Detection of Human Papillomavirus DNA in Invasive Carcinomas of the Cervix by in Situ Hybridization

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ABSTRACT

An examination of 27 invasive cancers of the cervix was performed using the technique of in situ hybridization using human papillomavirus DNA probes. Four tissues, previously found to harbor papillomavirus DNA by filter hybridization, were confirmed by in situ analysis. One further tissue never previously studied was also found to be positive by in situ hybridization. Overall, we found 33% of invasive cancers of the cervix to contain human papillomavirus DNA. In contrast, 55% of carcinoma in situ and severe dysplasia of the cervix were found to be positive for human papillomavirus DNA. These results confirmed that the sample population of patients in our studies have a relatively low association of human papillomavirus DNA with invasive cancers of the cervix and that in situ hybridization provides an effective complementation to filter hybridization for human papillomavirus-infected tumors.

INTRODUCTION

Recent studies have implied a close association of certain HPV with invasive cervical cancers. While HPV-18 has been found in cell cultures derived from patients with cervical cancer (1, 2), usually it has been HPV-16 that is detected in fresh biopsy samples (1, 3–7). To lesser extents, HPV-6 and HPV-11 have also been found in verrucous carcinomas of the penis and vulva (8, 9), in the vagina (10), as well as in an invasive cancer of the cervix (11). The portion of invasive cervical cancers with HPV DNA involvement varies from approximately 20% (7) to 90% (12). In some cases, variations of 35–80% can be observed in specimens from different geographical locations (5, 6). Results of our previously reported studies (7) tend to indicate a significantly lower proportion of HPV DNA-associated cervical cancers than some other laboratories. Although the filter hybridization technique used offered a sensitivity capable of detecting as little as one viral genome copy in 20 cells, we decided to reexamine our previously tested biopsies as well as new biopsies by a second technique (7). In situ hybridization, using tritium-labeled probes, offers the detection of as little as one to three viral genome copies per cell based upon 0.1 silver grain formed for each 3H disintegration; an 8-kilobase genome with a specific activity of 0.3–1.0 × 10⁸ dpm/µg, a 30-day exposure, and 10 grains above background (13). While on the average this is lower in sensitivity than can be achieved with filter hybridization assays, the ability to detect individual cells or foci of cells with a few copies per cell is indeed superior with in situ hybridization. Often such cells, diluted by many normal cells, may be missed by filter hybridization. We present here a survey of 27 invasive cancers of the cervix by this method.

MATERIALS AND METHODS

Biopsy samples were carefully taken to include only invasive cancer tissue while avoiding any adjacent warty or dysplastic tissue. This was confirmed by rigorous histopathological analysis of the paraffin block sections. The technique for in situ hybridization of the paraffin-embedded tissues is described in detail elsewhere (14, 15). Briefly, sections from formalin-fixed, paraffin-embedded tissues were floated on a warm 1% solution of Elmer’s glue mounted on microscope slides that had been acid washed, Denhardt’s (16) treated overnight and acetylated. Sections were dried at 45°C and successively incubated overnight at 37°C and then 2–4 h at 60°C. Following deparaffinization with xylene and alcohol dehydration, sections were treated sequentially with digoxin (0.05%, 23°C, 5 min), water, proteinase K (5 µg/ml), alcohol dehydration, and RNase A (100 µg/ml) and RNase T1 (10 units/ml) at 37°C for 30 min. Water-rinsed slides were fixed for 2 h at 23°C in the dark with 5% paraformaldehyde dissolved in 0.3 M NaOH in phosphate buffered saline and neutralized with HCl to pH 7. Subsequently, the slides were rinsed twice with 2× standard saline citrate (0.3 M sodium chloride-0.03 M sodium citrate) and once with water and dehydrated in preparation for the hybridizations. Tritium-labeled HPV DNA probes were prepared by nick translation and treated with DNase so that the probe size is under 240 nucleotides long as determined by acrylamide electrophoresis. The sections were hybridized using mixed probes of HPV-2, HPV-6, HPV-16, HPV-18, largely free of their plasmid vectors following restriction endonuclease cleavage, agarose gel electrophoresis, and electrolization of the HPV DNAs onto DE81 paper.

