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Very High Incidence of Germ Cell Tumorigenesis (Seminomagenesis) in Human Papillomavirus Type 16 Transgenic Mice

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Human papillomavirus type 16 (HPV16) is frequently found in carcinomas and precancerous lesions of the uterine cervix and is thought to be closely associated with carcinogenesis in these regions. However, the transforming activity of the E6 and E7 genes in vivo has not been characterized. To investigate this function, we produced transgenic mice carrying HPV16 E6 and E7 open reading frames. We obtained five transgenic founders and established three transgenic lineages. We observed testicular tumors of germ cell origin in mice of all three lineages. Morphological studies showed that these tumors were a type of seminoma. Both testes of all tumor-bearing mice were affected with this type of tumor. Strikingly, in one lineage, all of the male mice developed this tumor. On Northern (RNA) analysis, a high level of expression of HPV mRNA was detected in these tumors. These results suggest that transforming genes of HPV16 have transforming activity in vivo and preferential effects on germ cells in the testis.

The genomes of certain types of human papillomavirus (HPV) such as HPV types 16 (HPV16) and 18 are frequently found in carcinomas and precancerous lesions of the uterine cervix, indicating a causal relationship between infection and carcinogenesis in this region (31). In in vitro transformation assay systems, the E6 and E7 genes of HPV16 can fully transform cell lines of rodent fibroblasts and immortalize primary rat embryonic fibroblasts, human keratinocytes, and some other epithelial cells (1, 8, 15, 18, 27, 29). However, the transforming activity of the E6 and E7 genes in vivo has not been characterized. To investigate this function, we produced transgenic mice carrying HPV16 E6 and E7 open reading frames.

To produce transgenic mice expressing HPV16 E6 and E7 transforming genes in many organs, we constructed a fusion gene of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) and the E6 and E7 open reading frames of HPV16. The MMTV LTR has potent expression activity in many organs (23), and its expression can be regulated by glucocorticoid and is thought not to have a lethal effect during mouse embryogenesis. This construct also contains the HPV16 early promoter, and so more efficient expression was expected. We isolated a fragment containing only the MMTV LTR and the HPV16 E6 and E7 transforming genes and injected it into one-cell embryos of C57BL/6J mice (16). By screening 126 newborn mice, we obtained five transgenic founders carrying four to five copies of the transgene in a head-to-tail tandem arrangement and established three transgenic lineages, all of which transmitted the transgene in a Mendelian fashion.

During 8 to 10 months postdelivery, we observed obvious enlargement of the testes of male mice (Fig. 1a). In all mice that developed lesions, both testes were involved, although the onset and extent of involvement differed among the mice. At autopsy, their testes were found to be 10 times larger than those of age-matched normal mice. The tumors were relatively soft and fragile with a smooth surface and showed no evidence of invasion or macroscopic metastasis (this was confirmed by histological examination). For histological examination, tissues were fixed in 10% formaldehyde–90% phosphate-buffered saline. Sections were cut from tissues embedded in paraffin and stained with hematoxylin and eosin, periodic acid-Schiff stain, or silver. This examination showed that almost all of the testis was occupied by tumor cells with large immature nuclei, fine chromatin, and extensive eosinophilic cytoplasm. Many mitotic cells were seen (Fig. 1c and d). Some of the tumor cells were spindle shaped (Fig. 1e). The tumor cells were positive for glycogen, as shown by periodic acid-Schiff staining (data not shown). On silver staining, a characteristic chromatin pattern was observed, which was very similar to that of pachytene-stage spermatocytes in seminiferous tubules (Fig. 1f). Seminiferous tubules, in which spermatogenesis was in progress, were seen in some areas (Fig. 1c). Surprisingly, even in this state, mice were not sterile. All tumors examined had almost the same histological appearance. For electron microscopical examination, a piece of frozen tissue was fixed in 250 mM glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.4) for 1 week, stained with 0.01% tannic acid, and postfixed in 10 mM osmium tetroxide. Then it was washed with 250 mM sucrose solution, stained with 10 mM uranyl acetate, and embedded in epoxy resin. This examination showed the presence of numerous mitochondria, a well-developed smooth endoplasmic reticulum, and Golgi complexes (Fig. 1g). From the similarity in the features of these tumors to those of moderately differentiated seminomas in humans (17) and the pathological criteria for human seminomas, we concluded that these tumors were a type of seminoma. These tumors were transplantable only to the testis, not to other regions, of syngeneic C57BL/6 mice (data not shown).

