Human Papillomavirus 16 E5 Oncogene Contributes to Two Stages of Skin Carcinogenesis

John P. Maufort,1 Sybil M. Genther Williams,1 Henry C. Pitot,1,2 and Paul F. Lambert1

1Department of Oncology and the McArdle Laboratory for Cancer Research and 2Department of Pathology, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin

Abstract

High-risk human papillomaviruses (HPVs), which cause the vast majority of cervical cancer, other anogenital cancers, and a subset of head and neck squamous cell carcinomas, encode three oncogenes: E5, E6, and E7. To determine the oncogenic properties of HPV16 E5 in vivo, we previously generated K14E5 transgenic mice, in which expression of E5 was directed to the basal compartment of stratified squamous epithelia. In these mice, E5 induced epidermal hyperplasia and spontaneous skin tumors. In the current study, we determined how E5 contributes to tumor formation in the skin using a multistage model for skin carcinogenesis that specifies the role of genes in three stages: initiation, promotion, and malignant progression. Both initiation and promotion are required steps for papilloma formation. K14E5 mice treated with the initiating agent 7,12-dimethylbenz(a)anthracene (DMBA) developed more papillomas than like-treated nontransgenic mice, whereas neither K14E5 nor nontransgenic mice treated with the promoting agent 12-O-tetradecanoylphorbol-13-acetate (TPA) developed papillomas. K14E5 mice treated with both DMBA and TPA to induce large numbers of papillomas had a higher incidence and earlier onset of carcinoma progression compared with like-treated nontransgenic mice. Thus, HPV16 E5 contributes to two stages of skin carcinogenesis: promotion and progression. The progressive neoplastic disease in K14E5 mice differed from that in nontransgenic mice in that benign tumors converted from exophytic to endophytic papillomas before progressing to carcinomas. Initial genetic and immunohistopathologic analyses did not determine the underlying basis for this distinct morphology, which correlates with a highly penetrant neoplastic phenotype. [Cancer Res 2007;67(13):6106–12]

Introduction

Human papillomaviruses (HPV) are nonenveloped dsDNA viruses that infect stratified squamous epithelia and cause warts or other persistent but self-limiting proliferative lesions. A subset of the mucosotropic HPVs called the “high-risk HPVs,” including HPV16 and HPV18, are etiologic agents for the vast majority of cervical cancer, other anogenital cancers, and a subset of head and neck squamous cell carcinomas (1). HPV16, which is associated with ~60% of cervical cancer, encodes three oncogenes: E5, E6, and E7 (2–6). HPV16 E5, an 83-amino acid, hydrophobic membrane-associated protein that localizes to the endoplasmic reticulum (7, 8), can transform murine fibroblasts and keratinocytes in tissue culture (3, 5, 6), enhance the immortalization potential of E6 and E7 (9), and cooperate with E7 to stimulate the proliferation of human and mouse primary cells (10, 11). E5 is thought to act as an oncogene mainly through its ability to enhance the activation of the epidermal growth factor receptor (EGFR) in a ligand-dependent manner (6, 12, 13), although the exact manner by which it does so remains unclear (7, 14, 15).

To understand better the function of E5 in vivo, our laboratory generated HPV16 E5 transgenic mice in which a codon-optimized version of the HPV16 E5 gene was placed behind the human K14 promoter, which drives expression of the E5 gene to the basal compartment of the stratified squamous epithelia, such as the epidermis of the skin, the epithelial lining of the lower female reproductive tract, and the oral cavity. Initial characterization of K14E5 transgenic mice revealed phenotypes, including epidermal hyperplasia, hyperkeratosis, enhanced DNA synthesis, aberrant differentiation, and the formation of spontaneous skin tumors (16). These phenotypes were similar to phenotypes observed in mice that overexpress ligands of the EGFR in similar tissues (17–19).

