Human Papilloma Virus 5-DNA in a Carcinoma of an Epidermodysplasia Verruciformis Patient Infected with Various Human Papillomavirus Types

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ABSTRACT

An epidermodysplasia verruciformis patient suffering from generalized warts and a carcinoma at the forehead was found to be infected by at least six types and subtypes of human papillomaviruses. The central part of the carcinoma, however, harbored only human papillomavirus 5 DNA. The DNA persisted extrachromosomally in high genome copy number. In contrast, HPV 8 DNA was prepared from bacteria clones (13). Histology. Specimens were fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. Blot Hybridization of DNA. Tumor DNA was extracted by treatment with SDS, proteinase K, phenol, RNase, and phenol (18). One to 10 µg of DNA, either untreated or cleaved with various enzymes, were applied to agarose gel electrophoresis and transferred to nitrocellulose filters as described earlier (13, 15). For hybridization under stringent conditions, the filters were preincubated with 250 µg salmon sperm DNA per ml in 40% formamide, 0.2% Denhardt solution, and 3 x SSC for 5 hr at 37°C. Under the same conditions, the filters were subsequently incubated for 36 hr with 32P-labeled HPV 8 or HPV 5 DNA (0.5 x 106 cpm per slot). Filters were washed twice with 6 x SSC, 0.5% SDS for 2 hr at 68°C and 3 times with 2 x SSC. Hybridization under relaxed conditions was carried out in 10% formamide, 0.2% Denhardt solution, 1 M NaCl and 10 mM Tris, pH 7.5, for 48 hr at 37°C after preincubation with 0.2% Denhardt solution and 3 SSC for 5 hr at 68°C. These filters were washed twice with 4 x SSC at 42°C. After air drying, the filters were autoradiographed with Kodak XAR 5 X-ray film.

Labeling of DNA. One µg of cloned HPV 8 DNA or about 100 ng of HPV 5 DNA were labelled by the nick-translation procedure (14). The HPV 8 DNA preparation was described previously (13), and the HPV 5 DNA isolation followed a published protocol (12). Reactions were carried out at 17°C for 1 hr in 65 µl at the following final concentrations: 23 mM Tris, pH 7.5; 7.7 mM MgCl2; 0.1 mM dATP, dGTP, dCTP each; 2.6 mM [α-32P]dUTP (410 Ci/mmol); DNase I (50 ng/ml) (Worthington); and DNA polymerase I (50 units/ml). The reaction was terminated by the addition of 20 µl of 2.5% SDS, 60 µM EDTA, and salmon sperm DNA (2 mg/ml), and the solution was passed through a Sephadex G-50 column. The probe exhibited a specific radioactivity of about 106 cpm/µg DNA.

Restriction Enzyme Cleavage and S Digest. Restriction enzymes BamH1, EcoRI, HindIII, SalI, and PstI were purchased from BRL, and BclI and SacI were from Boehringer. They were used according to the

INTRODUCTION

EV3 was originally described as a genetic disease characterized by disseminated, persistent warts, usually arising during childhood, and by a high risk for developing skin cancer (5). The patients show congenital defects of cell-mediated immunity, which make them prone to infections with certain types of HPVs (7). At least 6 types can be isolated from EV lesions, namely HPVs 3, 5, 8, 9, 10, and 12 (Refs. 9 and 13 and Footnote 4). HPVs 3 and 10 lead to flat warts as frequently observed in children. The other types were derived from extremely flat warts, reddish plaques, and pityriasis versicolor-like lesions with a very typical histology which led to the name of the disease, dysplastic swollen cells with pale staining cytoplasm, clustered in the stratum spinosum and granulosum (2, 5, 10).

About one-third of the patients develop cancer between 2 and 60 years after the onset of verrucosis, with 24 years after onset being the average time of cancer development (for review, see Ref. 6). An etiological role of papillomaviruses in the progression from warts to carcinomas was first suggested by the observation that malignant conversion did not occur in connection with HPV 3 infections, but only in patients with papillomavirus-like warts (10). Orth et al. (8) furthermore demonstrated HPV 5-specific sequences in DNA from carcinoma material, and this was confirmed quite recently both for a primary carcinoma and a metastasis (11). These findings may point to a role of the virus both in development and maintenance of the malignant phenotype if the DNA persistence is not considered as a mere passenger effect.

