Validation of an HPV16-mediated Carcinogenesis Mouse Model

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Abstract. Background/Aim: Human papillomavirus Type 16 (HPV16) infection is a necessary but alone insufficient cause of invasive cervical cancer (ICC) and likely causes other genital cancers. Individual genetic variability influences the natural history of the neoplasm. Developing a variety of animal models to investigate HPV16-mediated carcinogenesis is important to Phase 1 trials for human cancer treatments. Materials and Methods: C57BL/6 mice expressing the HPV16-E7 transgene were treated with 100 nmoles of 7,12-dimethylbenz(a)anthracene (DMBA) on dorsal-thoracolumbar skin for ≤20 weeks. Results: Transgenic-HPV16E7 mice showed more tumors (14.1±1.49 vs. 7.2±0.73) that more quickly reached maximal size (17.53±0.53 vs. 28.75±0.67 weeks) than syngeneic controls. Conclusion: DMBA topically-treated C57BL/6-HPV16E7 mice developed chronic inflammation as well as benign and malignant lesions, many of which ulcerated. Histology showed that the HPV16-E7 transgene more than doubled the effect of complete carcinogenesis against a C57BL/6 background alone, strongly influencing the number, size, and time-to-maximal tumor burden for DMBA-exposed transgenic-C57BL/6 mice.

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Data show that the genetic background influences the sensitivity of murine models to neoplastic induction using cancer initiators and promoters such as benzoyl peroxide, benzo(a)pyrene (BP), 12-O-tetradecanoylphorbol-13-acetate (TPA), and 7,12-dimethylbenz(a)anthracene (DMBA) (6, 10, 17-20). Some strains are more vulnerable than others to the development of earlier and more numerous papillomas and invasive cancers (6, 10, 17-20). Data suggest C57BL/6 mice are sensitive to complete carcinogenesis using weekly topical DMBA, but resistant to the two-stage induction and promotion using single-dose DMBA followed by weekly TPA, respectively (18, 19). Complete carcinogenesis using DMBA in C57BL/6 mice causes more numerous cutaneous papillomas in comparison to other similarly-treated strains, including BALB/c, CD-1, and FVB/N mice (18, 21). The relative sensitivity of C57BL/6 mice to complete carcinogenesis using DMBA may provide an additional platform to evaluate HPV oncogenes in neoplastic induction in vivo. Dose-responsiveness to chemical carcinogenesis combined with the tumor promoter properties of the HPV16E7 transgene, may make the C57BL/6-HPV16E7 transgenic mouse a valuable screening tool for therapeutic agents and an informative model for the underlying mechanisms of human cancers.

Materials and Methods

Subjects and Setting. Nineteen C57BL/6 HPV16E7-transgenic mice, re-derived using frozen embryos that were previously developed (kindly donated by Dr. Paul Lambert, University of Minnesota, MN, USA (10, 15-17, 22-26)), using a standard protocol (UCLA Division of Laboratory Animal Medicine (DLAM) Assisted Reproduction Technology Lab), and 20 syngeneic controls were transferred to an Animal Research Committee-approved experimental protocol (ARC #2008-134-11). Development of K14-HPV16E7 transgenic mice is described extensively elsewhere (15). Briefly, C57BL/6-HPV16E7 mice showed unique phenotypic features (27) and tail biopsies at 10 days tested positive for E7 (27).

Study procedures. DMBA, 100 nmoles (DMBA; Thermo Fisher Scientific, Waltham, MA, USA) was dissolved in 200 μl acetone and administered weekly, 2-days following hair shaving using a standard protocol. DMBA treatment continued ≤20 weeks, followed by ≤10 additional weeks of observation. Mice were euthanized for signs/symptoms of distress unrelieved by symptomatic treatment, cumulative papilloma diameter of 2 cm, or 30 weeks following enrollment (28). Necropsy was performed. All skin papillomas and all treated dorsal-flank and untreated (negative-control) ventral-flank skin was preserved; half of each tissue was snap frozen and half was formalin-fixed and paraffin embedded (Electron Microscopy Sciences; Hatfield, PA, USA). Formalin-fixed papilloma samples from each animal were sectioned, stained with hematoxylin and eosin (H&E), and examined by two veterinary pathologists (GWL, LW).