Previously, our laboratory analyzed the oncogenic properties of HPV16 E6 and E7 in the skin. Both K14E6 and K14E7 transgenic mice developed spontaneous skin tumors. Whereas the spontaneous skin tumors in K14E7 mice were primarily benign, the tumors arising in the K14E6 mice were primarily malignant (20, 21). Three stages of tumorigenesis have been identified in a classic model for multistage skin carcinogenesis using chemical carcinogens: initiation, promotion, and progression (22). Initiation and promotion contribute to the formation of benign tumors or papillomas. Progression is defined as the process leading to malignant conversion. Using 7,12-dimethylbenz(a)anthracene (DMBA) as the initiating agent, 12-O-tetradecanoylphorbol-13-acetate (TPA) as the promoting agent, and assessing the potential synergy between these chemical carcinogens and E6 or E7, E6 was found to contribute to the stages of promotion and progression in skin carcinogenesis, whereas E7 contributed only to the promotion stage of skin carcinogenesis (20). These results together with the nature of the spontaneous tumors arising in these mice showed that E6 was the more potent oncogene in the skin of transgenic mice.

In this study, we evaluate the role of HPV16 E5 in skin carcinogenesis. In our previous report, it was noted that lines expressing high levels of E5 have high rates of spontaneous skin tumor formation (16). To assess further the nature and incidence of spontaneous skin tumor formation induced by HPV16 E5, lines of E5 transgenic mice expressing the transgene at either low or high levels were monitored for spontaneous skin tumor formation. All lines of E5 transgenic mice were found to display statistically significant induction of spontaneous tumors compared with
nontransgenic mice. To analyze HPV16 E5 in the context of skin carcinogenesis, experiments were carried out on K14E5 mice using various treatments with DMBA and TPA as were done previously with K14E6 and K14E7 mice. Based on these studies, E5 was found to contribute to both the stages of promotion and progression in skin carcinogenesis. These results show that, like E6, E5 is a potent oncogene in the skin. An interesting feature of tumor progression unique to the E5 transgenic mice was the observation that benign tumors arising in these mice evolved from exophytic lesions to endophytic lesions. These endophytic benign tumors were the tumors that progressed to malignant carcinomas. This finding indicates that E5 drives carcinogenesis through a unique histopathologic pathway. We hypothesize that this property likely reflects the mechanism of action of E5 in carcinogenesis.

**Materials and Methods**

**Mice.** The multiple lines of K14E5 transgenic mice used in this study were described previously (16). They were bred and maintained in the homozygous state on the FVB/N inbred genetic background in the Association for Assessment of Laboratory Animal Care–approved McArdle animal facility according to an animal protocol approved by the University of Wisconsin School of Medicine and Public Health’s Institutional Animal Care and Use Committee. Genotyping was carried out as described previously (16).

**Treatment with DMBA and TPA.** At 4 to 6 weeks of age, female mice were shaved on their backs and divided into three groups. The first group of mice was treated with DMBA, an initiating carcinogen, and TPA, which is a promoting agent. The second group of mice was treated with DMBA only, and the third group of mice was treated with TPA only. The DMBA plus TPA–treated group was topically given with 0.01 μmol DMBA dissolved in acetone to the shaved areas of the skin. One week later, the same areas of skin of these mice were topically treated with 15 nmol TPA dissolved in acetone twice weekly for 20 weeks. For the DMBA only group, the skin of the mice was topically given once with 0.3 μmol DMBA and monitored. For the TPA only group, the skin of the mice was topically given with 15 nmol TPA twice weekly for 20 weeks and monitored. All mice were examined every 2 weeks for the development of papillomas.

**Monitoring of malignant progression of papillomas from chemically treated mice.** Mice treated with DMBA and TPA were examined every 2 weeks for the development of papillomas. After TPA treatment was completed at week 20, mice were kept an additional 20 weeks to follow the malignant progression. Tumors that became flat, open, and invasive were provisionally classified as malignant carcinomas. Animals with lesions larger than 0.5 cm in diameter or that had become ulcerated were euthanized for humane reasons. Otherwise, animals were euthanized at the 40-week end point. At the time of euthanasia, tumors were collected for the preparation of genomic DNA, and the remaining tumors were fixed in buffered formalin and embedded in paraffin for histopathologic analysis.

**Histopathologic analysis of tumors.** Tumors that developed in the K14E5 mice were excised, fixed in buffered formalin, and embedded in paraffin blocks. The tumors were then sectioned and stained with H&E for histopathologic analysis. The tumors were classified as either benign or malignant. All classification was done by the same pathologist (H.C.P.).