Certainly, more patients must be analyzed to establish both the regular persistence of papillomavirus DNA in carcinoma tissue and the association of malignant conversion with certain virus types. In this paper, we describe another case of EV where striking heterogeneity of viruses from warts was in contrast to the homogeneity of papillomavirus DNA from the patient’s carcinoma. The virus type from the carcinoma is compared with the former carcinoma isolates.

MATERIALS AND METHODS

Materials. Wart biopsies and skin carcinoma investigated in this study were obtained from Patient E. S., born in 1941 in Turkey (3). Wart biopsies of EV Patient L. H.-S. were kindly provided by the Dermatological Department (Professor Schuppili), Kantonsspital Basel, Switzerland, and were the source of HPV 5 prototype DNA (8). HPV 8 DNA was prepared from bacteria clones (13).

Histology. Specimens were fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Blot Hybridization of DNA. Tumor DNA was extracted by treatment with SDS, proteinase K, phenol, RNase, and phenol (18). One to 10 µg of DNA, either untreated or cleaved with various enzymes, were applied to agarose gel electrophoresis and transferred to nitrocellulose filters as described earlier (13, 15). For hybridization under stringent conditions, the filters were preincubated with 250 µg salmon sperm DNA per ml in 40% formamide, 0.2% Denhardt solution, and 3 x SSC for 5 hr at 37°C. Under the same conditions, the filters were subsequently incubated for 36 hr with 32P-labeled HPV 8 or HPV 5 DNA (0.5 x 106 cpm per slot). Filters were washed twice with 6 x SSC, 0.5% SDS for 2 hr at 68°C and 3 times with 2 x SSC. Hybridization under relaxed conditions was carried out in 10% formamide, 0.2% Denhardt solution, 1 M NaCl and 10 mM Tris, pH 7.5, for 48 hr at 37°C after preincubation with 0.2% Denhardt solution and 3 SSC for 5 hr at 68°C. These filters were washed twice with 4 x SSC at 42°C. After air drying, the filters were autoradiographed with Kodak XAR 5 X-ray film.

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1 This work was supported by the Deutsche Forschungsgemeinschaft (SFB 31; Tumorentstehung und -Entwicklung).
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3 The abbreviations used are: EV, epidermodysplasia verruciformis; HPV, human papilloma virus; SDS, sodium dodecyl sulfate; SSC, standard saline citrate (0.15 M sodium chloride-0.015 M sodium citrate, pH 7.4).
4 G. Orth, personal communication.
5 Received July 13, 1982; accepted December 3, 1982.
recommendations of the manufacturer. To test for conversion of supercoiled papillomavirus DNA into linear molecules, carcinoma DNA was treated with various concentrations of single-strand-specific nuclease S1 (Sigma Chemical Co.) in 30 mM sodium acetate, pH 4.6, 0.5 mM zinc acetate, and 75 mM NaCl at 37°C for 10 min (1). After the reaction, samples were cooled in ice and neutralized by the addition of Tris-EDTA, pH 8.0, to a final concentration of 100 and 1 mM, respectively.

**Physical Mapping.** Physical mapping of HPV 5 was achieved by double cleavages of carcinoma DNA with several endonucleases. The virus-specific fragments were visualized by the Southern blotting technique with 32P-labeled HPV 5 DNA.

**RESULTS**

**Clinical Data.** Patient E. S. observed multiple verruca plana-like lesions on the dorsa of both hands (Fig. 1a), on neck, and on breast first at the age of 14. The flesh-colored or slightly brownish warts, 3 to 4 mm in diameter, gradually spread to the face, the trunk, and extremities paralleled by photosensitivity. At the age of 35, a left-sided frontotemporal forehead tumor had to be removed at the military hospital in Ankara. Four years later, a new tumor developed at the right frontotemporal forehead, which rapidly enlarged and ulcerated (Fig. 1b). The tumor was removed, and histological examination revealed a highly differentiated Bowen carcinoma (Fig. 2, a to c). Several wart scrapings were obtained at the same time. The lesions showed hyperkeratosis, hypergranulosis, moderate acanthosis, and highly characteristic nests of basophilic, foamy giant keratinocytes within stratum granulosum and stratum spinosum (Fig. 2d).