Exposure of interest. The effect of the HPV16E7 transgene on the C57BL/6 background was the primary exposure of interest. However, an original aim was to evaluate the effect of topical 0.5 mM solution of bovine α-lactalbumin made lethal for tumors (BAMLET), prepared and stored using a standard protocol, to 0.9% sodium chloride solution (NS) treatment (29-32). Data analyses showed no effect of BAMLET at this concentration.

Outcomes of interest. Time to first tumor, number of non-ulcerated versus ulcerated tumors, tumor diameter (Mitutoyo 8-0.0005 Digital Caliper; Mitutoyo Corporation, Kawasaki, JPN), tumor shape, color, and texture as well as body weight (Fast Weight MS-500-BLK Digital Pocket Scale; Vincennes, Indiana, USA) were recorded weekly for each animal into an electronic database (Office Access 2003, Microsoft Corporation, Seattle, WA, USA). Open lesions showing concavity of tumor masses and heightened vascularity were classified as ulcerated lesions. Treated dorsal skin was photographed and archived weekly (1024×768 pixels, 180 dpi horizontal and vertical resolutions, 24 bits, F/8, focal length, 7 mm, at 1/250 second exposure time) using a Canon Powershot G-5 digital camera (Canon USA, Inc., Melville, New York, USA).

Statistical analysis. Descriptive and tabular analyses contrasted the mean number of intact and ulcerated papillomas for weekly observations, and across genotypes (PROC GENMOD, SAS, Cary, NC, USA) (33). Generalized estimating equations (GEE) models were employed to determine the effects of time on study, and the presence of the HPV16E7 transgene. The GEE model used a negative binomial distribution with an autoregressive variance-covariance structure, and a compound symmetric covariance structure examined the effect of repeated weekly measurements using a mixed model. Cox Proportional Hazard (CHF) analyses were used to evaluate the time to appearance of the first papilloma, for all mice, comparing HPV16E7 transgenic to syngeneic mice (PROC PHREG, SAS, Cary, NC, USA) (34). Additionally, a generalized linear model was used to estimate the average tumor length at the time of euthanasia by genotype (PROC MIXED, SAS, Cary, NC, USA) (35). In all analyses, we controlled for the effect of gender, and observation time.

Results

The HPV16E7 transgene strongly affected overall survival, as well as the timing, size and number of intact and ulcerated tumors for DMBA-treated C57BL/6 mice. Although all DMBA-treated mice developed hyper-pigmented skin areas, the color, fluency and size of hyperpigmentation varied across mouse genotype. Transgenic mice showed discolored, scaly, dry skin; also, hair regrowth after shaving was more rapid. Syngeneic mice alone showed transiently pigmented, smooth skin with areas showing 5-100 pin-points, discrete and flat hyperpigmented lesions. Survival for transgenic mice was half that of syngeneic controls: μ=17.53 (16.5, 18.6) vs. 28.75 (27.4, 30.1) weeks (Table 1). Most syngeneic mice survived the 30 weeks of observation; only 30% (6/20) were euthanized due to a combined tumor diameter of >1.5 cm, invasive tumors or poor health. Comparatively, no transgenic mice survived beyond 22 weeks after DMBA treatment was initiated, and 79% (15/19) were euthanized due to cumulative tumor size >1.5 cm.
Transgenic mice showed quicker onset and more numerous papillomas that more frequently ulcerated, in comparison to syngeneic controls (Figure 1). On average, transgenic mice developed the first papilloma 6.69 weeks earlier than syngeneic controls: 11.26 (95% Confidence Interval: (10.4, 12.1)) vs. 17.95 (16.0, 19.9) weeks, respectively (Table I, Figure 1). Additionally, the total number of papillomas ranged wider for transgenic than syngeneic mice, 3-32 vs. 2-12, respectively (Table I, Figure 1). The maximum for total tumors was two-fold higher for transgenic than syngeneic mice: 14.11 (11.2, 17.0) vs. 7.20 (5.8, 8.6) (Table I, Figure 2A). Even after controlling for the effects of other covariates, the fully-adjusted model suggests HPV16E7 transgenic mice showed nearly 26 more papillomas per animal than similarly- treated syngeneic controls $[\mu=25.9 (13.8, 48.6)]$, (Table I, Figure 2A). Terminal measures showed ulcerated tumor beds for transgenic mice were 38% larger, on average, than for syngeneic controls: 0.11 (0.09, 0.13) vs. 0.08 (0.06, 0.10) cm, respectively (Table I, Figure 2B). The fully-adjusted model suggested transgenic mice showed 21 more ulcerated tumors than syngeneic controls, despite the overall longer survival of the latter group: $\mu=20.8 (12.9, 33.4)$.