**Statistical analysis of tumor data.** The statistical significance of differences in the frequency of chemically induced tumors that progressed to carcinomas in K14E5 mice and their controls was analyzed using the log-rank test. In tumor induction assays, the multiplicity of papillomas at a given time point for K14E5 mice and their controls was analyzed for their statistical significance using the Wilcoxon rank sum test.

**DNA isolation from paraffin-embedded tissues.** DNA was isolated from tumor sections from both K14E5 mice and nontransgenic mice. DNA was also isolated from normal skin sections to serve as a control. The protocol is similar to the one used in Nelson et al. (23). Briefly, paraffin was removed from tissue sections that were transferred to microcentrifuge tubes by two extractions of 1 mL of xylene. Excess xylene were removed through two additional extractions of 1 mL of 100% ethanol. Tissue samples were dried and resuspended in 100 μL of “K buffer” [50 mmol/L KCI, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L MgCl2, 100 μg of gelatin per mL, 0.45% Igepal, 0.45% Tween 20, 60 μg proteinase K per mL] and incubated at 35°C for 3 h and then at 95°C for 10 min. The tubes were centrifuged for 10 min at 14,000 × g and the supernatant was collected. The DNA was precipitated with 250 μL of ethanol and 10 μL of 3 mol/L sodium acetate and spun at 14,000 × g for 10 min. The pellet was washed with 70% ethanol, dried, and resuspended in 20 μL water.

**Analysis of codon 61 of H-ras by mutation-specific PCR.** DNA isolated from chemically induced tumors was subjected to PCR analysis described in Satomi et al. (24). Briefly, the PCR (50 μL) contained 1 μg tumor DNA, 1× PCR buffer with MgCl2, 0.3 μmol/L of each primer, 0.1 mmol/L dNTPs (deoxynucleotide triphosphates), and Taq DNA polymerase. Samples were denatured for 5 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. The final extension was 5 min at 72°C. PCR products (10 μL each) were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide. Primers used were 5‘ primer (5’-CTAGCGGTCGTTTTCGAGGCAG-3‘), 3‘ primer for amplification of normal H-ras (5’-CATGGGACTATATCTTCTTCTT-3‘), and 3‘ primer for amplification of mutant H-ras containing an A-T mutation at codon 61 (5’-CATGGGACTATATCTTCTTCA-3‘).

**Results**

**HPV16 E5 induces spontaneous skin tumor formation.** In our initial characterization of K14E5 mice (16), we noted the spontaneous onset of skin tumors in small groups of two lines of K14E5 mice (lines 614 and 615) that express the HPV16 E5 transgene at comparatively high levels to our other lines. One of these two lines (line 615) has a high morbid rate and short life span, limiting further study. To investigate spontaneous tumor formation further, we monitored tumor incidence over a 15-month life span in larger cohorts (Table 1) of K14E5 mice from the three other independent lineages, including lines expressing the transgene at comparatively low levels (lines 32 and 33) and high (line 614) levels, based on real-time PCR measurements of transgene-specific transcripts (16). The relative range of transgene expression among these three lines was 3.4-fold, with line 614 being 3.4-fold higher than that of line 32. The higher levels of E5 expression in line 614 correlated with severe gross phenotypes seen also in the highest expressing line, line 615 (8-fold higher than line 32), including curly whisker, scaly skin, alopecia, and morbidity (although less penetrant and later onset than in line 615; ref. 16). Conversely, the lower transgene expression in lines 32 and 33 (relative expression levels of 1.0 and 2.0) correlated with reduced

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**Table 1. Spontaneous tumor incidence in K14E5 transgenic mice**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mice with tumors/total</th>
<th>% Mice with tumor</th>
<th>Grade of tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontransgenic</td>
<td>0/248*</td>
<td>0.0</td>
<td>NA</td>
</tr>
<tr>
<td>K14E5 line 32</td>
<td>4/85</td>
<td>4.7</td>
<td>4/5 Benign</td>
</tr>
<tr>
<td>K14E5 line 33</td>
<td>5/81</td>
<td>6.2</td>
<td>5/5 Benign</td>
</tr>
<tr>
<td>K14E5 line 614</td>
<td>28/36</td>
<td>77.8</td>
<td>28/28 Benign</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not available.