Hybridization solution [50% formamide; 10 mM 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4); 0.6 M NaCl; 1 mM EDTA; 0.02% each of Ficol 400, polyvinylpyrrolidone, and bovine serum albumin; 200 µg/ml depurinated salmon sperm DNA; and 100 µg/ml yeast tRNA] containing 0.06 ng/µl of the ³H-labeled probe were pipetted onto a section and the section was covered with a siliconized coverglass which was then covered with mineral oil. DNA was denatured for 15 min at 80°C in an oven followed by quenching on ice for 10 min. The slices were then placed in the dark at room temperature for 60–70 h. Following this, the slides were washed with CHCl₃, the coverslips were removed, and the sections were washed for 3 days with a 50% formamide solution for hybridization under high-stringency conditions.

Following dehydration with ethanol containing 0.3 M ammonium acetate, the slides were air-dried and dipped in a Kodak NTB nuclear track emulsion, air dried for 1 h, and placed in a light-tight container with anhydrous calcium chloride as a drying agent for 18–32 days. The emulsion-covered slides were then developed in Kodak D-19 developer, fixed, counterstained with hematoxylin, and mounted with coverslips to protect the emulsion. Adjacent tissue sections were used for controls. These included a heterologous probe (pBR322), no heat denaturation of cellular DNA, or pretreatment with DNase I prior to hybridization.

Slides were scanned by at least two of us (D. A. M., R. S. O.), without prior knowledge of the filter hybridization results, and were spot checked by two other individuals (T. O., B. A. C.). Criteria for positive results included detection of silver grains directly over nuclei, detection of more than one cell per slide and cells in more than one subjacent section, detection of a reasonable signal:noise ratio, and no detection of grains in adjacent sections in controls (Fig. 1). Each individual batch of sections included an HPV DNA-negative endometrial tissue and an HPV-6 DNA-positive condyloma acuminateum sample or an HPV-16 DNA-positive carcinoma in situ tissue.

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3 The abbreviation used is: HPV, human papillomavirus.
RESULTS

Twenty-seven invasive cancers of the cervix were analyzed for the presence of HPV-2, HPV-6, HPV-16, and HPV-18 by in situ hybridization (Table 1). Many of these had also been screened earlier by filter hybridization analysis (7). An example of an HPV DNA-positive tissue and its control tissue sections is shown in Fig. 1. A total of nine of the samples were found to contain HPV DNA; sample fields of these are shown in Fig. 2. All positive nuclei appeared in the epithelial component. In one of these samples, however, strong hybridization was observed only in benign squamous cells contiguous to invasive squamous cells that were negative for HPV DNA (Fig. 2A). This sample was considered negative for HPV in invasive cells. In some cases, evidence for HPV DNA in the basal layers of the tumor nests, as well as in adjoining dysplastic areas, was present (Fig. 2B), indicating that cells in this layer may indeed be a target for viral infection. Each tissue positive for HPV DNA was also tested against the four individual HPV types used in the mixed probe study. The results showed that four tissues found to contain HPV DNA by filter hybridization were also found to have HPV DNA by in situ hybridization. As discussed below, lack of detection in tissues can be attributed to sampling error or a copy number below the detection limit of the procedure. We estimate that the HPV-16 DNA in these tissues was present in 0.1–10-genome copies per diploid cell equivalent based upon the filter hybridization and reconstruction experiments. The limit of the ability of the in situ technique to detect HPV DNA was at the level of about 5 genome copies per cell. The correlation of four tissues positive by filter analysis also being
positive with \textit{in situ} analysis implied strongly that those found positive by \textit{in situ} but not by filter analysis reflected the ability to detect foci of the HPV-positive cells. This was confirmed by the rigorous controls performed, which included tests of adjacent sections with a heterologous probe, pretreatments with DNase, no heat denaturation of cellular DNA, and duplicate positive observations.

Five specimens of squamous cell carcinoma were positive by \textit{in situ} hybridization. One of the specimens was well differentiated (Grade 1/3), three were moderately differentiated (Grade 2/3), and one was poorly differentiated (Grade 3/3). Background grain count was less than 1/cell in all specimens.