**Demonstration of Viral DNA in Benign and Malignant Tumors.** Benign warts and carcinoma material were tested for papillomavirus-specific DNA by Southern blot hybridization with 32P-labeled HPV 8 DNA. Both warts and carcinoma harbored viral sequences, the majority of which comigrated with Forms I, II, and III of free viral DNA (Fig. 3). In addition, high-molecular-weight DNA bands were observed in the carcinoma sample which did not appear with warts even after prolonged exposure (Fig. 3).

In order to obtain a more detailed picture of the DNA distribution in the carcinoma, adjacent cylinders were punched from the biopsy and examined histologically and biochemically, respectively. The epidermis part of the cylinder revealed a number of cells with cytopathogenic effects clearly indicative of HPV 5 or 8 infection (Fig. 2, a and d). The deeper layers were rather uniform with carcinoma islets and no signs of viral cytopathogenic effect (Fig. 2b). For DNA analysis, the second cylinder was cut into 1.0-mm slices, and DNA was extracted separately. All deeper slices contained high amounts of viral DNA, actually more than the epidermal and subepidermal layers. In contrast, cylinders at the edge of the malignant tumor were positive only in the 2 superficial slices, a fact which was in line with the histological picture with a few cells showing cytopathogenic effect in the epidermis and normal connective tissue below (data not shown). From these data, it seems likely that papillomavirus DNA persists in the carcinoma itself and is

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**Fig. 1.** a, multiple flat wart-like lesions on the dorsum of the left hand of Patient E. S. b, ulcerated Bowen carcinoma from the right frontotemporal region.
not due to surrounding virus-producing papilloma tissue.

Classification of Virus Types from Warts and Carcinoma. We examined several wart scrapings from small skin areas from hands, forearms (1, 2, 4), neck, and breast (6, 8) as well as the center and the edge of the carcinoma by restriction enzyme cleavage of the DNA. Virus-specific BamHI cleavage products of wart DNA were visualized by hybridization with HPV 8 DNA under stringent conditions which led to heterogeneous patterns due to different virus types or subtypes (Fig. 4). They had either no cleavage site, one cleavage site, or 2 cleavage sites, the latter leading to the following fragment pairs (M, x 10^-6): 4.4 and 0.4; 2.9 and 2.1; 2.7 and 2.2; 3.4 and 1.4. The different types can easily be identified by comparing quantitative and qualitative differences of individual wart scrapings.

Some wart samples contained up to 6 different subtypes (Fig. 4). In contrast, DNA from the center of the carcinoma revealed predominantly one subtype (more than 99%). EcoRI cleavage led to a corresponding result (data not shown); four subtypes were discernible, and again only one subtype prevailed in the carcinoma.

Cleavage data for the more frequent virus types are summarized in Table 1. Two can be identified as HPV 5 and HPV 8, respectively; the HindIII cleavage pattern of Isolate E is identical to that of HPV 8a (13), and both viruses differ only in an additional BamHI cleavage site. Isolate D shows BamHI and EcoRI fragments of the same size as HPV 5, described by Orth et al. (8), but its HindIII/Ill pattern is considerably different from that of the HPV 5 prototype (Fig. 5). As isolate D was detected in carcinoma material, it was characterized further by cleavage with additional restriction enzymes, and a physical map is shown in Fig. 5. The exact designation of Isolates B and C remains to be established.

In order to look for papillomavirus types which do not cross-hybridize with HPV 8 under stringent conditions, hybridization was also carried out at 50° below the melting temperature of the DNA, but no additional papillomavirus types were disclosed (Fig. 4).

Status of Viral DNA in Carcinoma Tissue. The organization of viral DNA was studied by the cleavage of total carcinoma DNA with restriction enzymes which cut HPV 5 DNA once (BclI), twice (BamHI, EcoRI), or not at all (SalI) (Fig. 3). Non-cutter SalI did not change the pattern of uncleaved carcinoma DNA, which consisted primarily of Forms I, II, and III of free viral DNA and some minor bands of higher molecular weight. All virus-specific DNA was converted into linear Form III by BclI, and cleavage with BamHI and EcoRI led to the known fragments of free viral DNA. These data argue for an extrachromosomal persistence of HPV 5 DNA without major deletions or insertions. The number of free viral genome equivalents per diploid cell is about 100 as deduced from reconstruction experiments (data not shown), and less than one viral genome equivalent per cell could be integrated into the cellular genome.