*Data taken from ref. 25.
gross phenotypes, including curly whiskers and mild scaly skin (see ref. 16 for complete phenotypic description of these lines of mice). Mice in these three lines were monitored on a weekly basis for the formation of spontaneous tumors. Line 614 animals (77.8%) formed at least one skin tumor by 15 months with an average age of onset at 9.6 months, whereas lines 32 and 33 had spontaneous skin tumor frequencies of 4.7% and 6.2% by 15 months, respectively (Table 1). The average age of onset for lines 32 and 33 was 6.8 and 10.4 months, respectively, but these averages were based on only a few mice with tumors in each line (Table 1). The frequencies of spontaneous skin tumors in the three K14E5 lines of mice were all significantly higher (P < 0.0005) than nontransgenic mice of the same FVB/N inbred genetic background, which do not develop spontaneous skin tumors (0 of 248 mice) over the same time period (25). The levels of E5 transgene expression correlate with the frequency of spontaneous tumor development, with lines 32 and 33 both being low expressing lines with similar low tumor frequencies and line 614 being the highest expressing line used in this analysis having the highest tumor frequency. Histopathologic analysis of the spontaneous skin tumors revealed that they were all benign. One tumor analyzed showed signs of early malignant transformation, indicating that if groups were allowed to age for a longer time period, some malignancies may have developed.

**Role of E5 oncogene in the context of a multistage model for skin carcinogenesis.** Based on prior studies using chemical carcinogens, skin carcinogenesis can be broken down into three stages: initiation, promotion, and progression. Initiation and promotion together lead to the development of benign papillomas, and progression leads to malignancy (22, 26). At appropriate doses, the mutagen DMBA acts as an initiator and TPA acts as a promoter. K14E5 transgenic mice were analyzed in context of this model to determine where E5 contributes to the oncogenic process. Because the level of E5 expressed in natural infections is thought to be quite low, we chose to carry out these studies with the line of K14E5 mice, line 32, which has the lowest level of E5 expression among our transgenic lineages.

To determine whether E5 contributes to the initiation stage of carcinogenesis, groups of K14E5 (line 32) mice and FVB nontransgenic mice were treated with the promoting agent TPA (15 nmol) twice weekly for 20 weeks and monitored over this period for tumor formation (Fig. 1). By this end point of 20 weeks, FVB nontransgenic mice treated with both DMBA and TPA developed on average six papillomas per mouse (data not shown). As expected, FVB nontransgenic mice treated with only TPA developed no tumors (Fig. 1), confirming that papilloma formation requires both initiating and promoting events. On average, only 0.05 tumors per mouse arose on the TPA only–treated K14E5 mice by the 20-week end point (Fig. 1), not significantly higher (P = 0.35) than the spontaneous incidence of tumors in age-matched K14E5 mice of this same line not treated with TPA (Fig. 1). These data show that E5 does not function at the initiation stage of skin carcinogenesis.

To determine if E5 is able to contribute to the promotion stage of carcinogenesis, groups of E5 transgenic mice and FVB nontransgenic mice were treated with a one dose of DMBA (0.3 µmol) and then monitored for papilloma formation. E5 transgenic mice had an increased incidence of papilloma formation compared with nontransgenic mice when treated with DMBA (Fig. 2). Papilloma formation in the DMBA-treated E5 transgenic mice was also significantly higher (P = 0.0003) compared with spontaneous tumor formation in the same line of mice not treated with DMBA. This cooperativity between DMBA and E5 indicates that E5 contributes to the promotion stage of skin carcinogenesis.