While some areas seemed to have rather uniformly high or low HPV DNA copy number, we have observed in these tissues as well as in othersootnote{S. squamous cell carcinoma; Ad, adenocarcinoma; AdSq, adenosquamous carcinoma; Undif, undifferentiated carcinoma; Cis, carcinoma in situ; ND, not determined; HPV, human papillomavirus.} that not all cells have the same number of HPV DNA copies. One specimen of well-differentiated carcinoma showed strong radioactivity of over 10 grains/cell in some clusters of the tumor cells. In one of three specimens of moderately differentiated carcinoma, most of the tumor cells showed strong radioactivity of over 10 grains/cell. Two specimens showed strong radioactivity in some clusters of cells. One showed weak radioactivity (less than 4 grains/cell) in some of the tumor cells, whereas the benign intermediate cells in an area contiguous to the invasive carcinoma showed very strong (over 50 grains/cell) radioactivity. One specimen of poorly differentiated carcinoma showed moderate radioactivity (3–18 grains/cell) in some foci. In fact, many cells had little or no HPV DNA as detected by this method. Also, in viewing large sections of malignant tissues, various foci of HPV DNA-positive cells were observed. As deeper sections of tissues were examined, variations in the number of positive cells and the intensity of the grain patterns were observed, nearly disappear-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Histological cancers$^a$</th>
<th>\textit{In situ} hybridization</th>
<th>Filter hybridization</th>
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<tr>
<td>UHB3-6114L</td>
<td>Sqc</td>
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<td>-</td>
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<tr>
<td>UHB3-3313L</td>
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<td>+ (HPV-16)</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UHB3-1695G</td>
<td>Sqc</td>
<td>+</td>
<td>+ (HPV-16)</td>
</tr>
<tr>
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<td>Sqc</td>
<td>+</td>
<td>+ (HPV-16)</td>
</tr>
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<td>-</td>
<td>-</td>
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<td>-</td>
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<td>+ (HPV-16)</td>
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<td>-</td>
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<td>-</td>
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<td>-</td>
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<td>+</td>
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<td>Undif</td>
<td>+ (HPV-16)</td>
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\footnote{R. S. Ostrow, unpublished observations.}

In contrast, when we examined premalignant CIN 3 lesions of the cervix consisting of carcinoma \textit{in situ} and severe dysplasia, we found much higher associations with HPV DNA. Mainly using \textit{in situ} hybridization and, in some cases filter hybridization, we found 6 of 11 (55\%) carcinoma \textit{in situ} and 4 of 7 (57\%) severe dysplasias (a total of 10 out of 18 for 55\%) positive for HPV DNA. This shows that we can, indeed, detect HPV DNA in premalignant cervical lesions, which act as a positive control for this study.

While we found this technique to be both sensitive and in some ways more informative about the distribution of infected cells, the protocol did have some drawbacks. We investigated the possibility of using low-stringency hybridization (17) in order to detect HPV types other than those in our probe. We hybridized HPV-16 and HPV-6 DNA probes to tissues known to be positive in large copy numbers for only HPV-16 or HPV-6 DNA using low-stringency hybridization conditions of 10\% formamide or the stringent conditions of 50\% formamide followed by appropriate washing conditions (Fig. 3). While hybridization of each tissue to the homologous probes gave large numbers of positive cells with many silver grains each, hybridization in an adjacent tissue section with a heterologous probe gave only a rather uniform 4–8 grains/cell and an occasional cell with several more grains. While we have observed about a 10-fold loss in sensitivity by similar filter hybridization tests, by \textit{in situ} hybridization we estimate the decrease in sensitivity to be about 2 orders of magnitude, rendering this approach marginally useful for screening purposes at this time. Hybridization of adjacent sections with a heterologous probe under stringent conditions resulted in no positive cells being observed, as was to be expected.

HPV-16 and HPV-6 are somewhat distantly related (5). HPV DNA types such as HPV-1, -5, and -6, which are even more evolutionarily distinct, showed no cross-hybridization at all under less stringent conditions, confirming a previous report (18). However, in contrast to that report which used biotinylated DNA probes, we could easily detect HPV-6 DNA in tissue using a tritiated HPV-11 DNA probe under stringent hybridization conditions with no more than a 2-fold loss of sensitivity compared to the homologous DNA probe.