The high-molecular-weight papillomavirus DNA is generally believed to represent oligomers (4, 16) either in catenated or concatemeric form. To differentiate between both possibilities, carcinoma DNA was treated with nuclease S1 which converts supercoiled papovavirus DNA into linear molecules (1). Catenates should lead to linear monomers whereas concatemers would result in monomers and oligomers. As shown in Fig. 6, 2 bands appear due to S1 treatment, a prominent one corresponding to linear monomers and a faint band at the position of linear dimers. This indicates that at least part of the oligomeric DNA persists in concatemeric form.

**DISCUSSION**

The EV patient of this study was infected by several types and subtypes of HPV, all of which cross-hybridized with HPV 8 under stringent conditions. Furthermore, they cross-hybridized with HPV 5 under stringent conditions but not with HPV types 1, 2, 3, 4, 6, 10, 11, or 13 (data not shown). Two of the isolates could be identified as subtypes of HPV 8 and HPV 5, respectively, both by their characteristic restriction enzyme cleavage patterns and by the hybridization efficiency with HPV 8. The comparison of ethidium bromide staining intensity with autoradiography signals (data not shown) and the comparison of autoradiography signals after stringent and relaxed hybridization conditions (Fig. 4) demonstrate the low cross-reactivity between HPV 5 and HPV 8. BamHI cleavage types A (resistant), B (1 recognition site), C (fragments, 4.4 x 10^6 and 0.4 x 10^6), and F (fragments, 3.4 x 10^6 and 1.4 x 10^6) also showed stronger signals after relaxed conditions, indicating a more remote relationship with HPV 8, but a further characterization was not within the scope of this paper and remains to be established.

As described in 3 earlier cases (8, 11), HPV 5 DNA could be demonstrated in the central part of the carcinoma. In this study, the corresponding regions were histologically characterized as connective tissue with carcinoma islets and without cells with virus-specific cytopathogenic effects. In another one (11), the viral DNA was also present in a metastasis. Both arguments...
favor the association of HPV 5 DNA with the carcinoma itself and make it unlikely that the DNA is due to virus production in nearby papilloma cells.

It was interesting to find only HPV 5 in our patient's carcinoma although wart scrapings from very small skin regions harbored many types and subtypes. This may theoretically point to a monoclonal origin of the carcinoma from a cell which happened to be infected with HPV 5 or to an increased carcinogenic potential of HPV 5. The latter assumption would be in line with the preferential association of HPV 5 DNA and cancers observed by other authors (Refs. 9 and 11 and Footnote 4). From this point of view, the virus could not be regarded as a mere passenger but would be at least partially responsible for induction and/or maintenance of the malignant state.

Three HPV 5 subtypes from carcinomas are now characterized in more detail by restriction enzyme analysis (Refs. 8 and 11 and this paper). They seem to be closely related as far as some rare cutting enzymes are concerned, but all 3 differ considerably in the HindIII cleavage pattern. This may reflect varying homology in different genome regions. The available data suggest a highly conserved part within the EcoRI B fragment, and it will be interesting to identify it as early or late function.

The state of the viral DNA in the carcinoma and in warts was similar. As described repeatedly for nonproducing, papillomavirus-induced tumors (for review, see Ref. 17), the DNA persists extrachromosomally without evidence for integration into the cellular genome. The only difference was that aggregates of viral DNA were observed in the carcinoma probe and were missed in wart DNA blots even after prolonged exposure. SSH treatment showed that at least part of them represents concatemeric dimers. A quantitative difference in the relative amount of viral DNA oligomers between benign and malignant lesions was also described for the Shope papillomavirus system (16). Most probably, however, this difference is due to DNA replication in persistently infected cells versus productively infected cells and will not reflect an important step in malignant conversion.

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HPV 5 in an EV Carcinoma

hybridization with HPV 8

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