To determine if E5 contributes to the progression stage of skin carcinogenesis, E5 transgenic mice and FVB nontransgenic mice were treated once with DMBA (0.03 µmol) and twice weekly for 20 weeks with TPA (15 nmol/treatment) to induce efficiently papilloma formation. By the end of the TPA treatment, all groups of mice had a similar incidence of papillomas, ranging from five to seven papillomas per mouse (data not shown). The mice were then allowed to age an additional 20 weeks so progression from papillomas to carcinomas could be followed. The incidence of progression to malignancy was monitored in lines 32, 33, and 614 K14E5 transgenic mice. K14E5 mice in both the low and high expressing lines displayed statistically significant increases in their incidence of carcinomas compared with nontransgenic mice (Fig. 3). In addition, the time of onset of carcinomas correlated with the level of transgene expression among these three lines of K14E5 mice, with line 614 mice, which has the highest relative level of E5 expression, having the earliest time of onset, and line 32, which has the lowest relative level of E5 expression, having the latest time of onset (Fig. 3). Thus, we conclude that E5 contributes to progression.

**E5 leads to the formation of endophytic papillomas, which are the precursors to carcinomas.** In the course of carrying out the skin painting studies, we observed that the DMBA/TPA-treated K14E5 transgenic mice had an unusual progression of papillomas to carcinomas that is distinct from like-treated nontransgenic mice or transgenic mice that express another HPV16 oncogene, E6 (20). In nontransgenic and K14E6 mice, the papillomas that form are exophytic (protruding outwards) in nature (20). Over time, carcinomas arise, arguably at the sites of these benign tumors.
Progression is distinct in K14E5 mice. Early in the tumorigenesis process, exophytic papillomas form, but with time, these exophytic papillomas flattened out. Histologic analysis of these flattened tumors indicated they were endophytic or sessile papillomas. Longitudinal monitoring of these K14E5 mice led us to suspect that carcinomas were arising specifically from these flattened papillomas. To monitor more closely tumor progression, a detailed time course study was carried out in which K14E5 transgenic mice were treated with both DMBA and TPA to induce papilloma formation. These papillomas were photographed weekly to identify the pattern and position of tumors (which also assisted in our monitoring the morphology of individual tumors over time; Fig. 4A). In addition, tumors were collected from subgroups of mice at different time points for detailed histologic analysis (Fig. 4B). Using this histologic data, we plotted the percentage of tumors that showed possible early signs of malignant transformation (Fig. 4C) over this same time course. At the 14-week time point, when papillomas first arose (and, therefore, the time point at which tumors were first harvested for histologic analysis), none were endophytic (i.e., all tumors were exophytic in nature at the time they first appeared). By the 24-week end point, all of the papillomas had converted to endophytic papillomas, and 70% of these endophytic tumors had possible early signs of malignant transformation. These data show that malignancy in the K14E5 mice arises through a unique morphologic and histopathologic progression characterized by an intermediate endophytic papillomatosis.

Nature and frequency of H-ras mutations in tumors arising in DMBA/TPA-treated nontransgenic and K14E5 mice are indistinguishable. The initiating event in DMBA induced carcinogenesis in the mouse skin is thought to be the induction of activating mutations in H-ras, specifically an A→T transversion at the second position of codon 61 (27). To determine whether the unique morphologic/histopathologic features and heightened frequency of malignant progression in K14E5 mice might be associated with differences in the nature or frequency of H-ras mutations, we analyzed tumors for the presence of an A-T mutations at codon 61 of H-ras using mutation-specific PCR. Both papillomas and carcinomas were analyzed from nontransgenic mice, whereas exophytic papillomas, endophytic papillomas, and carcinomas were analyzed from E5 transgenic mice. There was no difference in the frequency of H-ras mutations at codon 61 between chemically induced tumors in E5 transgenic mice and in nontransgenic controls (Fig. 5). Tumors from both K14E5 and nontransgenic mice showed the same high frequency (11 of 12 or 92% in both tumor sample sets) of codon 61 mutations as observed by us previously in the FVB/N genetic background (20).