\textbf{DISCUSSION}

We observed that 6 of 27 (22\%) invasive cancers of the cervix had HPV DNA involvement as determined by \textit{in situ} hybridization analyses. These results were compared to those we have
Fig. 2. Detection of HPV DNA in two invasive cervical cancers by \textit{in situ} hybridization. A mixed-HPV DNA probe was hybridized to each tissue section and exposed for either 17 or 32 days. A, sample 833-HH; B, sample 931A. Original magnification, $\times$ 275.

Fig. 3. Detection of a heterologous HPV DNA using conditions of lowered stringency. A tissue known to have HPV-16 DNA was hybridized \textit{in situ} in a solution containing (A, C) 10\% formamide (relaxed conditions) to HPV-6 or (B, D) 50\% formamide (stringent conditions) with HPV-16. The adjacent sections were each exposed for 11 days (A, B) or 22 days (C, D).

found by filter hybridization (7 of 20 or 35\%). The overall result by both techniques was 33\%, including one sample containing carcinoma \textit{in situ}. There was also one sample in which HPV DNA was observed only in a small area of benign intermediate cells contiguous to invasive carcinoma. These results were confirmed by duplication of the study using $^{35}$S-labeled probes (data not shown). In addition, we have been able to detect HPV-18 at 8–10 grains/cell (data not shown) in Hela cells where we have found about 4–6 copies/cell by filter hybridization analysis (1, 2, 19, 20), thus confirming the sensitivity of this method. By rigorous analysis, we have shown that our study population seems to have a low association of HPV DNA with invasive
cancers of the cervix. This is in contrast to several reports that have shown a much higher association of HPV with cervical cancers (1, 5, 12). However, it is in agreement with several other studies that demonstrated a low association of HPV with cervical cancer (5, 6). Clearly geographical differences of the study population may be responsible for these discrepancies. Perhaps in our study population, HPV may not be directly involved in as large a portion of invasive cervical cancers. Additional factors may be responsible for these observations. Also, it may be that a new type of HPV is responsible for the disease in this area, one that, on the one hand, is present in too low a copy number to be detected by the Southern blot technique and, on the other hand, is sufficiently distinct from the HPV DNA probes that it would not be detected under the highly stringent conditions of hybridizations used in the in situ technique. It is also conceivable that a significant genetic divergence from our prototypical HPV probes may make their detection difficult even by low-stringency hybridization analysis on filters. Biopsies are also mosaics of normal, inflammatory, premalignant, and malignant cells. Careful isolation of malignant cells by physical separation or by specific in situ detection may be eliminating the majority of premalignant cells that have high HPV DNA copy numbers from our assays. Alternatively, the HPV DNA genome may not be necessary for maintenance of the malignant state. Support for this hypothesis lies in a recent report that alimentary tract cancers of cattle were found to lack BPV DNA, although the virus was necessary for the induction of the cancer-associated benign papillomas and was present in the benign lesions (21). The implication was that the continual presence of the viral genome was not necessary for progression or continuation of the transformed cells.

Even in those tissues where we detected HPV DNA by either technique, the viral DNA copy number was quite low. Perhaps what was observed was the final stage of an initial papillomavirus infection which acted as fertile foundation for an interaction with a transforming cofactor. The resulting proliferation of transformed cells which have lost the HPV genome now represent the bulk of the tissue and in nearly 70% of our tissue samples now has all but eliminated the presence of HPV-infected cells. Because this is not the case in malignancies due to HPV-5 infection (22–24), several different oncogenic mechanisms may exist which are specific for species, tissue, or HPV type.

This in situ hybridization technique offers several advantages over the Southern blot procedure. As we have shown, in situ hybridization is both sensitive and informative and complements filter hybridizations for HPV DNA screening. Screening can be accomplished using minute amounts of tissue that have been stored for at least 8 years. In prospective studies, a very small biopsy can be taken that minimizes the effect of the biopsy upon the natural course of development of the tumor. We have also used variants of this technique to detect either RNA transcripts or the simultaneous detection of HPV nucleic acids and structural gene proteins (14). In addition, the use of tritium-labeled probes significantly reduces the exposure of the researcher to high energy nuclides. However, there are limitations in the detection of HPV DNA in tissues having fewer than 5 copies per cell. In short, in situ hybridization provides an effective complement to filter hybridization as well as providing information that would be otherwise unattainable.

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