The construction of the pKT2/CLP-AKT plasmid has been described previously (30). The plasmid was purchased from Addgene (plasmid #20281). To generate Pkt2-c-myc, mouse c-myc was first amplified by PCR using pCAGMKOSiE (from Addgene, # 20865) as a template and the following set of primers: 5'-AAATCTAGAGCCACCTGCG- CTTCAAGTGAACCT-3' and 5'-TTTAGATCTTATTGACCCA- GAGTTTCGAAGCT-3'. The PCR product was cloned into the Xbal and Bgl II sites of the Pkt2/clp-akt vector (from Addgene, #20281).

**In vivo tumor formation experiments**

To generate cervicovaginal tumors in C57BL/6NTac mice, mice (5 mice/group) received intraperitoneal injection of monoclonal anti-CD3 antibody (200 µg/mouse) for three consecutive days prior to plasmid electroporation. Mice were anesthetized via intramuscular injection of 80 µL of a solution of 16.7% ketaset (100 mg/mL), 16.7% anased (20 mg/mL), and 66.6% PBS. Mice were then injected with plasmids, dubbed AME-16 plasmids, that encoded luciferase and HPV16-E6/E7 or luciferase and HPV18-E6/E7, combined with plasmids encoding myrAKT, c-myc, and SB100 (10 µg DNA of each plasmid in 20 µL total injection volume) in the lower third of their vaginal canal, and plasmids were locally electroporated into the site of injection. Mice then received two subsequent doses of anti-CD3 antibody (200 µg/mouse) weekly for 2 weeks after plasmid electroporation. For comparison, mice (n = 5) were injected with DNA plasmids encoding only luciferase with HPV16-E6E7 combined with SB100 (10 µg DNA of each plasmid) or with plasmids encoding AKT, c-myc, and SB13, which contains a luciferase reporter gene (10 µg DNA of each plasmid). Mice were sacrificed when (i) tumor diameter >15 mm, (ii) abdomen enlarged because of intraperitoneal metastasis, or (iii) body mass reduced 10% compared with age-matched mice.

**In vivo electroporation**

Mice were electroporated using the ECM 830 Square Wave Electroporation System (BTX). Mice were anesthetized via intramuscular injection of 80 µL of a solution of 16.7% ketaset (100 mg/mL), 16.7% anased (20 mg/mL), and 66.6% PBS. Using 3 mm electrode tweezers, mice were electroporated at the site of plasmid injection in the lower third of the vaginal tract. Settings: 72 V, 200 ms (interval), 20 ms (duration), 8 pulses.

**In vivo bioluminescence imaging**

In vivo bioluminescence imaging was performed using the IVIS Spectrum In Vivo Imaging System Series 2000 (PerkinElmer). Weekly after electroporation, mice were injected intraperitoneally with 200 µL of α-luciferin (Goldbio) substrate. Mice were anesthetized in an isoflurane chamber and bioluminescence imaging for luciferase expression was conducted on a cryogenically cooled IVIS system using Living Image Acquisition and Analysis Software (Xenogen). Images were acquired 10 minutes after α-luciferin injection. The levels of light from the bioluminescent cells were detected by the IVIS imager, integrated, and digitized. The region of interest from displayed images was quantified as total flux using Living Image 2.50 Software (Xenogen).

**Histology and IHC staining**

After euthanizing the tumor-bearing mice, the cervicovaginal tumors were surgically removed, isolated, and placed into 10% neutral buffered formalin solution for adequate fixation with a minimum of 48 hours at room temperature. The tumor samples were then sent to the Johns Hopkins University Oncology Tissue Services for subsequent procedures including paraffin embedding, tissue sectioning, hematoxylin and eosin (H&E) staining, and IHC staining.
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Histology slides were reviewed by two board-certified gynecologic pathologists (Dr. T.C. Wu and Dr. Deyin Xing, The Johns Hopkins Medical Institutions, Baltimore, MD) of the Pathology Department in the Johns Hopkins University School of Medicine (Baltimore, MD).