Comparison of differentiation and proliferative properties of neoplastic lesions at different stages in progressive disease in K14E5 mice. To assess whether differences in the exophytic and endophytic papillomas might be manifested at the level of the differentiated state of the tumors, or their proliferative index, we monitored the pattern of staining for a marker for the undifferentiated basal (keratin 14) versus the differentiated suprabasal (keratin 10) compartments of the epidermis. Although carcinomas showed reduced staining for keratin 10, there was no gross change in the pattern of K14 or K10 staining between the exophytic and endophytic papillomas (Supplementary Fig. S1). However, in the small number of endophytic papillomas analyzed by immunofluorescence in which there were signs of early malignant transformation, we observed reduced K10 staining. This would suggest that the dedifferentiated state found in carcinomas may begin to arise in the context of the endophytic papillomas. We detected no clear difference in the proliferative index or pattern of proliferation between the exophytic and endophytic papillomas (Supplementary Fig. S1).
Discussion

The better-studied HPV16 E6 and E7 oncogenes have been shown previously to contribute to distinct stages of skin carcinogenesis: E7 to promotion and E6 to promotion and progression (20). In the latter case, the role of E6 in promotion and progression correlates, respectively, with its ability to bind cellular PDZ scaffolding proteins, such as DLG and Scribble, and its ability to associate with α-helix cellular protein partners, such as the E3 ligase E6AP (28). Thus, different biochemical properties of E6 contribute to its role at different stages of carcinogenesis. In this study, using the carcinogens DMBA and TPA, we show a role for HPV16 E5 in both the promotion and progression stages of carcinogenesis. Therefore, it is reasonable to consider that, like E6, distinct biochemical properties of HPV16 E5 may contribute to its role in multiple steps in carcinogenesis. Studies previously done in cell culture have given us clues into the mechanism by which E5 transforms cells. The first is through activation of the EGFR. Like BV1, HPV16 E5 is thought to transform cells through activation of growth factor receptors. In the presence of HPV16 E5, increased numbers and phosphorylation of EGFR were observed following the addition of the EGF ligand (6, 12, 13). E5-expressing cells also have increased activity of downstream signaling pathways of the EGFR, such as the mitogen-activated protein kinase (MAPK) pathway, when treated with EGF, further supporting the hypothesis that E5 enhances EGFR activity (29).

Studies previously done in animal models have also given us clues that the EGFR could be playing a role in the oncogenic potential of HPV16 E5. In our previous study, we showed a requirement of the EGFR for the hyperplasia induced by E5 (16). In addition, transgenic mice that express transforming growth factor-α, a ligand of the EGFR, treated with a DMBA/TPA protocol had enhanced sensitivity to tumor growth and development compared with nontransgenic mice, which is consistent with what we have found in E5 transgenic mice (30).

Figure 4. Tumors arising in DMBA/TPA-treated K14E5 transgenic mice display a unique morphologic and histologic pattern of malignant progression. A, sequential photographs of two DMBA/TPA-treated K14E5 mice. Left and middle left, one mouse at weeks 18 and 22. White arrowheads, an exophytic papilloma at week 18 that converts to an endophytic papilloma by week 22. Right and middle right, another mouse at weeks 22 and 26. Black arrowheads, an endophytic papilloma at week 22 that converts to a carcinoma by week 26. B, subgroups of mice were sacrificed at weekly time points (starting at 14 wks after DMBA treatment at which time papillomas had begun to arise and extending to the 24-wk end point). All tumors were excised at weekly intervals and subjected to histologic analysis. Representative histologic images at ×40 magnification of exophytic papilloma (1), endophytic papilloma (2), endophytic papilloma with area of possible early malignant transformation (3), and carcinoma (4). 5, ×200 magnification of possible early malignant transformation area. C, red, percentage of the endophytic tumors that showed signs of possible early malignant transformation over the 24-wk time course.
The mechanism by which E5 enhances EGFR-dependent signaling is still unclear. HPV16 E5 can bind to the 16-kDa subunit of the v-ATPase H+-pump and, through this interaction, delay endosomal acidification in human keratinocytes (7, 31). The ability of E5 to delay endosomal acidification has been suggested to be a reason for enhanced EGFR phosphorylation in keratinocytes because a failure to acidify endosomes may result in decreased receptor degradation and increased receptor recycling to the cell surface. Correspondingly, E5-expressing cells express more EGFR molecules on the cell surface (6, 13). It has also been recently reported that E5 can block ubiquitin-mediated degradation of the EGFR by inhibiting the function of c-Cbl, an E3 ubiquitin ligase (15). Thus, E5 may be enhancing EGFR activity by inhibiting two distinct steps in the normal down-regulation of activated receptor. It is interesting to note that these same two steps in down-regulation are also shared among other cell surface receptors; therefore, E5 could have pleiotropic effects on enhancing the activity of other signal transduction pathways that are initiated at the cellular membrane.

