Human Papillomavirus DNA Sequences in Adenocarcinoma of the Uterine Cervix in Indian Women

B. C. Das, M.Sc., Ph.D., V. Gopalkrishna, M.Sc.,* D. K. Das, M.D., Ph.D.,† J. K. Sharma, M.Phil.,* V. Singh, M.D.,‡ and Usha K. Luthra, M.D., Ph.D.†*

Background. **Infection with human papillomavirus (HPV) is considered to be the principal causal agent in the development of squamous cell carcinoma of the uterine cervix. Although adenocarcinoma of the cervix originates adjacent to the squamous epithelial neoplastic lesions, the etiopathogenesis of adenocarcinoma is not yet clearly understood. Recent studies have raised more controversy, rather than answering the question of whether specific HPV infection also plays a role in the development of adenocarcinoma of the cervix. Molecular DNA hybridization techniques were used to detect HPV types prevalent in both adenocarcinoma and squamous cell carcinoma of the uterine cervix, which is the most common cancer in Indian women.**

Methods. **Histologically confirmed, formaldehyde-fixed, paraffin-embedded tissue sections from 12 cases of adenocarcinoma and 30 cases of squamous cell carcinoma of the uterine cervix were analyzed retrospectively for the presence of HPV DNA types 6b, 11, 16, and 18 by both Southern blot hybridization and in situ hybridization.**

Results. **Of 12 adenocarcinomas, 5 (41.67%) tumors were positive for HPV DNA. All five cases were positive for HPV 16, and two (16.6%) of these were hybridized again to the HPV 18-specific DNA probe. All tumors were negative for HPV 6b and 11. In addition, no biopsy specimens were positive after hybridization with a mixed probe of HPV 31, 33, 35, 39, and 45. These results were compared to those obtained for 30 squamous cell carcinomas of the cervix. Although 20 (66%) were exclusively positive for HPV 16 and 6 (20%), more tumors were of HPV 16 related types as detected under nonstringent conditions of hybridization, only one (3%) was positive for HPV 18. The results of in situ hybridization were found to be in good agreement with those of Southern blotting.**

Conclusions. **HPV 16 is the type present almost exclusively in squamous cell carcinoma of Indian women. A higher frequency of HPV 16 in adenocarcinoma of Indian women, in contrast to HPV 18, as reported from other regions, may be attributed to geographic variation rather than to histologic differences only, and both HPV 16 and 18 may be present in adenocarcinoma of the uterine cervix. *Cancer* 1993; 72:147–53.**

Key words: human papillomavirus, cervical cancer, adenocarcinoma, squamous cell carcinoma, in situ hybridization, Southern blot hybridization.

The association of human papillomavirus (HPV) infection with cervical carcinogenesis is well documented.[1–2] Distinct HPV DNA types, particularly HPV 16 and HPV 18, are demonstrated in more than 95% of invasive squamous cell carcinomas (SCC) of the uterine cervix.[3–8]

Invasive carcinomas of the uterine cervix mainly comprise three histologic subtypes, namely, SCC, adenocarcinoma (ADC), and adenosquamous carcinoma (ADSC). The occurrence of the latter two is relatively low, and the frequency varies between only 3% to 10% of all malignant cervical tumors.[9,10] Unlike SCC, however, the risk factors for ADC of the cervix are not well understood. HPV DNAs, particularly types 16 and 18, are detected frequently in high-grade cervical intraepithelial neoplastic lesions that often arise adjacent to adenocarcinoma of the cervix.[11] Recently, there have been several reports showing the presence of HPV DNA, predominantly HPV type 18, in ADC and ADSC of the cervix.[12–16] This is in sharp contrast to invasive SCC, in which the incidence of HPV 16 is very high.[4–7,17] The high prevalence of HPV 18 in ADC has been attributed mainly to histologic differences rather than to geographic variation.[15,16]

From the Divisions of *Molecular Oncology, †Cytopathology, and ‡Clinical Research, Institute of Cytology and Preventive Oncology (Indian Council of Medical Research), Maulana Azad Medical College Campus, New Delhi, India.

Address for reprints: B. C. Das, M.Sc., Ph.D., Division of Molecular Oncology, Institute of Cytology and Preventive Oncology (Indian Council of Medical Research), Maulana Azad Medical College Campus, New Delhi 110002, India.