**ISH to detect the expression of HPV16 oncogenes**

ISH was performed using the RNAscope 2.0 HD Brown Chromogenic Reagent Kit (Advanced Cell Diagnostics) using the supplied protocol and a target probe against HPV16-E6 (Advanced Cell Diagnostics #450591). In short, fresh-cut, formalin-fixed, paraffin-embedded slides were baked at 60°C for 1 hour. After deparaffinization, slides were air-dried, circled with a hydrophobic barrier pen, and then exposed to pretreatment solutions 1, 2, and 3. Target probes were hybridized on slides for 2 hours at 40°C in oven, followed by a series of signal amplification and washing steps. The signals were detected by chromogenic reactions using DAB chromogen followed by counterstaining of hematoxylin (mixed 50/50 with distilled water Sigma-Aldrich).

**RT-PCR to verify HPV16 oncogene expression**

Cell lines [Tal3, TC-1 (positive control) and HEK293 (negative control)] were cultured in vitro and lysed in TRIzol. Cellular RNA was isolated using Direct-zol MicroPrep Kit (Zymogen) and cDNA was made using iScript Reverse Transcription Supermix for qRT-PCR (Bio-Rad) according to the supplied protocols. Gene transcript was amplified using the isolated RNA as a template and HPV16 E6 primers (forward, ACAAACCGTTGTGTGATTTGTT; reverse, CAGTGG-

**Isolation of lymphocytes from tumor-draining lymph nodes**

When tumor-bearing mice were sacrificed to analyze tumor immune infiltrate, the inguinal lymph nodes were also harvested for further analysis. Lymph nodes were suspended in 2 mL of MACS buffer and then ground through a 70 μm nylon filter to achieve a single-cell suspension. Cells were then washed and stained with the tumor infiltrate and tumor exhaustion panels as indicated in Tables 1 and 2.

**Establishment of Tal3 cell line**

The Tal3 cell line was derived from an intraperitoneal metastasis of an AMES-16-plasmid--induced tumor. The intraperitoneal tumor was excised, sliced into small pieces, then filtered through a 70 μm Falcon Cell Strainer (Thermo Fischer Scientific). Tal3 cells were then cultured in complete DMEM media supplemented with 10% FBS, sodium pyruvate, MEM non-essential amino acids solution, l-glutamine, penicillin–streptomycin, and 2-mercaptoethanol (all purchased from Thermo Fisher Scientific). The cells were then harvested using 0.05% Trypsin-EDTA (Thermo Fisher Scientific). The polyclonal cell population was then expanded across three cell passages. The tumor cells (1 million cells/mouse) were injected subcutaneously into C57BL/6 mice to induce tumor outgrowth (first in vivo passage). A resultant tumor from the first Tal3 in vivo passage was excised, processed and expanded as before, and then challenged once again in new C57BL/6 mice (second in vivo passage). A resultant tumor from the second in vivo passage was excised, processed, and expanded as before.

**Isolation of tumor-infiltrating lymphocytes**

Tumor-bearing mice were euthanized, and the tumor tissues were surgically removed and placed into C tubes (Miltenyi Biotec) containing 3 mL of MACS buffer (Miltenyi Biotec) supplemented with magnesium and calcium per the manufacturer's recommendation. Tumors were then cut into 1–2 mm pieces and digestive enzymes were added (0.05 mg/mL collagenase I, 0.05 mg/mL collagenase IV, 0.25 mg/mL DNase I). The tumor/enzyme mixture was incubated at 60°C for 1 hour. After deparaffinization, slides were air-dried, circled with a hydrophobic barrier pen, and then exposed to pretreatment solutions 1, 2, and 3. Target probes were hybridized on slides for 2 hours at 40°C in oven, followed by a series of signal amplification and washing steps. The signals were detected by chromogenic reactions using DAB chromogen followed by counterstaining of hematoxylin (mixed 50/50 with distilled water Sigma-Aldrich).

**T-cell flow cytometry**

To evaluate the kinetics of T-cell recovery post T-cell depletion, peripheral blood was collected via facial vein bleeding into 1.5 mL Eppendorf tubes with 100 μL of 0.05 mol/L EDTA. RBC Lysis Buffer (ChemCruz) was used to lyse red blood cells and then peripheral blood mononuclear cells (PBMC) were subsequently washed in MACs buffer. PBMCs were then stained with the antibodies as described in Supplementary Table S1 and characterized by flow cytometry analysis.

