Development of Skin Tumors in Mice Transgenic for Early Genes of Human Papillomavirus Type 8

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Abstract
The cutaneous human papillomavirus (HPV) 8 is clearly involved in skin cancer development in epidermodysplasia verruciformis patients and its early genes E2, E6, and E7 have been implicated in cell transformation in vitro. To examine the functions of these genes in vivo we integrated the complete early region of HPV8 into the genome of DBA/Bl6 mice. To target their expression to the basal layer of the squamous epithelia the transgenes were put under the control of the keratin-14 promoter. Transgenic mice were back-crossed for up to six generations into both FVB/N and Bl6 mouse strains. Whereas none of the HPV8 transgene–negative littermates developed lesions in the skin or any other organ, 91% of HPV8-transgenic mice developed single or multifocal benign tumors, characterized by papillomatosis, acanthosis, hyperkeratosis, and varying degrees of epidermal dysplasia. Squamous cell carcinomas developed in 6% of the transgenic FVB/N mice. Real-time reverse transcription-PCR showed highest expression levels for HPV8-E2, followed by E7 and E6. There was no consistent difference in relative viral RNA levels between healthy or dysplastic skin and malignant skin tumors. Whereas UV-induced mutations in the tumor suppressor gene p53 are frequently detected in human skin carcinomas, mutations in p53 were not observed either in the benign or malignant mouse tumors. Nonmelanoma skin cancer developed in HPV8-transgenic mice without any treatment with physical or chemical carcinogens. This is the first experimental proof of the carcinogenic potential of an epidermodysplasia verruciformis–associated HPV-type in vivo. (Cancer Res 2005; 65(4): 1394–400)

Introduction
Nonmelanoma skin cancer (NMSC) is the most frequent human cancer in the Caucasian population (1). Basal cell carcinoma is the most common NMSC, whereas squamous cell carcinoma (SCC) accounts for 20% of all cutaneous malignancies. The incidence of NMSC has increased continuously over the last decades, and at present it represents about 30% of all cancers (2). This is partially due to greater sun exposure and to the rising number of persons experiencing acquired immunosuppression. Transplant recipients have an up to 100-fold increased risk of SCC and a 10-fold increased risk of basal cell carcinoma.

A role of human papillomavirus (HPV) infection in skin cancer development is well established in patients with the rare, inherited disorder of epidermodysplasia verruciformis (EV; refs. 3, 4). EV is characterized by the life-long occurrence of multiple flat warts and macular lesions, which are induced by a group of at least 20 HPV types, so-called EV-HPV (5). There is a high risk of developing SCC later in life. In contrast to the plurality of EV-associated HPVs in benign tumors, only a few virus types, predominantly HPV5 or HPV8, are found in the cancers and are regarded as a high risk. Sensitive detection techniques frequently identified EV-HPVs also in healthy skin and NMSC of the general population. They are particularly prevalent in immunosuppressed organ transplant recipients (6–8). The meaning of these findings for skin carcinogenesis is still uncertain because no single high-risk HPV types predominate in NMSC of non-EV patients and low viral loads suggest that only a minority of tumor cells contain HPV DNA (reviewed in ref. 1). However, the presence of antibodies against HPV8 capsids has recently been shown to be associated with cutaneous SCC (refs. 9, 10) and with the development of large numbers of precancerous actinic keratoses (11), which may suggest that this EV-type also plays a role in NMSC in the general population.

Extensive studies have linked the efficiency of mucosal HPV types (e.g., HPV16) in promoting cancer development to activities of their major oncoproteins E6 and E7, which interfere with the regulation of both the cell cycle and apoptosis. Comparatively, little is known about cutaneous HPV and much of the data suggests that the oncogenes of HPV from skin and mucosa have only a few shared properties. Thus, in contrast to HPV16, the E6 proteins of the EV-associated HPV5 and HPV8 do not interfere with p53 (12–14); however, they can still induce morphologic transformation and anchorage-independent growth in rodent cells (15, 16). A contribution to NMSC development may be expected from the inhibition of apoptosis by E6 proteins of, for example, HPV5 (17). If cells with DNA damage caused by transient exposure to the UV component of sunlight cannot be eliminated by apoptosis, somatic mutations may accumulate and eventually lead to cancer. The E7 proteins of HPV5 and HPV8 have a much weaker interaction with the tumor suppressor pRB than that of HPV16, and only transform rodent cells in collaboration with an activated H-ras gene (18). Expression of the HPV8 E2 protein led to anchorage-independent growth of rodent fibroblast and the human skin keratinocyte line HaCat (19). Given these in vitro differences between the cutaneous and mucosal HPV types, one might expect a lower carcinogenic activity of cutaneous viruses.