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Table 1. Analysis of HPV DNA in Adenocarcinoma of the Uterine Cervix

		In situ hybridization			Southern blot hybridization		
Slide no.	Case no.	*HPV 16*	*HPV 18*	*HPV 6 + 11*	*HPV 16*	*HPV 18*	*HPV 6 + 11*
1	H-84/125	+	–	–	+	–	–
2	H-82/117	–	–	–	–	–	–
3	H-88/399	–	–	–	–	–	–
4	H-88/334	+	+	–	+	+	–
5	H-88/237	–	–	–	–	–	–
6	H-86/82E	+	–	–	+	–	–
7	H-88/369	–	–	–	–	–	–
8	H-81/106	–	–	–	–	–	–
9	H-84/320	+	+	–	+	+	–
10	H-88/420	–	–	–	–	–	–
11	H-89/54	+	–	–	–	–	–
12	H-84/107	–	–	–	–	–	–
Total		5/12 (41.66%)	2/12 (16.66%)	Nil	4/12 (33.33%)	2/12 (16.6%)	Nil

In India, cancer of the uterine cervix is the most common cancer in women, and more than 90,000 women have this cancer annually.[18–20] The prevalence of HPV 16 and its related types was found to be very high (> 90%), whereas the frequency of HPV 18 and other types is extremely low or absent.[5] So far, however, there have been no reports on HPV prevalence in ADC of Indian women. The current study therefore was carried out in 12 histologically confirmed cases of ADC of the cervix, using both in situ hybridization and Southern blotting techniques to detect different HPV types and to compare the results with those obtained for SCC of the uterine cervix.

Materials and Methods

Twelve histologically confirmed ADC and 30 SCC of the cervix were selected for retrospective analysis of HPV DNA sequences by both in situ hybridization and Southern blotting. These cases were diagnosed during regular screening for cervical carcinoma from 1988 to 1990 at the Division of Cytopathology of our Institute. Biopsy specimens were obtained from gynaecological outpatient department (cancer clinic) of the Lok Nayak Jai Parkash Narayan (LNJPN) Hospital, New Delhi. Tissue sections from paraffin blocks were used for in situ hybridization as well as to extract DNA for Southern blot hybridization.

Table 2. Prevalence of HPV DNA in Adenocarcinoma of the Uterine Cervix

Method employed	HPV 16 positivity	HPV 18 positivity	HPV 6 + 11 positivity	Total HPV positivity
Tissue in situ hybridization (n = 12)	5 (41.6%)	2 (16.6%)	Nil	7 (58.3%)
Southern blot hybridization (n = 12)	4 (33.3%)	2 (16.6%)	Nil	6 (50%)

In Situ hybridization

Formaldehyde-fixed, paraffin-embedded tissues were cut into 5-μm sections, mounted on slides that were prewashed in acid and sodium dodecyl sulfate, and coated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO). After deparaffinization with xylene and dehydration through alcohol grades, sections were treated with proteinase K (100 μg/ml; Boehringer Mannheim, Germany) at 37°C for 30 minutes. The slides were then washed in phosphate-buffered saline (PBS; pH 7.2) three times for 5 minutes each, dehydrated through alcohol grades, and air dried. The slides were desiccated at 4°C until use.

Probe Preparation and Hybridization

Molecularly cloned HPV 16,[21] 18,[22] 6b,[23] and 11[24] DNAs were the kind gift of Profs. H. zur Hausen, L. Gissmann and E. M. deVilliers (Deutsches Krebsforschungszentrum, Heidelberg, Germany). The HPV plasmids were purified by cesium chloride–ethidium bromide gradient centrifugation methods. The 8-kilobase (kb) viral inserts were isolated from the vector DNA by digestion with cloning enzyme, separated in agarose gel, and subsequently eluted in DEAE ion-exchange membrane (Serva 40362, Heidelberg, Germany). Vector-free HPV DNAs were nick-translated with ^{3}H-dCTP, ^{3}H-dATP, and ^{3}H-dTTP (specific activities: 66,

Table 3. Prevalence of HPV DNA in Squamous Cell Carcinoma of the Uterine Cervix

Method employed	HPV 16 positivity	HPV 18 positivity	HPV 6 + 11 positivity	HPV 16* positivity	Total HPV positivity
Tissue in situ hybridization (n = 30)	19 (63.4%)	1 (3.3%)	1 (3.3%)	—	21 (70.0%)**
Southern blot hybridization (n = 30)	20 (66.7%)	1 (3.3%)	1 (3.3%)	6 (20.0%)	28 (93.4%)

* Positivity under nonstringent hybridization conditions.
** This excludes related HPV types because no in situ hybridization was done under nonstringent conditions (Tm −40°C).