**Table 1. Tumor infiltrate panel.**

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**Table 2. Tumor exhaustion panel.**

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expanded Tal3 tumor cells were diluted into a single-cell dilution and expanded to develop a clonal population of Tal3 cells. Tal3 cells were then used to induce tumor via submucosal injection into the reproductive tract or subcutaneous injection into naive C57BL/6 mice.

Statistical analysis
The statistical analyses were performed with GraphPad Prism V.8 Software (La Jolla). Data were expressed as means ± SD. Kaplan–Meier survival plots were constructed to estimate either tumor-free rate or survival percentage. The log-rank test was used to compare survival times between treatment groups. Comparisons between individual data point were analyzed by two-tailed Student t test. A P < 0.05 was considered statistically significant.

Results
Transfection of plasmids encoding high-risk HPV E6E7 oncogenes with AKT and c-myc oncogenes in the reproductive tract of C57BL/6 mice in the presence of transient T-cell depletion induces tumor growth

To induce tumor outgrowth in C57BL/6 mice, we mixed together 10 μg of each AMES-16-plasmid (Fig. 1B; 20 μL total injection volume), injected them submucosally in the lower third of the vaginal tract of C57BL/6 mice and subsequently electroporated the mice at the site of injection (Fig. 1A). We found that administration of AMES-16 plasmids was not able to induce tumor growth in naive immunocompetent C57BL/6 mice. However, we previously reported that HPV− oral tumor outgrowth via the integration of oncogenes was achievable in conjunction with transient T-cell depletion via administration of anti-CD3 antibody (28). Similarly, when mice were treated with subsequent doses of anti-CD3 antibody (200 μg/mouse/injection) for three consecutive days prior and two weekly doses after plasmid electroporation (Fig. 1A), cervicovaginal electroporation of AMES-16 plasmids induced HPV16− tumorigenesis 3–5 weeks post-electroporation (Fig. 1C), which is monitorable via in vivo bioluminescence imaging (Fig. 1D). In comparison, mice were injected with either HPV16-E7E6 luciferase and SB100 plasmids alone, or injected with AKT, c-myc, and a SB13-luciferase plasmids alone, which failed to induce tumorigenesis, demonstrating the importance of including all four AMES-16 plasmids to achieve tumor outgrowth (Fig. 1D and E). Tumor penetrance among AMES-16 challenged mice was 80% (4 of 5 mice; Fig. 1E). Interestingly, resultant AMES-16 tumors metastasize to the intraperitoneal cavity in approximately 40% of mice, and manifest as poorly differentiated SCCs (Supplementary Fig. S1A–S1D).

Our methodology offers unique flexibility in which oncogenes are utilized for tumorigenesis. As such, we constructed a plasmid that encodes HPV18E7E6 (Fig. 1B) and found that cervicovaginal electroporation of myrAKT, c-myc, SB100, and HPV18E7E6Luc (AMES-18) plasmids in transiently CD3-depleted C57BL/6 mice also induced tumorigenesis (Fig. 1D) in 80% of mice. Ultimate survival at 12 weeks postplasmid injection was similar for AMES-18 tumors.

Figure 1. Generation of spontaneous HPV− cervicovaginal carcinoma model in C57BL/6 mice. Mice received transient CD3 cell depletion at day −3, −2, −1, 7, and 14. On day 0, C57BL/6 mice received 10 μg of each DNA plasmid (total injection volume 20 μg) as a submucosal injection in the vaginal tract, followed by electroporation. A, Schematic of experimental design. B, Schematic of plasmids used to induce HPV− cervicovaginal tumors via oncogene integration. Arrows, direction of plasmid integration. C, Representative images of the development of AMES-16-induced cervicovaginal tumor post electroporation as measured by IVIS Spectrum imaging. Bioluminescence was recorded by IVIS Spectrum after intraperitoneal injection of luciferin solution. D, Tumor growth as monitored by bioluminescence imaging. E, Survival rate shown by percentage. Mice were considered dead due to tumor when tumor diameter >15 mm, and the mice were subsequently sacrificed. **, P = 0.0023; ****, P ≤ 0.0001.
compared with AMES-16 tumors ($P = 0.3039$), and tumor penetrance upon injection of AMES-18 followed by electroporation was likewise 80% (4 of 5 mice; Fig. 1E). Such findings demonstrate that our methodology can assess tumorigenic properties across different high-risk HPV strains and may be appropriate for assessing HPV-targeted therapies for high-risk strains beyond HPV16. In addition, transfection of AMES-16 DNA caused tumor growth, leading to shorter survival compared with mice treated with transfection with E7E6Luc and SB plasmids alone ($P < 0.0001$), and likewise AMES-18 caused tumor growth, resulting in shorter survival compared with mice treated with E6E7 plasmids alone ($P = 0.0023$), suggesting the importance of including AKT and c-myc plasmids to achieve appreciable oncogenicity (Fig. 1E).