Multivariate analyses suggest the cumulative effect of topical weekly DMBA on C57BL/6 (mouse) skin was approximately 1 papilloma $[\mu=1.30 (1.25, 1.35)]$, consistent with the findings of others employing the same treatment protocol (19). Additionally, analyses showed that no tumors are expected in the absence of DMBA exposure, consistent with our observation of syngeneic and transgenic mice housed in our breeding colony $[\mu=0.005, (0.002, 0.015)]$.

Although both transgenic and syngeneic mice developed tumors with weekly DMBA treatment, tumor features and onset characteristics varied between groups. Invasive squamous cell carcinomas (SCC) were more often evident on histology for HPV16E7-transgenic than syngeneic mice: 89% (17/19) vs. 40% (8/20), respectively ($p=0.001$); however, carcinoma in situ (CIS) was detected nearly as often: 21% (4/19) vs. 15% (3/20), respectively (Figure 3A and 3B). Keratoacanthomas, a low-grade tumor resembling SCC, were detected statistically significantly more often in syngeneic controls: 60% (12/20), $p=0.04$ (36). Albeit comparisons are not statistically significantly different, sarcoma was detected among syngeneic mice alone and evidence of epithelial hyperproliferation was more often seen among transgenic mice on histology (Figure 3A and 3B).

**Discussion**

These C57BL/6-HPV16E7 mice developed chronic epithelial inflammation and benign and malignant lesions, many of which ulcerated, after weekly topical DMBA-treatments. Some data suggest that the observed skin hyperplasia may
be due to T-lymphocyte-induced inflammation (37). Others have reported dry scaly skin in both HPV16-E6 and -E7 transgenic mice (15). Some HPV16E7-effects we report may be due to independent effects of retinoblastoma protein (pRB); for example, 28% of estrogen-treated FVB/129/C57-K14HPV16E7 mice and 0-3.4% of otherwise similar animals develop skin malignancies in the absence of topical carcinogen exposure (38). Others report airway and esophageal hyperproliferation and external skin dysplasias for K14HPV16E7 mice when crossed with Cre-lox induced Rb-knock-out mice, suggesting independent activities beyond the abrogation of pRb (39). Thus, a C57BL/6-HPV16E7 mouse model adds to other HPV16-E7 transgenic models for studying HPV16 pathogenesis and treatments.

Histology showed the HPV16-E7 transgene more than doubled the effect of complete carcinogenesis against a C57BL/6 background alone. In HPV16-mediated human tumors, HPV-E6 and -E7 oncogene expression strongly predicts dysplastic characteristics and cell proliferation that increase after HPV integration into the host genome (15, 40-45). Nonetheless, differences in our carcinogenesis method complicate comparisons of our findings to other published reports. Data suggest HPV16-E7 expression poorly promotes cancers when initiated by single-dose DMBA; however, two-stage carcinogenesis initiated using DMBA, TPA or co-expressed HPV16E6, followed by HPV16-E7 expression efficiently promotes neoplasia (6, 8, 25). For example, Song et al. report an average of approximately 0.8 papillomas and no SCCs in FVB/HPV16E7 mice initiated with a single 300 nmole DMBA dose after 20 weeks of observation (6, 8, 25). However, FVB/HPV16E7 mice treated with topical DMBA and followed by 20 weekly TPA doses induced about 20 papillomas/HPV16E7-mouse, also without carcinomas (6). Effectively, our findings suggest topical, weekly DMBA exposure as a complete carcinogen, within the context HPV16-E7 transgene, more successfully promotes malignancies in C57BL/6 mice when compared to other two-stage carcinogenesis protocols. Nonetheless, like others using HPV16E7-transgenic inbred mouse strains, these findings show a strong effect of the (E7) oncogene (7, 10, 17, 27).