65, and 100 Ci/mmol, respectively; Amersham International plc, Amersham, United Kingdom) to specific activities of not less than 1×10^8 disintegration per minute/μg DNA. The hybridization mixture contained 50% formamide, 10% W/v dextran sulfate, 10% (V/V) 20× 3M sodium chloride plus 0.3M sodium citrate (SSC), 0.02% (W/V) Ficoll, 0.02% W/V bovine serum albumin (pentax fraction V), 0.02% W/V polyvinylpyrolidone, 250 μg/ml RNA (4S), and the labeled HPV DNA probe. The hybridization solution with HPV probe was added to the tissue sections on slides processed earlier, and hybridization was carried out at 42°C for 48 hours after denaturation at 90°C for 10 minutes and chilling in ice-water for 5 minutes. After washings in 50% formamide in 2× SSC and 0.5× SSC

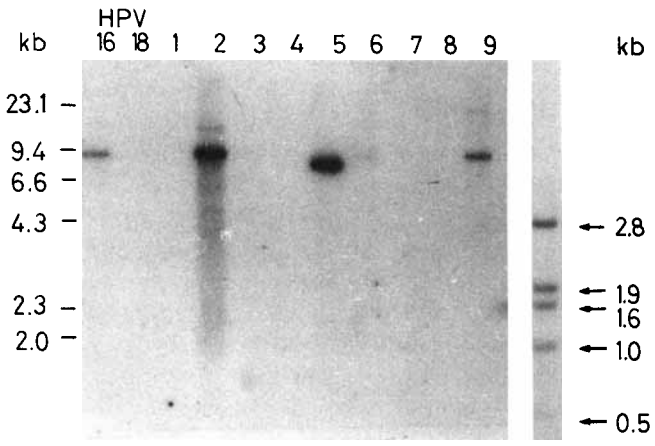

Figure 1. Southern blot hybridization analysis of HPV DNA sequences in ADC of the uterine cervix. Seven to 8 μm of cellular DNA were digested with (left) Bam HI and (right) Pst I, electrophoresed in 1% agarose gel, and hybridized with ^{32}P-labeled HPV 16 DNA probe under stringent conditions (Tm −20°C and 50% formamide). (Left) The results from nine specimens (lanes 1–9), of which four were positive (lanes 2, 5, 6, and 9), showing 8-kb bands for HPV 16. In the first two lanes, 10 pg of HPV 16 and 18 DNA were used as positive controls, and there is no signal for HPV 18 because the probe used was HPV 16. (Right) The characteristic Pst I cleavage pattern of HPV 16 is shown (arrows), and sizes are indicated in kilobases.

solutions at 42°C for 5 minutes each, the slides were again washed twice in 0.5× SSC at room temperature, alcohol dehydrated, and air dried. The slides were then coated in the dark with Kodak NTB 2 nuclear track emulsion (Eastman Kodak, Rochester, NY) and exposed for 4 to 6 weeks at 4°C. After developing in D19b developer, the slides were stained with hematoxylin and eosin and examined by oil immersion microscopy for the presence of silver grains over the cell nuclei.

DNA Extraction and Southern Blotting

DNA extraction was done as described earlier,[25,26] with certain modifications. About 15 to 20 tissue sections from each of the formaldehyde-fixed paraffin blocks were placed in an Eppendorf tube. After deparaffinization in xylene and dehydration in absolute ethanol, the sections were rehydrated and washed three times in PBS. These tissue sections and biopsy samples of SCC, stored at −70°C, were either homogenized in mortar and pestle with a small amount of sea sand and 0.5 ml of 1× TE (10 mmol/l Tris-HCl, and 1 mmol/l EDTA, pH 8.0), or minced with a fine blade in a Petri dish, and the tissue homogenate was transferred to a centrifuge tube containing lysis (3% SDS) buffer and proteinase K (100 μg/ml). The tubes were incubated at 37°C or 50°C overnight, and DNA was extracted using conventional phenol–chloroform methods.