**AMES-16–induced cervicovaginal tumors present as SCCs, express relevant tumor markers, and are infiltrated by diverse immune cell populations**

Tumors induced by cervicovaginal electroporation of AMES-16 plasmids present as SCC with morphologic features showing either well-differentiated (Fig. 2A) or poorly differentiated (Fig. 2B) carcinomas, reflective of what is seen clinically. AMES-16 plasmids induced cervicovaginal tumors that display expression of HPV16 E6 as measured by RNA ISH (Fig. 2C and D); integrated AKT and c-myc oncogenes; and typical carcinoma marker, CK14 (Fig. 2E–H).

After transient CD3 depletion, mouse T-cell populations gradually recover over the course of tumor outgrowth and neared the CD3 T-cell levels of naïve mouse by week 6 (Fig. 3A). Lymphocytes were identified via flow cytometry using a gating strategy as shown in Supplementary Fig. S2. AMES-16–induced tumors are infiltrated with a variety of immune cells (Fig. 3B), and the tumors display strong expression of immune exhaustion markers (Fig. 3C), suggesting these tumors may be amenable for study of novel immunotherapies.

**AMES-16–induced cervicovaginal tumors progress from high-grade lesions to invasive carcinoma**

Because cervical cancer progresses through HSIL before forming SCC, we sought to investigate whether our AMES-16–induced tumors similarly progressed through HSIL. Weekly after AMES-16-plasmid electroporation and correlating with specific luminescence values, mice were sacrificed ($n = 3$) and their reproductive tracts were harvested for histologic analysis. Remarkably, we saw that transfection of AMES-16 DNA produced HSIL in the cervicovaginal tract by week 3 (Fig. 4A–D), and we discovered that AMES-16 DNA–induced tumors progress through HSIL to invasive SCC in a stepwise fashion (Fig. 4E). HSIL, illustrated by near-full–thickness cytologic atypia and increased nuclear-to-cytoplasmic (N:C) ratio, present as early as 2 weeks post electroporation and had progressed to invasive SCC by week 4 (Fig. 4E). Similar to squamous intraepithelial lesions in some immunocompromised patients in a clinical setting, multiple HSIL lesions could be found in the mouse reproductive tract as early as week 2 post AMES-16 electroporation, appearing both on the murine cervix and along the vaginal wall. In mice electroporated with only HPV16–E6E7 and SB100 DNA constructs, HSIL were identified at week 3 post electroporation (Supplementary Fig. S3A–S3C). Furthermore, HSIL appearance reliably correlates with luminescence values of around $1 \times 10^6$ in mice electroporated with AMES-16 plasmids (Fig. 4F). These results suggest that this model may be appropriate for assessing novel treatments for HSIL prior to SCC progression.

