

## Involvement of Human Papillomavirus Type 20 in Epidermodysplasia Verruciformis Skin Carcinogenesis

MASUO YUTSUDO,<sup>1\*</sup> TAKEHIKO TANIGAKI,<sup>2</sup> RYOJI KANDA,<sup>3</sup> TOSHIYUKI SASAGAWA,<sup>1</sup>  
TADA0 INOUE,<sup>1</sup> PAN JING,<sup>1</sup> HWANG YONG-II,<sup>1</sup> AND AKIRA HAKURA<sup>1</sup>

*Department of Tumor Virology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita 565,<sup>1</sup> Kinki Central Hospital in Hyogo, 3-1 Kurumazuka, Itami 664,<sup>2</sup> and Osaka Rosai Hospital, 1179-3 Nagasonecho, Sakai 591,<sup>3</sup> Japan*

Received 29 October 1993/Returned for modification 21 December 1993/Accepted 18 January 1994

**The involvement of human papillomavirus (HPV) in skin carcinogenesis in a patient with epidermodysplasia verruciformis was studied. This patient had disseminated pityriasis versicolor-like lesions, flat warts, and a malignant skin carcinoma. HPV types 3, 17, 20 (HPV-20), and 38 were isolated and molecularly cloned from the benign skin lesions of this patient. Of these HPVs, only HPV-20 was detected in the malignant skin carcinoma. Transcripts of HPV-20 were also expressed in the carcinoma. These findings suggest that HPV-20 was involved in the skin carcinogenesis in this patient.**

Epidermodysplasia verruciformis (EV) is a rare chronic skin disease characterized by disseminated red plaques and pityriasis versicolor-like lesions (2). As various kinds of human papillomaviruses (HPVs), such as types 5, 8, 9, 12, 14, 15, 17, and 20 (HPV-20), have been isolated from the lesions, EV is now thought to be caused by these HPVs (6). In addition to infection with these EV-associated HPVs, alteration of a recessive gene, possibly a gene involved in some cellular immunity, is also related to induction of EV, because EV preferentially appears in children of consanguineous marriages and in immunosuppressed renal recipients (6, 10). The most serious problem about this disease is its high risk for development of malignant skin carcinomas: in about 50% of EV patients, squamous cell carcinomas develop, with a long latency in areas of the skin exposed to sunlight (6, 10). This fact suggests that skin carcinomas may be induced by EV-associated HPVs though mutation of cellular genes induced by some mutagenic agents such as UV rays. Indeed, HPV type 5, 8, 14, and 17 genomes have been detected in EV skin carcinoma tissues (3, 7-9, 11).

Here, we examined the HPVs in benign and malignant lesions of an EV patient. Our results suggest that HPV-20 may be involved in the development of skin carcinoma.

The patient (male) studied developed EV lesions before the age of 10, and since then, the skin lesions have enlarged, now covering almost all the body without regression. When the patient was in his mid-thirties, a tumor appeared in the left *angulus oculi nasalis*. This tumor was removed surgically and subjected to histological and molecular biological studies. Histologically, the tumor was identified as a squamous cell carcinoma (data not shown).

To evaluate the role of HPVs in carcinogenesis, we first attempted to isolate and molecularly clone HPV DNAs from the benign lesions of this patient. His benign lesions were pityriasis versicolor-like macules on the body and flat warts on the arms. We scraped off these benign skin lesions, homogenized them, and purified virus particles by ultracentrifugation. We then extracted the viral DNA with phenol-chloroform,

partially digested it with *Bam*HI, and cloned the fragments into the *Bam*HI site of pBR322. Plasmid DNA was obtained from each transformed colony, and the type of the HPV was determined by restriction enzyme digestion and Southern blot hybridization with standard HPV types, including types 3, 17, 20, and 38. As shown in Fig. 1, we identified three types of HPV, types 17, 20, and 38, from the pityriasis versicolor-like lesions. No other types were detected on examination of more than 100 plasmid clones. However, we identified HPV type 3 (HPV-3) in samples from flat warts on the arms (data not shown). All these HPVs have often been isolated from other EV patients and so are considered EV-associated types, although HPV-3 has also been isolated from flat warts of non-EV patients.

Next, we examined the carcinoma tissue of this patient for the presence of HPV DNA, because the absence of an HPV genome in the carcinoma tissue would imply that HPV is probably not involved in skin carcinogenesis, whereas the presence in the carcinoma of one of four HPVs identified in benign lesions in this patient would strongly suggest that this HPV contributes to skin carcinogenesis. The carcinoma DNA was digested with various restriction enzymes and subjected to Southern blot hybridization, using the four types of HPV DNA as probes. As shown in Fig. 2a, HPV-20 DNA was detected in the skin carcinoma tissue, but the other types of HPV DNA were not detected. By reconstitution experiment with the cloned DNA, we estimated that there were about 100 copies of HPV-20 DNA per cell. Almost all, if not all, the HPV-20 DNA existed in the episomal state, and oligomers as well as monomers were also present. A similar physical state of HPV DNA has been observed in many other cases of EV carcinoma (7-9, 11). Judging from the sizes of the HPV-20 DNA fragments obtained by digestion with restriction enzymes, this HPV does not have a large deletion or rearrangement.