Some data suggest that wild-type C57BL/6 mice show greater sensitivity to some chemical and biological cancer initiators and promoters than other murine models (18, 21). For example, Reiners et al., reported C57BL/6 mice show greater sensitivity to complete carcinogenesis using topical DMBA, but lower sensitivity to similar administration of Benzoyl Peroxide (BP) than do SENCAR mice (19). In a separate study using a lower DMBA dose of 39 nmole, 9% of papillomas on SENCAR mice, and 38% on CD-1, 23% on BALB-C, 50% on FVB and 15% on C57BL/6 mice converted to malignancy following up to 20 weeks of topical DMBA (21). This suggests the possibility of using different doses in creating a range of varying dysplastic characteristics in the hope of studying cancers of different severities. Lastly, no published findings to date evaluate the effect of FVB-HPV16E7 mice using a complete carcinogenesis model employed herein, making direct comparisons of HPV16E7-transgene effects for these two genetic backgrounds difficult.

Some reports suggest HPV16E7 can be detected in differentiating suprabasalar cells of HPV16E7-transgenic mice, and that higher cell cycling increase the proliferating cell nuclear antigen (PCNA) in spinous and granular cells of primary human keratinocytes in raft cultures (46). Our data suggest constitutive epithelial expression of HPV16E7 with repeated DMBA-initiation induces sufficient genetic instability in the stratified epithelium to cause cancers (7, 27).

Figure 2. Comparison of C57BL/6 HPV16-E7 transgenic and syngenic mouse survival showing the average number of papillomas, total (2A) and ulcerated (2B), observed at weekly intervals during the treatment period.
Genetic characteristics of C57BL/6 mice-alone may contribute significantly to differences between observations reported herein and other published reports. For example, Reiners et al. report that 10 and 100 nmoles of DMBA topically applied weekly caused a less than additive effect on tumor formation in C57BL/6 mice: <1 vs. about 1 papilloma/mouse at 20 weeks, with 1 vs. approximately 4 at 35 weeks, respectively (19). Our data are consistent with findings from weekly 100 nmoles DMBA and our fully-adjusted multivariate analyses show an increase of almost 1.3 papillomas/week across the study period. Although HPV16E7 strongly influenced tumor formation in our study, we could not formally evaluate synergy between DMBA and HPV16E7 in these analyses.

Our study may be limited by certain factors. The small sample size may limit our power to detect some effects important in tumorigenesis. The HPV16E7 transgenic mouse model described herein may poorly-mimic natural infection of HPV16 in humans and findings may be poorly-generalized to human populations. Nonetheless, findings from animal models are important to the development of randomized human clinical trials.

In summary, our experiments explore the effects of DMBA and HPV16E7 on carcinogenesis using a C57BL/6 mouse model. While many of our findings that relate to DMBA-mediated carcinogenesis are consistent with the published works of others, our data suggest that the HPV16E7 transgene more strongly affects the natural history of papillomas than might be expected with lower-risk HPVs that are often associated with skin warts (47).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

<table>
<thead>
<tr>
<th>Histology</th>
<th>Transgenic (%)</th>
<th>Syngeneic (%)</th>
<th>p-Value*</th>
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</thead>
<tbody>
<tr>
<td>Invasive SCC</td>
<td>17 (89)</td>
<td>8 (40)</td>
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<tr>
<td>CIS</td>
<td>4 (21)</td>
<td>3 (15)</td>
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<tr>
<td>Keratoacanthoma</td>
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<td>12 (60)</td>
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<td>Hyperproliferation</td>
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<td>1 (05)</td>
<td>0.003</td>
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*Fisher’s exact test performed due to small sample sizes.

Histological exemplars show squamous cell carcinomas detected in mice from each genotype group. Pictures were taken using 40× magnification: (A) C57BL/6 HPV16E7 (B) C57BL/6 syngeneic. The Table reports the type and frequency of cutaneous lesions detected in mice for each genotype and treatment group and compares frequency of each lesion-type across genotypes within the treatment groups.

Figure 3. Hematoxylin and eosin stained C57BL/6 (A) HPV16-E7 transgenic and (B) syngeneic mouse skin tissues Treated with 100 nmoles DMBA weekly for up to 20 weeks with comparison of histology findings.

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