**Tal3 cell line derived from the AMES-16–induced tumor cells forms SCC in challenged immunocompetent mice**

Transplantable tumor cell lines are still widely used and can be useful for evaluating certain cancer treatments. As such, we sought to create an HPV$^+$ cell line that displayed carcinoma morphology and was accompanied by a PI3K/AKT pathway mutation, characteristics not displayed by current HPV–E6E7–expressing cell lines. To this end, a single-cell clone derived from an intraperitoneal metastasis from a
cervicovaginal AMES-16 tumor, designated Tal3, was selected and expanded for further analysis. To test tumorigenicity, Tal3 was subcutaneously injected into C57BL/6 mice at different doses. We observed that Tal3 cells achieved 100% tumor penetrance at a dose of $1 \times 10^5$ (Fig. 5B and C; Supplementary Table S2). Upon sacrifice, Tal3 tumors were analyzed histologically and found to express relevant tumor markers including c-myc, AKT, HPV16 oncogenes (Fig. 5A; Fig. 6B and C) and proliferation marker Ki-67 (Fig. 6D) and found to display SCC morphology (Fig. 6A). In addition, we measured the tumor-infiltrating leukocytes frequencies by flow cytometry. As shown in Fig. 6E, Tal3 tumors were infiltrated with a variety of immune cells, and the tumor-infiltrating lymphocytes were shown

Figure 3.
T-cell characterization in HPV16+ spontaneous cervicovaginal tumor. A, Time course of T-cell recovery post depletion. Mice received transient T-cell depletion via administration of intraperitoneal injection of 200 μg anti-CD3 antibody daily 3 days prior to AMES-16 electroporation and weekly for 2 weeks after electroporation. B, Tumor-bearing mice were sacrificed 6 weeks post AMES-16 electroporation and tumor immune infiltrate was evaluated by flow cytometry. Bar graph summary of AMES-16–induced cervicovaginal tumor-infiltrating lymphocytes. C, Bar graph summary of exhaustion markers expressed by tumor-infiltrating lymphocytes (TIL) 6 weeks post AMES-16 electroporation.

Figure 4.
Anatomical orientation and lesion progression from HSIL to SCC in spontaneous HPV+ cervicovaginal carcinoma model. At each time point following the AMES-16-plasmid electroporation, three mice were sacrificed and their reproductive tracts were harvested and fixed in formalin for sectioning and histologic analysis. Representative images of the histologic examination were selected. A, Magnification (> 2) of mouse reproductive tract 3 weeks post AMES-16 DNA electroporation anatomically labeled. B, Magnification (> 40) showing cervical HSIL in black box with orientation as shown by cervix in A. C, Magnification (> 40) showing vaginal HSIL in dotted black box as shown in lower left corner of A. D, Magnification (> 20) showing vaginal HSIL in dashed black box and carcinoma in black circle with orientation as shown in A. E, Development of SCC from HSIL. Left, emergence of HSIL (see box) corresponding with luminescence values approximately $1 \times 10^6$. Middle, appearance of SCC (see box) and corresponding with luminescence values approximately $1 \times 10^7$. Right, appearance of HSIL and invasive carcinoma corresponding with luminescence values approximately $1 \times 10^8$. F, Left, representative H&E of HSIL lesion 2 weeks post AMES-16 DNA electroporation. Right, HPV16 RNA (black arrows) colocalizes with HSIL lesion at week 2 post AMES-16 DNA electroporation with no evidence of HPV RNA expression in dermal compartment. HPV16 RNA was performed by RNAscope ISH.
to display exhaustion markers (Fig. 6F). In addition, we challenged C57BL/6 mice with Tal3 cells submucosally in the cervicovaginal tract of C57BL/6 mice. Mice achieved 100% tumor growth and demonstrated SCC morphology resembling that of the Tal3 tumor generated by orthotopic tumor challenge (Supplementary Fig. S4A–S4D). These findings suggest that Tal3 may serve as a potentially important transplantable preclinical tumor model for the studies of novel treatments for HPV16+ cancer.

Discussion

Preclinical models of cervical cancer should recapitulate critical aspects of the human malignancy. Here, we present the development of a novel preclinical model of cervical cancer that utilizes SB100 to integrate clinically relevant AMES-16 oncogenes to induce tumor outgrowth in the reproductive tract of mice. Remarkably, our model recapitulates key features of (i) gynecologic tumor outgrowth,
(ii) progression through precancerous lesions to SCC, (iii) and local expression of HPV-oncogenes. Such innovation has been sought after in the field and is particularly important upon documentation of the inadequacy of existing preclinical models to predict clinical outcomes (15–17).