HPV16 E5 has also been shown to affect human keratinocytes through EGFR-independent activities, including its ability to (a) modulate the sorbitol-dependent activation of MAPK p38 and extracellular signal-regulated kinase 1/2 (32), (b) impair the gap junction-mediated cell-cell communication coupled with connexin 43 (33), and activate endothelin-1, allowing human keratinocytes to undergo DNA synthesis under growth factor–starved conditions (34). E5 has also been shown to induce anchorage-independent growth in immortalized human keratinocytes by stimulating c-jun and junB expression (35). Any one of these activities, or a combination of them, may be responsible for the ability of E5 to transform cells and contribute to tumor formation in vivo. Further studies are necessary to ascertain the contribution of each of these activities to the role of E5 in carcinogenesis.

Role of E5 in the carcinogenesis process. Our observation that E5 can induce spontaneous skin tumors and contribute to multiple stages of skin carcinogenesis, together with its transforming properties in tissue culture, supports the hypothesis that E5 contributes to HPV-associated human cancers. Our data specifically show that E5 contributes not only to the onset of benign tumors but also to the progression to frank (i.e., malignant) cancer. This demonstration is consistent with studies in which E5 was found expressed throughout the different stages of progressive human cervical disease, including cervical cancer (36). In that study, E5 expression correlated with the retention of the HPV16 genome as an extrachromosomal, nuclear plasmid. However, in approximately half of human cervical cancers, the HPV16 genome is found integrated into the host genome, leading to the increased expression of E6 and E7 and the loss of E5 expression. This integration event is thought to arise as early as the cervical intraepithelial neoplasia III stage of the progressive disease, although it is commonly only detected in a subset of frank cancers. If E5 is contributing to the genesis of these HPV-associated cancers, then there must arise genetic/epigenetic changes that obviate the need for E5 in later stages of malignant progression.

Unusual tumor progression in K14E5 mice. Chemically induced tumors in E5 mice follow a morphologic and histopathologic pattern of progression that is distinct from that seen in nontransgenic mice and mice transgenic for another HPV16 oncogene, E6. Both E5 and E6 contribute to the same two stages of skin carcinogenesis: promotion and progression. They are also far more potent oncogenes in the skin than is E7, which only contributes to the promotion stage (20). The unique morphologic and histopathologic progression pattern of skin tumors in the K14E5 mice suggests that, although both E5 and E6 are potent oncogenes in the skin, carcinogenesis mediated by these two oncogenes is quite different.

There is an interesting parallel between the role of sessile or endophytic papillomas in progressive neoplasia of the skin in K14E5 mice and a similar relationship of sessile villous adenomas in colon cancer. Sessile villous adenomas of the colon have similar histopathologic features to the endophytic papillomas in the K14E5 mice and, interestingly, are also at increased risk of malignant progression to adenocarcinomas when compared with other more common forms of adenomas that tend to grow exophytically into the lumen of the colon (37). We have done some preliminary studies to ascertain what differences exist between the exophytic and endophytic papillomas that might explain their propensity to progress to carcinomas. No differences in the nature of ras mutations (Fig. 4), proliferative indices (Supplementary Fig. S1), or differentiation state (Supplementary Fig. S1) could be discerned. Further study is needed to define what events are triggering the exophytic to endophytic conversion of papillomas in K14E5 mice and why the latter are more prone to develop into carcinomas.

Acknowledgments

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Figure 5. Detection of codon 61 mutations in H-ras. For each group of mice [line 32 E5 and nontransgenic (NTG) mice], 12 tumors (exophytic papillomas, endophytic papillomas, and carcinomas) and 3 samples of normal skin (controls) were analyzed for the presence of wild-type and mutant H-ras. Primers specific to the wild-type H-ras were used in each lane marked (W), whereas primers specific to the mutant H-ras were used each lane marked (M). Mutant H-ras is not detected in the normal skin controls compared with the tumors. There was no difference in H-ras mutation frequency in tumors collected from E5 mice compared with nontransgenic mice.
References


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