To study the effects of the early genes of HPV8 in vivo, we established a transgenic mouse model with the complete early region of HPV8. To direct the expression to the basal layer of squamous epithelia the genes were put under the control of the...
human keratin-14 promoter. Single or multifocal tumors developed in 91% of the transgenic animals and SCCs in 6% of all examined mice, which points to an impressive oncogenic potential of the EV-associated HPV8.

Materials and Methods

Construction and Preparation of the Transgene. The HpaI-BamHI DNA fragment of the HPV8 genome spans nucleotides 1 to 5,111 and contains the complete early region of HPV8 and parts of the noncoding region and the L2-open reading frame (ref. 20; Fig. 1A). It was ligated to BamHI octamer linkers, cleaved with BamHI and inserted into the BamHI site of the K14CreERTam plasmid (21), located between the sequence of the second intron of rabbit β-globin gene and the K14 polyadenylation sequence (Fig. 1B).

The resultant recombinant plasmid K14HPV8 was digested by EcoRI and SmaI and sequenced to verify the sense orientation of the HPV8 insert and its intact state. Plasmid K14HPV8 was digested with HindIII and SacI followed by purification of the 8.3-kb fragment via agarose gel electrophoresis and millipore membrane filtration.

Generation of Transgenic Mice. The linearized transgene was microinjected into the pronucleus of fertilized DBA/Bl6-F2 oocytes (Harlan, Borchen, Germany), which were implanted into pseudopregnant surrogate mothers as described by Hammes and Schedl (22) to produce putative founder mice expressing the K14 HPV8 transgene. Founders bearing the transgene were back-crossed into FVB/N and C57BL/6J (Bl6) wild-type mice to generate progeny. Mice were checked every 3 days to monitor the development of skin tumors. If animals became moribund due to tumor burden, they were euthanized. An autopsy including preparation of the skin tumors was done. Part of tumor tissue was fixed in 4% buffered formalin, subsequent embedded in paraffin, cut into 4-μm-thick sections, and stained with hematoxylin and eosin for histologic analysis. Another part was snap frozen in liquid nitrogen and stored at −80°C for preparation of genomic DNA and total RNA.

PCR and Southern Blot Analysis. Genomic DNA was isolated from tail biopsies of 3-week-old mice using the QIAmp Tissue Kit (Qiagen, Hilden, Germany). Samples of genomic mouse DNA were analyzed for presence of the transgene by PCR, using primers that bind outside the early genes E6 and E7 (5-CAATTTCCTAACCAATGGAC and 3'-CAGCTATCGACGCTTCTAAAATACA). The PCR reaction conditions consisted of a 3-minute denaturation step (95°C) and 35 cycles of amplification (95°C, 45 seconds; 50°C, 1 minute; 72°C, 1.5 minutes). To check the quality of all DNA samples, the β-globin gene was amplified by PCR as described by Konkel et al. (23). For detection of transgene integration, 10 μg of the genomic DNA were analyzed, uncleaved, or digested with restriction enzymes by 1% agarose gel electrophoresis and Southern blot hybridization. An HPV8 DNA fragment which completely covers the E6 and E7 genes (nucleotides 136-964; ref. 20) was 32P-labeled with the random primers DNA labeling kit from Invitrogen (Karlsruhe, Germany) and used as probe.