The purified DNA, amounting to about 7 to 8 μg, was cleaved with Bam HI (a single-cut restriction enzyme for HPV; Boehringer Manheim) according to the manufacturer's protocol. One of the five positive samples in which the yield of DNA was sufficient was also digested with a multicut enzyme, Pst 1 (Boehringer Manheim). Digested DNA was separated in 1% agarose gel electrophoresis and blotted onto Gene-Screen Plus membrane (NEN Research Products, Dupont, Boston, MA) as described by Southern,[27] with some modifications.[5,28] Vector-free HPV inserts were nick-translated[29] or random-primed[30] with ^{32}P-dTTP or ^{32}P-dCTP (specific activity, 3000–4000 Ci/mmol; Amersham UK, and

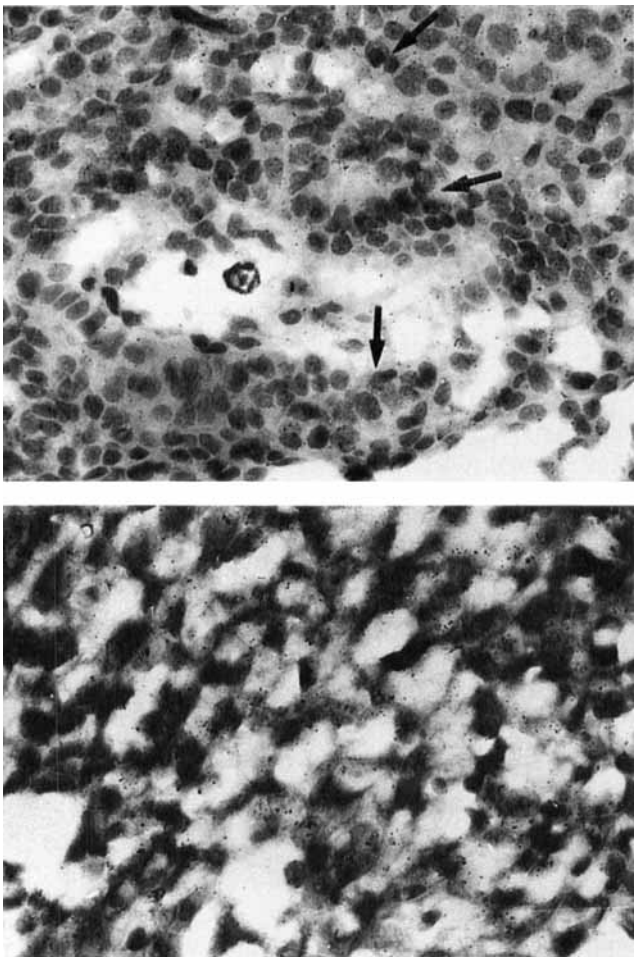

Figure 2. In situ hybridization of ADC tissue sections with radiolabeled HPV 16 and 18 DNA probes using the single-copy gene localization method. The presence of (top) HPV 16 and (bottom) HPV 18 in areas of well differentiated ADC (arrows) was shown by deposition of silver grains over the nuclei. Hybridization was done with ^{3}H-labeled dTTP, dCTP, and dATP, with exposure at 4°C for 5 weeks after coating with Kodak NTB2 nuclear track emulsion.

Bhabha Atomic Research Centre, Bombay, India) to a specific activity of more than 10^8 dpm/μg of DNA. Blots were hybridized with radiolabeled HPV 16, 18, 6b, and 11 DNA under stringent (melting temperature −20°C) as well as nonstringent (melting temperature −40°C) conditions at 42°C (50% formamide) for 16 to 18 hours. After washings, the filters were air dried and exposed to Kodak X-OMAT-AR x-ray film with intensifying screen at −70°C.

Results

HPV types 16, 18, 6b, and 11 DNAs were used as probes either individually or in combination to detect the type of HPV DNA sequence present in 12 pure ADC and 30 SCC of the uterine cervix. The results of in situ hybridization as well as Southern blot hybridization are presented in Tables 1 through 3. Using in situ hybridization, HPV 18 DNA was detected only in two cases, whereas HPV 16 DNA was found in five cases of ADC. The results of in situ hybridization were in good agreement with those of Southern blotting, except for one HPV 16 in situ-positive case that could not be detected by Southern blotting. This disparity between the two methods might be because of low copy of HPV 16 DNA or an inadequate amount of cellular DNA used for Southern blot analysis. Figure 1 shows Southern blot hybridization analysis of nine ADC DNA samples. Lanes 2, 5, 6, and 9 are the four samples, each of which shows a single 8-kb band after digestion with a single-cut enzyme, Bam H1, for HPV 16. Figure 1 (right) shows a characteristic Pst 1 cleavage pattern of HPV 16 (arrows) for the same sample shown on lane 2 in Figure 1 (left).