Although transient T-cell depletion by anti-CD3 mAb is necessary to induce tumor formation via integration of AMES-16 plasmids, the T-cell population gradually recovers over the course of tumor growth (Fig. 3A). In addition, the resultant tumors are infiltrated with a variety of immune cells and express various exhaustion markers resembling cervical cancer (Fig. 3B and C). Thus, this model may be appropriate for the evaluation of novel cancer therapies that require a functioning immune system, including therapeutic HPV vaccines and/or checkpoint blockade therapies. This preclinical tumor model may be potentially useful due to high clinical and translational interest in using immunotherapy for HPV+–associated cancers (32, 33). To better determine whether such a preclinical tumor model has clinical relevance, further studies need to be conducted to evaluate whether outcomes of the therapeutic interventions tested in this model correlate with responses of the therapeutic interventions in clinical trials.

It was an interesting and unexpected finding that our AMES-16 model metastasizes to the intraperitoneal cavity in 40% of mice (Supplementary Fig. S1A). All intraperitoneal tumors present as poorly differentiated squamous carcinomas. The squamous morphology of the intraperitoneal tumors suggest that these tumors likely represent true metastases derived from the reproductive tract. Furthermore, because there is no route from uterine lumen to intraperitoneal cavity via fallopian tube because the bursa covers ovary and fallopian tube, the tumors in the peritoneal cavity likely represents a metastasis from uterine serosa to intraperitoneal cavity. It is noteworthy that although most advanced human cervical cancers directly extend to vagina and/or uterine corpus, parametrium, bladder and rectum, they only occasionally spread to the peritoneal cavity. This aberrant metastasis site may represent a limitation of murine models to anatomically mimic metastatic human cancers.

Our preclinical model’s ability to demonstrate tumor progression from HSIL to SCC is an unprecedented strength in modeling cervical cancer. HSIL lesions are detected by pap smears and are treated by surgical or ablative therapies with the success of such therapies reaching 75%–85% (6, 7). However, such surgical therapeutic approaches may potentially result in cervical incompetence, leading to preterm birth (34). Thus, the availability of such a model will allow the development of medical interventions for control of HSIL, such as vaccines, in preclinical models. Novel therapeutic approaches that minimize potential detrimental reproductive effects are particularly important for women who wish to bear children. Here, we show that AMES-16 plasmid–induced cervicovaginal tumors progress through HSIL, and these HSIL are potentially monitorable via bioluminescence (Fig. 3A), opening an unprecedented opportunity for preclinical evaluation of novel treatments for HSIL. One potential limitation of our model for the assessment of novel HSIL therapies is that the model HSIL progresses to SCC within about a week (Fig. 4A). Thus, this model may not be suitable for assessing treatments for HSIL that require a lengthy mechanism of action.

To gain insight on the role of T cells in HSIL development, we electrooporated fully immunocompetent mice with AMES-16 DNA plasmid without CD3 depletion and sacrificed them at different time points to evaluate their reproductive tracts for HSIL. We found that HSIL lesions could be found in fully immunocompetent AMES-16 DNA electroporated C37BL/6 mice by week 3 post plasmid injection, suggesting that the presence of T cells does not prohibit normal epithelium from developing into HSIL (Supplementary Fig. S5A–S5D). However, no lesions were observed on week 4 post AMES-16-injection, suggesting that the presence of T cells eventually eliminates the HSIL lesions and precludes cancer formation (data not shown).

In our studies, HSIL and SCC were found to express HPV16 oncogenes by RNA ISH. The RNA ISH stain for HSIL (Fig. 4F) demonstrated mainly nuclear speckle staining pattern, probably due to weak cross-hybridization with integrated HPV DNA and relatively lower levels of HPV E6 RNA in the cytoplasm. In comparison, the RNA ISH stain for SCC (Fig. 4C and D) demonstrated both nuclear and cytoplasmic staining pattern, probably due to relatively higher levels of HPV E6 RNA in the nuclear and cytoplasm in cancer. It has been demonstrated that HPV-associated malignancies tend to upregulate E6/E7 RNA due to multiple mechanisms related to HPV genome integration (reviewed in ref. 35).