Reverse Transcription-PCR Analysis. Total RNA was extracted from frozen samples of tumors and normal skin with the RNeasy mini kit with
Determination of Transgene Copy Number and Quantification of mRNA. To quantitate DNA or cDNA we designed real-time PCR protocols for HPV8-E2, HPV8-E6, and HPV8-E7 and the mouse β-actin gene using the LightCycler (Roche, Mannheim, Germany) system with Sybr Green (Invitrogen) as a fluorophor. The quantitation of the single copy gene β-actin was used to calculate the transgene copy number per cell, which was defined as HPV-E6 DNA copies per two β-actin gene copies. To differentiate for the analysis of gene expression cDNA from residual DNA after DNase digestion, the copy number of HPV8-DNA in a given volume of RNA without reverse transcription was subtracted from the copy numbers of cDNA with reverse transcription. Resulting cDNA copy numbers were normalized to equivalently determined cDNA copies of β-actin as a cellular reference point. Amplification was done in 20 μL with 0.8 units Platinum Taq DNA polymerase and the associated buffer (Invitrogen) containing nonacetylated bovine serum albumin (500 ng/μL; Sigma), DMSO (5% Sigma), deoxynucleotide triphosphates (200 μmol/L each), primers (0.5 μmol/L), and 2 μL of a 1:1000 dilution of Sybr Green. Copy numbers were determined in duplicate. Calculation of initial copy numbers in samples was done by the LightCycler software (version 3.1.102) using a standard curve generated in the same PCR run with a 10-fold dilution series of HPV8-DNA or mouse DNA. Standard dilution series were based on 5-fold repeated absorbance measurements at 260 nm of five DNA aliquots, which were then pooled and adjusted with water to the desired initial concentration. HPV8 serial dilutions included 40 ng/μL human placental DNA (Sigma) to mimic cellular background. Sensitivity was ≤20 copies and quantification was linear from 20 to 10^7 copies for all HPV-protocols and from 20 to 10^4 for β-actin. Cycling conditions were 60 seconds at 95°C, followed by 45 cycles of 1 second at 95°C (20°C/s), 5 seconds at T_m (20°C/s), and 12 seconds at 72°C (E2: 5°C/s; E6, E7, and β-actin: 20°C/s). Fluorescence was measured once per cycle at the end of the elongation step (HPV8-E2, β-actin) or at an increased temperature (78°C) following the elongation step (HPV8-E6 and HPV8-E7). For β-actin, a 10-cycle touchdown protocol preceded the above described amplification protocol. Here, the annealing temperature was decreased stepwise by 1°C/cycle from 65°C to 56°C. The following primers and Mg^2+ concentrations were used: β-actin-fw (5’-CCAGGACCAAGAGGTATCTCTGCAC-3’, 3 mmol/L Mg^2+), β-actin-bw (5’-CATTGTAGAAGTTGTGTCGCCAG-3’, 3 mmol/L Mg^2+), HPV8-E2-fw (5’-AACAGCCCACAACAACCCG-3’, 3 mmol/L Mg^2+), HPV8-E2-bw (5’-CGTATCCAGCTCCGCTTCTC-3’, 3 mmol/L Mg^2+), HPV8-E6-fw (5’-GGCGGTATTTGAATCTTCCGTGC-3’, 4 mmol/L Mg^2+), HPV8-E6-bw (5’-GCTCACACAAAAACACCAAGACAGACG-3’, 4 mmol/L Mg^2+), HPV8-E7-fw (5’-CCTGAGGGTCTGTTACAGTTGCACG-3’, 4 mmol/L Mg^2+), HPV8-E7-bw (5’-CAGTTGCTGTGACAAAAGACG-3’, 4 mmol/L Mg^2+).

Analysis of p53 Status in Mouse DNA. Exons 4, 5, 7, and 8/9 of p53 from mouse DNA were amplified and sequenced. Amplifications were done in 50 μL with 1 unit Taq-polymerase (Amersham, Freiburg, Germany) and the associated buffer containing deoxynucleotide triphosphates (200 μmol/L each), primers (0.3 μmol/L, 1.5 mmol/L Mg^2+), and 1 μL of template DNA. Cycling conditions were 3 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 1.5 minutes at 60°C, and 1.5 minutes at 72°C, with a final elongation step for 10 minutes at 72°C.