We also tested for the presence of HPV transcripts in this carcinoma tissue. On Northern (RNA) blot hybridization with HPV-20 DNA, two prominent bands and a few weak bands were detected in the carcinoma tissue (Fig. 2b) but not in control tissue of the same patient (data not shown). Because of the limited amount of carcinoma tissue available, we could not identify the genes encoding these transcripts. However, this finding of expression of HPV-20 clearly indicates that the presence of HPV-20 DNA in the carcinoma tissue was not due

\* Corresponding author. Mailing address: Department of Tumor Virology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita 565, Japan. Phone: 06-875-2249. Fax: 06-876-2678.

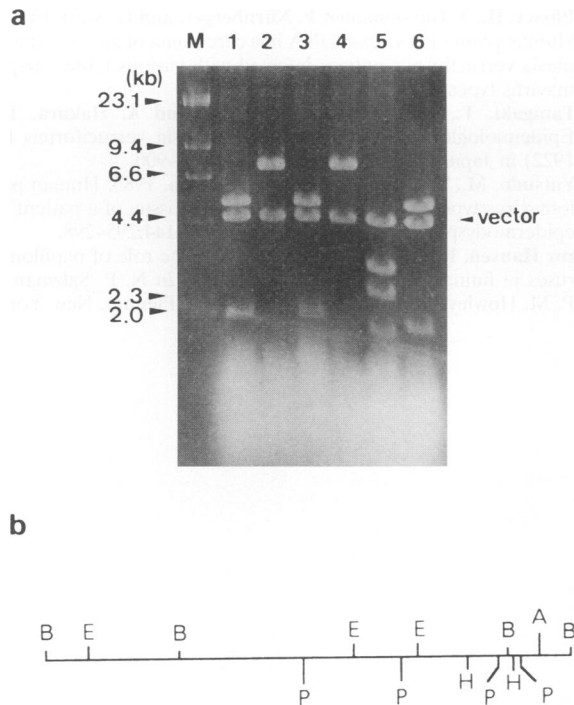


FIG. 1. Cloning of HPVs from benign lesions of the EV patient. (a) Agarose gel electrophoresis of cloned HPVs. Minipreparations of plasmid DNAs from transformed colonies were digested with *Bam*HI, subjected to 0.8% agarose gel electrophoresis, and stained with ethidium bromide. Lanes: 1, 3, and 6, HPV-20; 2 and 4, HPV-17; 5, HPV-38. (b) Restriction map of HPV-20 isolated from the benign EV lesions. Restriction sites: A, *Ava*I; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I.

to contamination of the tissue with virus particles but to its infection with HPV-20.

In this study, we identified the HPV types associated with benign skin lesions and a skin carcinoma of an EV patient. This patient was infected with four types of HPV (types 3, 17, 20, and 38), but only one of these types, HPV 20, was present in the skin carcinoma. As the number of DNA copies of this HPV in the carcinoma was high, it is difficult to explain the presence of this HPV in the carcinoma as being due to contamination with a small amount of tissue from benign lesions. Furthermore, some of the HPV-20 DNA in the carcinoma was present as oligomers, and transcripts of HPV-20 were also found in the carcinoma, excluding the possibility of contamination of the carcinoma sample with virus particles. These results strongly suggest that HPV-20 was involved in skin carcinogenesis in this EV patient.

This conclusion is supported by the following observations. The risk of malignant conversion is very high in EV patients (skin carcinoma appears in about 50% of EV patients) and almost all EV carcinomas examined contain DNAs of certain types of EV-associated HPV. Furthermore, some other types of HPVs, such as types 16, 18, and 31, have been shown to be causative agents of cervical carcinoma (12). These HPVs transform established rodent cells completely and immortalize primary human and rat cells. Although the transforming activities of EV-associated HPVs on cultured cells are very weak, some EV-associated HPVs have been reported to transform primary rat embryo fibroblasts in cooperation with activated *H-ras* (5) and to induce peculiar morphological changes of rat 3Y1 cells (1).