Furthermore, we evaluated whether in vivo electroporation of HPV16-E6E7Luc and SB100 DNA constructs alone could form HSIL in the absence of the AKT and Myc oncogenes. We found that this treatment could induce HSIL 2–3 weeks post electroporation but that no lesions were observed by week 4 post electroporation (Supplementary Fig. S3A–S3C), suggesting the importance of AKT and Myc oncogenes for cancer formation. P16INK4A is frequently overexpressed in HPV-associated malignancies. To test whether our model additionally overexpresses p16, we stained AMES-16 DNA electroporated tumors with mouse p16-specific antibody using Abcam antibody Ab51243. While we observed upregulation of p16 protein in HSIL as well as SCC in mice, the p16 staining pattern only show the “block” staining pattern (diffuse nuclear and cytoplasmic staining) that is typically encountered in human samples infected with HR-HPV (Supplementary Fig. S6A–S6D). Our findings are consistent with those reported by other investigators (13).

In our AMES-16 model, multiple HSILs occur in different regions of the lower reproductive tract of AMES-16 DNA electroporated mice, including on the cervix and on the vaginal wall (Fig. 4). Because of the small size of the murine reproductive tract, our electroporation methodology cannot induce HSIL on only the cervix. This may represent a limitation of our model in capturing clinical features of typical cervical cancers. However, in humans although high-risk HPV most often affects squamous epithelial cells in the cervix, it can also cause squamous intraepithelial lesion and carcinoma in the vagina, vulva, and other locations, suggesting a common HPV-related oncogenic mechanism in the reproductive tract was recapitulated in our model system.

As cancer therapies become more personalized, it is important to have preclinical models that represent diverse clinical scenarios. To that end, the methodology presented in this study offers unique flexibility in which oncogenes are used to induce tumorigenesis. Here, we showed evidence that this methodology can be used to induce tumor outgrowth using both HPV16 E6E7 and HPV18 E6E7 (Fig. 1). Although HPV16 and HPV18 are responsible for causing the majority of cervical cancers, there are at least 13 other high-risk HPV types that cause cervical cancer (1). This study suggests that this methodology may be amenable to evaluating targeted therapies for diverse high-risk HPV genotypes. Of additional interest, this methodology may also be able to lend insight to mechanistic differences in tumorigenesis between the high-risk HPV genotypes.

In addition, we utilized myrAKT and overexpression of c-myc to help drive tumorigenesis in our model. However, the AMES-16 plasmid combination can be easily altered to reflect the oncogene...
profile of interest by adding or replacing relevant plasmids encoding different oncoproteins to investigate their roles. Furthermore, this methodology could be expanded in mouse strains beyond C57BL/6. A previous study has shown that FVB/n mice have increased susceptibility to HPV16 E6E7–driven malignancy compared with C57BL/6 mouse. It would be of interest to investigate whether FVB/n mice would require the same degree of immune suppression to induce tumorigenesis compared with C57BL/6 mouse. It would be also of particular interest in evaluate this methodology in different human MHC class I transgenic mice or genetically outbred mice and subsequently evaluate their immune responses to novel treatments such as E6E7-targeted vaccines. Such information may be important for future clinical translation.

Although our methodology of HPV+ tumor induction via the integration AMES-16 oncoproteins in the reproductive tract of C57BL/6 mice may be the preclinical model most closely related to human cervical cancers to date, we also found merit in developing the Tal3 cell line, which we derived from an intraperitoneal metastasis of an AMES-16–induced cervicovaginal tumor. Despite the previously discussed limitations of transplantable cell lines for the study of cervical cancer, which remain for Tal3, transplantable tumor-forming cell lines are still useful in rapid screening of novel drugs or vaccines. Prior to the development of Tal3 cell line, there were no HPV16 E6E7–expressing cell lines that demonstrated SCC phenotype, a clinically important parameter in modeling cervical cancer. As Tal3 displays these important characteristics, it may serve as an appropriate preclinical tumor model for the studies of HPV+ cancer. Together, our AMES-16-plasmid-induced cervicovaginal tumors and Tal3 cell line represent marked advancements in the preclinical modeling of HSIL and/or cervical cancer and hold promise to more reliably predict clinical outcomes of novel therapeutic interventions.

Authors’ Disclosures

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Authors’ Contributions


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References

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