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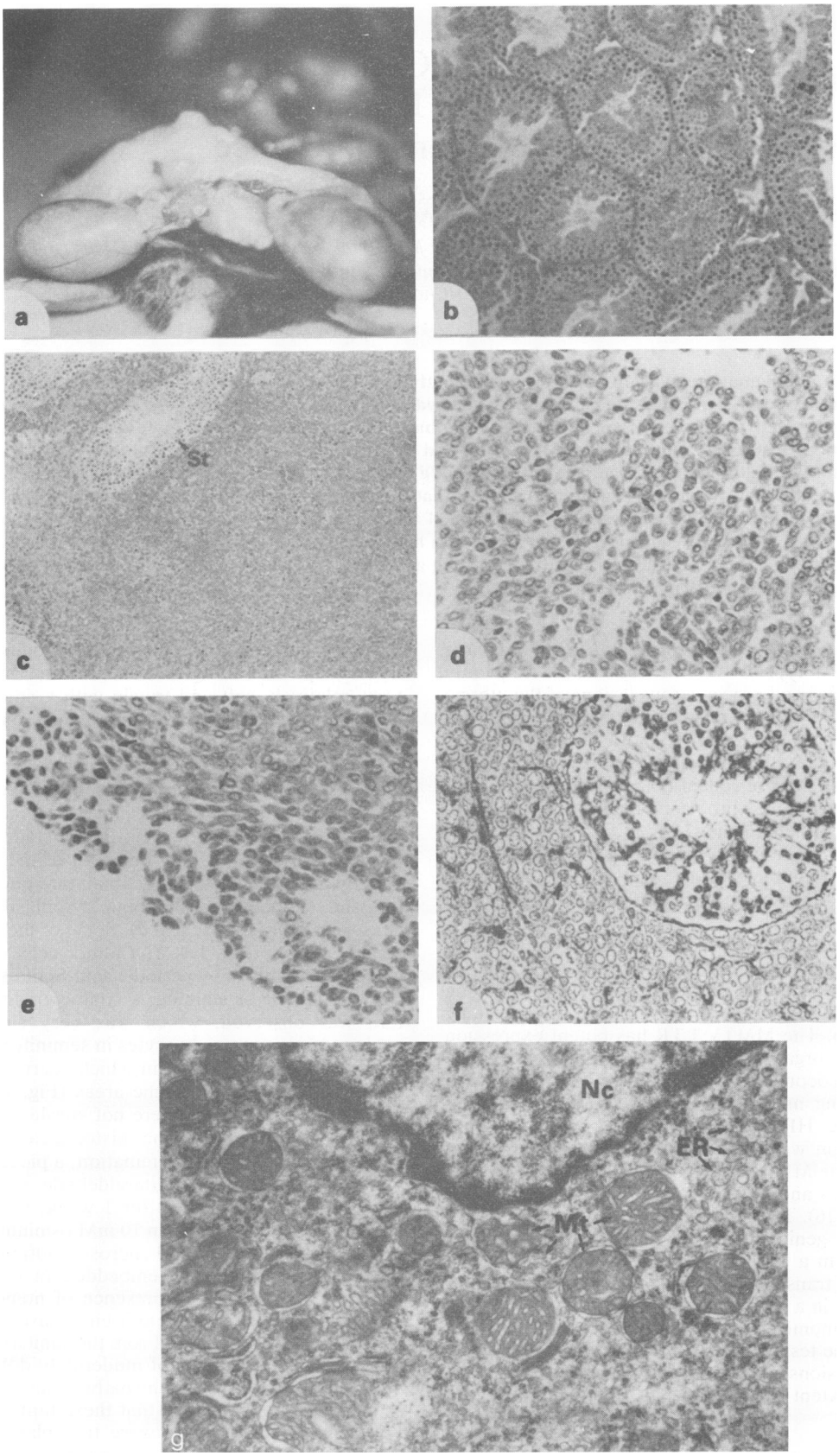
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FIG. 1. Pathology of testicular tumors in HPV16 E6-E7 transgenic mice. (a to f) Macroscopic and histological appearance. (a) Massive enlargement of both testes. No invasion or metastasis was detected. (b) Normal testis of age-matched syngeneic mouse. Fine structure with many seminiferous tubules can be seen. ×168. (c) Tumor cells occupying almost the whole organ. Seminiferous tubules (St) show normal spermatogenesis. Hematoxylin-eosin staining; ×84. (d) Tumor cells with large immature nuclei, fine chromatin, and extensive eosinophilic cytoplasm. Many cells are in the mitotic phase (arrows). Hematoxylin-eosin staining; ×378. (e) Spindle-shaped cells (arrows). Hematoxylin-eosin staining; ×378. (f) Characteristic chromatin pattern (arrows) like that of pachytene-stage spermatocytes (arrowheads). Silver staining; ×378. (g) Ultrastructure of a tumor cell. Many mitochondria (Mt) and scattered profiles of smooth endoplasmic reticulum (ER) are seen around the nucleus (Nc). ×16,800.