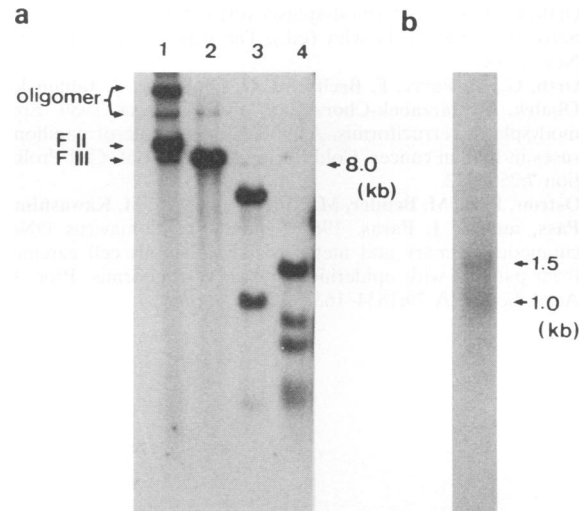


FIG. 2. Detection of HPV-20 DNA and transcripts in carcinoma tissue of the EV patient. (a) Southern blot hybridization. Samples of carcinoma DNA (4  $\mu$ g) were undigested (lane 1) or digested with various enzymes (*Ava*I [lane 2] *Bam*HI [lane 3], or *Bam*HI and *Eco*RI [lane 4]) and subjected to Southern blot hybridization with  $^{32}$ P-labeled HPV-20 DNA as a probe (4). F II, open circle DNA; F III, linear DNA. (b) Northern blot hybridization. A sample of total carcinoma RNA (15  $\mu$ g) was denatured with formamide and subjected to Northern blot hybridization with radiolabeled HPV-20 DNA as a probe (4).

To date, DNAs of HPV types 5, 8, 14, and 17 have been detected in EV carcinomas (3, 7–9, 11), and here we reported the detection of HPV-20. Of these HPVs, types 5 and 8 have been detected in skin carcinomas most frequently, and the others less frequently. This seems reasonable because HPV types 5 and 8 are the HPVs most frequently detected in benign EV lesions.

We conclude that HPV-20 may be a member of the group of oncogenic EV-associated HPVs.

We are grateful to G. Orth and H. zur Hausen for providing HPV types 3, 17, and 20 and HPV type 38, respectively.

This work was supported in part by grants for cancer research from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare of Japan.

#### REFERENCES

- Hiraiwa, T., T. Kiyono, K. Segawa, K. R. Utsumi, M. Ohashi, and M. Ishibashi. 1993. Comparative study on E6 and E7 genes of some cutaneous and genital papillomaviruses of human origin for their ability to transform 3Y1 cells. *Virology* **192**:102–111.
- Lewandowsky, F., and W. Lutz. 1922. Ein Fall einer bisher nicht beschriebenen Hauterkrankung (Epidermodysplasia verruciformis). *Arch. Dermatol. Syphilol.* **141**:193–203.
- Lutzner, M. A., C. Blanchet-Bardon, and G. Orth. 1984. Clinical observations, virological studies and treatment trials in patients with epidermodysplasia verruciformis. A disease induced by specific human papillomaviruses. *J. Invest. Dermatol.* **83**:185–255.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Nishikawa, T., T. Yamashita, T. Yamada, H. Kobayashi, A. Ohkawara, and K. Fujinaga. 1991. Tumorigenic transformation of primary rat embryo fibroblasts by human papillomavirus type 8 E7 gene in collaboration with the activated *H-ras* gene. *Jpn. J. Cancer Res.* **82**:1340–1343.

6. **Orth, G.** 1987. Epidermodysplasia verruciformis, p. 199. *In* N. P. Salzman and P. M. Howley (ed.), *The papovaviridae II*. Plenum, New York.
7. **Orth, G., M. Favre, F. Breitburd, O. Croissant, S. Jablonska, S. Obalek, M. Jarzabek-Chorzelska, and G. Rzeska.** 1980. Epidermodysplasia verruciformis. A model for the role of papillomaviruses in human cancer. *Cold Spring Harbor Conf. Cell Proliferation* **7**:259–282.
8. **Ostrow, R. S., M. Bender, M. Niimura, T. Seki, M. Kawashima, F. Pass, and A. J. Faras.** 1982. Human papillomavirus DNA in cutaneous primary and metastasized squamous cell carcinomas from patients with epidermodysplasia verruciformis. *Proc. Natl. Acad. Sci. USA* **79**:1634–1638.
9. **Pfister, H., A. Gassenmaier, F. Nürnbergger, and G. Stüttgen.** 1983. Human papilloma virus-5 DNA in a carcinoma of an epidermodysplasia verruciformis patient infected with various human papillomavirus types. *Cancer Res.* **43**:1436–1441.
10. **Tanigaki, T., R. Kanda, M. Yutsudo, and A. Hakura.** 1986. Epidemiologic aspects of epidermodysplasia verruciformis (L-L 1922) in Japan. *Jpn. J. Cancer Res.* **77**:896–900.
11. **Yutsudo, M., T. Shimakage, and A. Hakura.** 1985. Human papillomavirus type 17 DNA in skin carcinoma tissue of a patient with epidermodysplasia verruciformis. *Virology* **144**:295–298.
12. **zur Hausen, H., and A. Schneider.** 1987. The role of papillomaviruses in human anogenital cancer, p. 245. *In* N. P. Salzman and P. M. Howley (ed.), *The papovaviridae II*. Plenum, New York.