The following primers were used: exon4fw (5’-TAGCTGGAGAAGCATCGTCCTCGG-3’), exon5bw (5’-GCCTGAAAGGTCCACAGACGACG-3’), exon5fw (5’-CCTGTAGGTTCTCGGCTCTCTG-3’), exon5bw (5’-CACTGTCCTCTGGGACGAGGAG-3’), exon5fw (5’-CCTGTAGGTTCTCGGCTCTCTG-3’), exon5bw (5’-CACTGTCCTCTGGGACGAGGAG-3’), exon6fw (5’-GGATGCAGGTACACACCTGATTTG-3’), and exon 6bw (5’-GGATGCAGGTACACACCTGATTTG-3’), and exon 8 + 9 bw (5’-GGAGAGAAGCGACGACTGG-3’).

Statistical Analyses. Statistical analyses were done with SPSS 10.0.7 (SPSS, Inc., Chicago, IL).

Results

Construction of the Transgene and Generation of Transgenic Mice. The complete early region of HPV8 was cloned into the BamHI site of the K14-CreERT2 plasmid under the control of the keratin-14 promoter (Fig. 1B). The purified HindIII-SacI Fragment of K14-HPV8 plasmid was microinjected into DBA/Bl6 F2 oocytes. The offspring were analyzed for the presence of the transgene by amplifying the early genes E6 and E7 through PCR. In the founder generation, 11 mice of 92 (12%) were identified to be transgene positive. Three transgene-positive mice (nos. 9, 61, and 85) were back-bred with FVB/N (nos. 9 and 85) and/or Bl6 (nos. 61 and 85) wild-type mice. FVB/N mice were used because previous studies showed the susceptibility of the FVB/N in-bred mouse strain to epidermal carcinogenesis (24–26), whereas Bl6 mice were used to compare the influence of the genetic background. We established four different lines with 4 (line 61 Bl6), 5 (line 9 FVB/N, 85 Bl6) and 6 (line 85 FVB/N) generations. The HPV positivity in offspring of generations F1 to F6 ranged between 29% and 83% as expected for Mendelian transmission from heterozygous ancestors. Integration of the transgene was shown by Southern blot hybridization. Ten micrograms each of genomic DNA were analyzed, uncleaved, or digested with BamHI or EcoRV. Integrated uncleaved HPV8-DNA was expected to migrate with the high molecular weight cellular DNA, whereas cleavage with BamHI should reveal a 5111 fragment (Fig. 1B). Southern blots confirmed integration of the transgene and showed that it is intact at least between position ~1014 of the K14 promoter and position 5111 of the HPV8 insert (Fig. 1C). To quantify transgene copy number, we did real-time PCR for the early gene E6. Considering each line and generation 25 mice were tested. We detected on average about 50 copies in lines 9 and 61 and about 70 in lines 85 FVB/N and Bl6.

Total RNA was isolated from skin tissue of euthanized mice. Following cDNA synthesis, real-time PCR was done using primer pairs for the early HPV8 genes E2, E6, and E7. The cDNA copy numbers of the HPV8 genes were normalized to β-actin cDNA copy numbers. Expression of each of the tested genes could be detected. Expression levels were similar in skin of different founder mice and back-crossed generations and were highest for the E2 gene followed by E7 and E6 (Table 1). The E2/E7 transcript ratio was about 10 and the E7/E6 transcript ratio was about 2.

Tumor Development in HPV8-Transgenic Mice. Three of 11 transgene-positive mice of the founder generation developed tumors (27%). First phenotypic alterations of mouse 9 started about 14 weeks of age. It showed tumor like growth on the back with hair loss, hyperkeratosis and ulceration. The lesions were irregularly distributed and displaceable (Fig. 2A). Mouse 85 developed circumscribed lesions on the laterodorsal trunk with a maximal diameter of 3 cm (Fig. 2B). In mouse 61, tumor development started periventrically and at the tail root after 81 weeks.