The same type of tumor was observed in all the lines, but the incidences of tumors in these lines were different. In one line, named 181, all four F_1 mice and the founder (F_0) developed this type of tumor. We also observed this lesion in several F_2 mice 7 months postdelivery, but we have not yet checked all of the F_2 mice. We have not, however, found any abnormalities, including ovarian tumors, in female mice of this line. Line 274 also showed a relatively high incidence of testicular tumorigenesis (two of three mice, or 67%) and also a low incidence of some other types of tumors, such as salivary gland tumors and sweat gland tumors, even in females (3 with salivary gland tumors and 1 with a sweat gland tumor among 11 transgenic mice). In line 263, only one of six mice (17%) exhibited tumorigenesis.

To determine whether expression of the HPV16 E6 and E7 genes is essential for this tumorigenesis, we analyzed HPV gene transcripts in the tumors and other organs by Northern (RNA) blot analysis. We observed distinct expression of HPV mRNA in all tumors analyzed. Comparable expression was also observed in normal submandibular gland but not in other organs (Fig. 2A and B). RNA of the expected size was detected, suggesting that no rearrangement or unexpected splicing had occurred. To confirm this, we checked the state of the transgene in tumors (Fig. 2C). No rearrangement or amplification of the transgene was detected, indicating that high expression of HPV genes was caused by transcriptional activation of the transgene itself, not by rearrangement or amplification.

These results suggest that the transforming genes of HPV16 have transforming activity in vivo and preferentially transform germ cells in the testis. These preferential effects on testicular germ cells seem to be caused not by a preferential function of the MMTV LTR on these cells but by the transforming genes of HPV16 themselves, because this type of tumor has not been reported in transgenic mice with other oncogenes using the MMTV LTR. In adenovirus type 12 E1a and E1b transgenic mice, gastric cancer and neuroblastoma were observed (9, 10), while in *neu* transgenic mice, mammary gland tumors, lymphomas, and some other tumors were found (2, 14). Mammary gland tumors were also observed in *int-1* and *int-2* mice (13, 26). H-*ras* mice developed mammary tumors, salivary gland tumors, lymphoma, and Hardeman gland dysplasia (19). c-*myc* mice and simian virus 40 (SV40) T-antigen mice also developed various tumors in several organs, including the testis, but the incidences and histological types of these testicular tumors were very different from those in our mice. Our mice developed testicular tumors of germ cell origin, whereas the stromal cell (Sertoli cell) tumor observed in a c-*myc* mouse (one mouse among five F_0 and F_1 mice) and mesenchymal cell (Leydig cell) tumor in an SV40 T-antigen mouse (one F_0 mouse) were not of germ cell origin (3, 11, 24). These findings suggest that the HPV16 E6 and E7 genes induce tumors preferentially in the testis, especially in germ cells. There are no previous reports of mouse strains developing