Under nonstringent conditions of hybridization (Tm −40°C and 20% formamide) using HPV 16 DNA as probe, no positive tumor was found of the seven negative ADC; in addition, none of these were found positive for HPV 16 or 18 by polymerase chain reaction (data not given). The two HPV 18-positive tumors also contained HPV 16 DNA. Figure 2 shows HPV positivity by in situ hybridization of histologic sections of ADC of the cervix. In the current study, the frequency of HPV 16 positivity was found to be 41.67% (5/12), which is higher than the frequency of HPV 18 positivity (16.67%, 2/12) in ADC of the uterine cervix. The difference, however, was found to be statistically insignificant because of the small number of cases analyzed. No ADC was found to be positive for HPV 6b and 11 DNA by either in situ or Southern blot hybridization. Also, no positive signal was obtained when hybridization was carried out using a mixed probe of other prevalent genital HPV types (31, 33, 35, 39 and 45).

The results obtained in 30 SCC samples by both in situ and Southern blot hybridization are presented in Table 3. Representative in situ hybridization photomicrographs (Fig. 3) show clear positivity for HPV 16, as revealed by high grain concentrations in squamous cell nuclei. These samples were used again for Southern blot hybridization (Fig. 4), which showed authentic Pst I fragments (arrows) for HPV 16. There also was good correlation between the results obtained by the above two techniques. With in situ hybridization, 63% (19/30) were positive for HPV 16, whereas 66% (20/30) were positive by Southern blotting. Total positivity for HPV was 93%, which includes nonstringently (Tm −40°C) detected related HPV types (20%). Other HPV types were completely absent, except one specimen each positive for HPV 18 (3.3%) and HPV 11 (3.3%). Because no nonstringent in situ hybridization could be

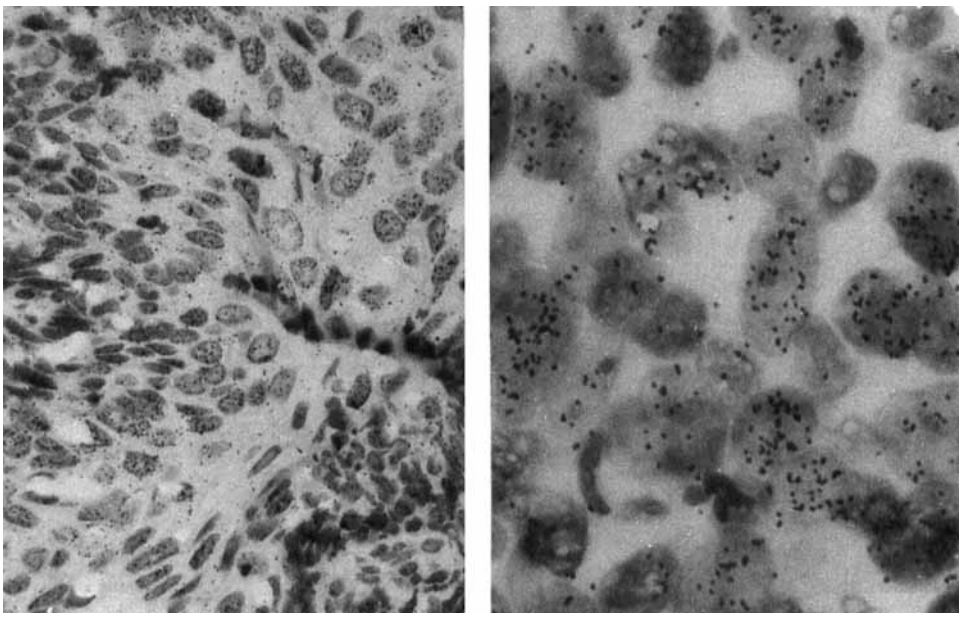

Figure 3. In situ hybridization of SCC tissue sections with ^{3}H-labeled HPV 16 DNA probe. High grain concentrations can be seen in most of the cell nuclei under (left) low (hematoxylene and eosin, original magnification ×100) as well as (right) high (hematoxylene and eosin, original magnification ×400) magnification.

done, no related or unknown HPV type was detected by this technique.