In generations F1 to F6 altogether, 217 of 239 transgenic mice (91%) developed tumors when observed for at least 60 weeks. The time course of tumor development in the four transgenic mouse lines is depicted in Kaplan-Meier tables (Fig. 3). Mice that died for other unknown reasons before developing a tumor were not included. There was no difference in location and morphology of lesions in mice from different lines and generations. Tumor growth generally started in the dorsocaudal region of the trunk and at the
tail, with alopecia and hyperkeratosis, continued to become more hyperkeratotic and spread diffusely or multifocally to the cranial and ventral regions of the body. Two to more than five individual foci could be distinguished in about 80% of the animals from the four lines. No skin tumors appeared in the 128 nontransgenic littermates, which were also observed for at least 60 weeks. In line 9, the age at which mice developed tumors decreased with the number of back-cross generation (Fig. 3A), log-rank test, \( P < 0.0001 \).

In the F1 generation, the median age for the development of tumors was 47.4 weeks (95% confidence interval, 42.5-52.3) and by the F5 generation only 8.4 weeks (95% confidence interval, 6.9-9.9). The same trend was observed with generations 1 and 2 of the lines 85-FVB/N and 85-Bl6 but was not consistent for generations 3 to 5 of line 85-FVB/N.

Male mice developed tumors earlier than females. For example, 75% of the tumor bearing animals of generations F1 to F5 of line 85 FVB/N were males at the age of 60 weeks compared with 56% males at the age of 96 weeks.

Histologically, tumor-positive mice (\( n = 143 \)) exhibited papillomas with hyperkeratosis, dyskeratosis, and parakeratosis (Fig. 4A). The epidermis showed multiple cavities filled with lamellated concentric keratin masses. In the upper layers, intracytoplasmatic keratohyaline granules were present and in some areas koilocytosis occurred. These changes were often also found in the epithelium surrounding the hair shafts. There were different degrees of dysplasia in the papillomas of 61 mice. In 30 mice, mild dysplasia occurred. These tumors showed atypia in the basal layer with decreased nucleus/plasma ratio, hyperchromatosis of nuclei and anisonucleosis. Nuclei were pleomorphic and showed an increased number of partly atypical mitotic figures. Only rarely was lymphocytic infiltration detected. Distant metastases to the lung were found in one mouse. The histologic changes are summarized in Table 2.

The HPV8 genes E2, E6, and E7 were continuously expressed in benign and (pre) malignant tumors. Transcript levels relative to \( \beta \)-actin mRNA appeared up to 20-fold increased in some cases compared with normal skin from the same animal (data not shown); however, due to the varying ratios of keratin-14 promoter permissive cells in papillomas and normal skin, this may only be taken as an approximation. It is noteworthy that the relative abundance of E2 transcripts did not change during tumor progression. The E2/E7 transcript ratio was 10 to 30 and the E7/E6 transcript ratio was 6 to 7 in the tumors.

To test for \( p53 \) mutations, we amplified and sequenced \( p53 \) exons 4, 5, 7, and 8/9 from mouse DNA. Neither in DNA extracted from the skin of HPV8-positive mice with and without tumors, nor in DNA from a carcinoma of a HPV8-transgenic mouse could any \( p53 \) mutation be observed.
Discussion

HPV8-transgenic mice were generated by pronuclear injection of DNA without homology to mouse sequences, which is followed by random integration. Each transgenic mouse can therefore be expected to display an individual transgene integration pattern (27, 28). Because 3 of 11 HPV8-positive mice of the founder generation developed tumors later in life, the possibility of tumor induction due to insertional mutagenesis is negligible. These three animals were mated with FVB/N and/or Bl6 wild-type mice. To date, 47% to 53% of the offspring within the four lines were transgene positive, which fits to the expected 50% transgenic offspring from crosses of a wild-type mouse and a transgenic animal heterozygous for one chromosome.