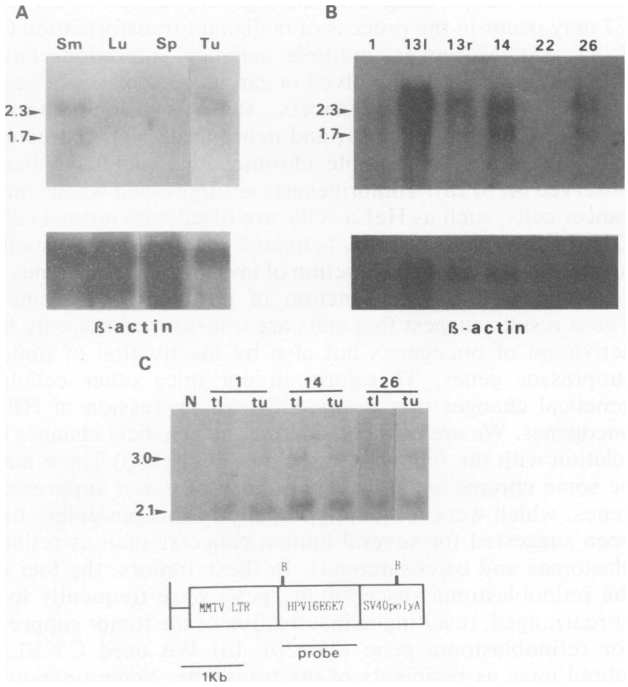

FIG. 2. HPV16 E6 and E7 expression and state of the MMTV-HPV16 E6-E7 transgene in tumors of line 181 mice. RNA from different tissues was prepared by the method of Gilson et al. (6). (A) Poly(A)$^+$ RNA was selected from 40 μg of total RNA, using oligotex-dT30 (Japan Rosche), denatured with 50% formamide–5% formaldehyde in boiling water for 5 min, and separated on a 1% agarose gel containing 5% formaldehyde in 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer. Hybridization was carried out by the procedure of Southern (20). Expression of the β-actin gene is shown as an internal control. Sm, submandibular gland; Lu, lung; Sp, spleen; Tu, testicular tumor (mouse 26). (B) A sample of 15 μg of total RNA from tumor tissues was subjected to formaldehyde gel electrophoresis and hybridized as for panel A. Numbers at the top are numbers of tumor-bearing mice. The expression patterns of the left testis (13l) and right testis (13r) of mouse 13 are shown. Mouse 22 was transgene negative and had no histological abnormalities. There was no obvious difference in mRNA level among the three lineages. (C) Samples (10 μg) of genomic DNAs from the tail (tl) and tumor (tu) of each of the indicated mice were digested with *Bam*HI, separated in a 1% agarose gel, and subjected to Southern blot hybridization (20). Construction of the transgene with the *Bam*HI (B) site is shown at the bottom. SV40polyA, polyadenylation sequence of the SV40 early gene. Numbers of mice are the same as for panel B. N, tail DNA from a nontransgenic mouse. The *Sph*I-*Kpn*I 1.3-kb fragment and human β-actin fragment in Northern analysis were radiolabeled and used as a probe.

the high incidences of seminomas observed here. Thus, these transgenic lineages may be useful for analyzing transformation of testicular germ cells.

As mentioned above, the incidence of tumors in the testes was very high. In particular, all male mice of transgenic line 181 developed testicular tumors. Moreover, tumors developed in both testes in all of these mice. We observed high expression of HPV mRNA in all of the tumors, but this may not be sufficient alone for tumorigenesis because there was a relatively long latency (about 7 months) before tumor development and incidences of tumors differed in the three lineages. This possibility may also be supported by the fact that comparable amounts of HPV mRNA were observed in normal submandibular gland. Although the E6 and E7 genes of HPV can immortalize primary cells, some other genetical alterations cooperating with the high expression of E6 and E7 may occur in the process of malignant transformation (7, 8, 12, 15). Moreover, multiple genetical alterations have been suggested to be involved in carcinogenesis. For example, in several human cancers, such as colon cancers, small-cell lung carcinomas, and neuroblastomas, characteristic alterations of multiple chromosomal loci have been observed (4, 5, 28). Tumorigenesis is suppressed when some cancer cells, such as HeLa cells, are fused with normal cells (21). In these hybrid cells, activated oncogenes are usually not suppressed, but dysfunction of impaired chromosomes is complemented by the function of normal chromosomes. These results suggest that cells are transformed not only by activation of oncogenes but also by inactivation of tumor suppressor genes. Therefore, in our mice other cellular genetical changes may cooperate with expression of HPV oncogenes. We are now considering the genetical changes in relation with the following three possibilities. (i) There may be some chromosomal loci, like those of tumor suppressor genes, which were frequently impaired. This possibility has been suggested for several human cancers, such as retinoblastomas and osteosarcomas. In these tumors, the loci of the retinoblastoma susceptibility gene were frequently lost or rearranged, resulting in inactivation of the tumor suppressor retinoblastoma gene (25, 30). (ii) We used C57BL/6 inbred mice as recipients of the transgene. Some unknown recessive mutations inherent in this strain might cooperate with HPV genes. For example, this kind of recessive mutation was suggested to be present in 129 strains that developed testicular teratocarcinomas at frequencies of less than 1%. If the so-called Steel locus was transferred to this strain, the incidence of teratocarcinogenesis was increased about 10-fold (22). (iii) The transgene may have been integrated near the chromosomal loci that regulate growth of germ cells. During puberty, promoters of the transgene may have activated and deregulated gene expression at these loci. This could explain the difference in incidences in the three lineages.

The transgenic lineages that we produced should be useful for genetic analyses of tumor generation by HPV16 and transformation of testicular germ cells.

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