Discussion

Squamous cell carcinoma is the predominant type of cancer of the uterine cervix, and HPV 16 is the most common type of HPV DNA in these tumors, being detected in 80% to 90% of cases, whereas HPV 18 has been found only in 1% to 25% of cervical carcinomas.[1,3-8,31] In contrast, several reports demonstrate that HPV 18 is the most prevalent and HPV 16 is less frequent in ADC and ADSC of the uterine cervix.[12-16]

The current retrospective study in Indian women demonstrates a higher frequency of HPV 16 rather than HPV 18 in ADC, as revealed by both in situ and Southern blot hybridization (Tables 1, 2). This is in good agreement with the recent report of Giffin et al, who used polymerase chain reaction.[32] Of 12 cases, 5 (41.67%) were positive for HPV 16, whereas only 2 (16.67%) contained HPV 18 DNA. Again, these two cases with HPV 18 also contained HPV 16 DNA. This may raise the question that this HPV 16 DNA might have been derived from adjacent neoplastic squamous epithelium. Such an assumption is not warranted, because all cases were histologically confirmed as pure ADC, and there was no sign of neoplastic squamous epithelium in the sections stained with hematoxylin and eosin (Fig. 2), which were examined thoroughly by cytopathologists before being selected for HPV DNA analysis. Nevertheless, presence of both HPV 16 and HPV 18 in ADC of the cervix has been reported recently.[16,33] The existence of HPV 16, although in low frequency, already has been demonstrated in ADC of the cervix by several earlier authors.[12-16,34] In sharp contrast to findings from other regions, the high frequency of HPV 16 in ADC of the cervix in Indian women may be attributed to geographic variation

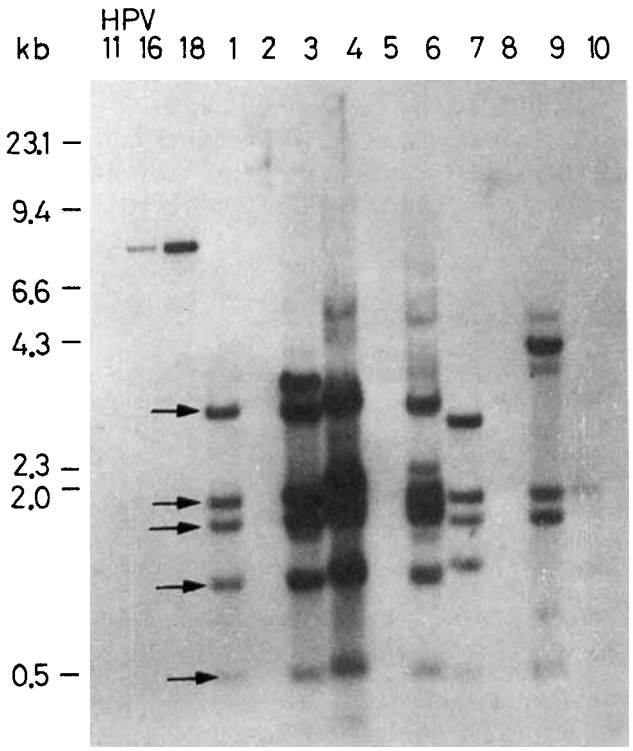

Figure 4. Southern blot hybridization of Pst I-digested DNA samples of SCC. Arrows indicate authentic Pst I-specific bands for HPV 16.

rather than solely to histologic differences. This argument gains support from the recent United Kingdom study using polymerase chain reaction that also demonstrated a higher frequency (4/16) of HPV 16 as opposed to HPV 18 (1/16) in ADC of the cervix.[32] Furthermore, the frequency of HPV 18 in SCC in Indian women was found to be extremely low (3%), whereas the frequency of HPV 16 was more than 90% (Tables 2, 3).[4,5] Using biotinylated probes in in situ hybridization, Young et al. have shown a complete absence of HPV in ADC from other parts of the United Kingdom.[35] These observations strongly suggest that the prevalences of HPV types in ADC of the cervix vary in different geographic regions, just as has been reported for SCC.[3] The frequent coexistence of adenomatous lesions with squamous lesions[11] suggests that both squamous and columnar neoplasm may originate from the same precursor "reserve cells."[35] Depending on the carcinogenic stimulus (e.g, viral infection) and the type of viral receptors present, the cervical cells may transform into neoplasia of different histologic types such as SCC, ADSC, and ADC.[12] Alternatively, if HPV infection is responsible for transformation, it is possible that there may be subclinical benign lesions in endocervical cells that give rise to SCC through cervical intraepithelial neoplasia.[15] There is good agreement, however, between the results with Southern blotting and in situ hybridization. To resolve the issue of which HPV type is more prevalent in ADC, more studies on the prevalence of HPV in cervical ADC are needed from different geographic regions.

Nevertheless, the presence of both HPV types 16 and 18 DNA sequences in ADC confirms others findings and suggest that both of these "high-risk" HPV types may coexist in ADC of the uterine cervix.

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