The transgene copy number per cell was within the published range of usually 1 to 150 (28). The HPV8 transgenes are under control of the cytokeratin-14 promoter, which is well known to target expression to the epidermis and developing hair follicles of mouse embryos and to basal and spinous layers of the adult skin (29–31). The early HPV genes are transcribed as multiple polycistronic messages generated by alternative splicing (32, 33). The relative expression levels of HPV8 genes E2, E6, and E7 were similar in the three independently generated lines 9, 85, and 61, thus were the absolute levels relative to the amount of β-actin RNA. These results are in contrast to keratin-14 promoter-HPV16 transgenic mice, where E6 and E7 are much more strongly expressed than E2 (31).

The similar expression of HPV8 genes in lines 9, 85, and 61 both in the FVB/N and the Bl6 background corresponded to a comparable incidence of papillomas and dysplasias. Whereas tumors were never observed in transgene-negative mice, papillomas occurred mostly multifocally and disseminated in 91% of HPV8-positive mice, suggesting they arose from independent precursor cells. About 25% of the papillomas finally showed signs of moderate or severe dysplasia such as disturbed stratification, suprabasal and atypical mitotic figures, nucleus hyperchromatosis, and anisonucleosis. It is noteworthy that papillomas occurred earlier in male mice in all lines. This is probably due to their frequent dominance fights (34), resulting in injuries primarily in the dorsal and caudal regions, where papillomas generally started to develop. This observation suggests that complex cellular processes during wound healing may accelerate tumor development.

The high penetrance of papillomatosis followed by progression to dysplasia in a substantial proportion in HPV8-transgenic FVB/N
and BL6 mice is remarkable and in contrast to many other HPV-transgenic mice. The early genome region of HPV1, which induces benign plantar warts, has been expressed under control of the keratin-6 promoter in CBA/C57Bl-transgenic mice but this only led to a transient hyperproliferation (35). Transgenic IRCxFVB mice with keratin-1 promoter–driven E6/E7 genes of the cervical cancer associated HPV18 developed papillomas that remained benign for up to 12 months (36). The K14-promoter–driven oncogene E6 of the Mastomys natalensis PV, which causes keratoacanthomas in its natural host, did not perturb normal skin differentiation per se but favored malignant progression of chemically induced tumors (30). Only the high penetrance of papillomatosis and dysplasia in K14-HPV16 transgenic mice (37) is comparable with HPV8 transgenics.

Skin cancer occurred in 6% of the FVB/N back-crossed HPV8 transgenics. It could thus be shown for the first time that expression of an EV-associated HPV leads to skin cancer development without exposure to any further physical or chemical carcinogens. As our mice were never irradiated by UV light, it was not surprising to find wild-type p53 gene even in a skin cancer. Although the p53 protein is not degraded by HPV8-E6 in contrast to HPV16-E6 the wild-type p53 gene was obviously not able to prevent carcinoma development. As with HPV16 transgenic mice, SCC did not develop thus far in BL6 back-crossed HPV8 transgenics. However, the small number of BL6 mice analyzed to date does not yet allow a valid comparison with FVB regarding the susceptibility to malignant conversion.

It is remarkable that HPV8-induced carcinogenesis takes place in spite of large amounts of E2-specific mRNA in contrast to the findings from HPV16-transgenic mice. The selective advantage of the frequent loss of E2 in cervical cancers induced by HPV16 or 18 has been explained by E2-mediated suppression of the viral E6/E7 promotor, which is not relevant in transgenic mice, and by the induction of senescence or apoptosis (38–40). Interestingly, HPV8 E2 expressing keratinocytes do not undergo apoptosis (41), which may suggest that it has at least no inhibitory effect on carcinogenesis. Further studies are under way to test whether the increased levels of E2 may promote transformation.

The carcinogenic potential of HPV8 in the transgenic mice was surprising given the lack of some of the biochemical activities of E6 and E7 regarded as central to oncogenesis by HPV16. It clearly points to other highly tumorigenic and as yet unknown properties of HPV8 early proteins. The rapidity of skin tumor development in the mice is certainly related to the permanent expression of the HPV8 early genes in all proliferation competent keratinocytes driven by the K14 promoter. In humans, HPV8 early genes are only weakly expressed from their natural promoter, the episomal DNA may finally get lost with

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References

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