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Absence of Human Papillomavirus Sequences in Ovarian Pathologies

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The role of human papillomaviruses (HPVs) in ovarian epithelium (either normal or neoplastic) remains controversial. We have investigated by PCR the presence of HPV DNA in fresh tissue from ovarian neoplasms and in primary cell cultures established from either normal, benign, or malignant ovarian surface epithelia. None of the fresh samples and primary ovarian cultures contained HPV DNA sequences.

Ovarian epithelial cancer is the most lethal gynecologic malignancy in North America (4). Most of these ovarian cancers originate from the surface epithelium or the crypts of the ovary. Although human papillomaviruses (HPVs) have been associated with benign and malignant proliferative lesions of the genital tract (7), published reports of biopsy sample analyses have drawn conflicting conclusions on their potential role in ovarian cancer (3, 10, 11, 15–18). Analysis of cervical cancer-derived cell lines helped in defining the association between HPV and cervical cancer (6, 7) and was a determinant in the characterization of the transformation mechanism of oncogenic HPV types (7, 9, 22, 23). Such an approach has not been reported for ovarian cancer.

In this study, we investigated the potential role of HPVs in ovarian pathologies by testing fresh clinical specimens and also primary cell cultures derived from ovarian epithelia. A consensus PCR assay was used to detect most genital HPV types and also novel HPV types (2, 8).

Fresh ovarian material. Fresh neoplastic and normal ovaries were collected aseptically during laparotomies performed at Hôpital Notre-Dame, Montréal, Canada. Histological reports were obtained for all specimens. A portion of each sample was used to extract DNA and to derive primary cultures.

Tissues were cut into small pieces and suspended in 500 μl of extraction buffer containing 10 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 400 mM NaCl. 10 μg of RNase was added, and the solution was incubated at room temperature for 15 min. Following the addition of proteinase K and sodium dodecyl sulfate (400 ng and 0.5%, respectively), the mixture was incubated at 55°C overnight and extracted with phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was then precipitated and resuspended in 10 mM Tris-HCl (pH 7.5)–0.1 mM EDTA prior to PCR.

Culture methods. Normal ovarian surface epithelium cultures were obtained by the scrape method and maintained in OSE medium consisting of a 50:50 mixture of medium 199 and medium 105 (Sigma) supplemented with 15% fetal bovine serum (FBS) as previously described (13). OSE medium was consistently supplemented with 2.5 μg of amphotericin B (Fungizone) per ml and 50 μg of gentamicin per ml.

Protocols for establishing primary cultures of solid tumors (benign and malignant) are described elsewhere (17). Briefly, tissues were minced with scissors into 2- to 4-mm-thick explants in OSE medium (without FBS) and then subjected to enzymatic dissociation with 1,000 U of collagenase for 4 to 12 h. After the collagenase treatment, aggregates were dissociated by gentle pipetting and the remaining undigested solid material was allowed to sediment and then discarded. The cellular fraction was diluted 1:5 in OSE medium supplemented with 10% FBS, placed in 100-mm-diameter dishes (Nunc), and incubated undisturbed at 37°C in 5% CO_2–air for 24 to 48 h. After cells had adhered to the plastic, they were washed once in phosphate-buffered saline (PBS).

Subsequently, cultures were regularly fed every 3 to 4 days and subcultured at a 1:2-to-1:3 split ratio by detaching cells after one PBS wash with 1 ml of trypsin solution (0.05% trypsin and 0.53 mM EDTA in Hanks' balanced salt solution without calcium and magnesium; Gibco-BRL).

Cultures were characterized by a number of evaluation criteria, including morphological, immunological, and molecular parameters (17). By all criteria, the cells in culture were representative of the original clinical material. We have noted, however, that early-passage cultures can contain a variable number of contaminating fibroblast-like cells. These cells disappear after a number of passages, and testing by the L1 consensus PCR assay for HPV detection (see below) was done on later passages when cultures were found to harbor mainly (>80%) epithelial cells from the primary lesion.

Cultured cells were trypsinized and washed with PBS, and an aliquot was resuspended in 10 mM Tris, pH 8.3, at a concentration of 10^7 cells per ml. Cells were lysed with Tween 20 and Nonidet P-40, each at a final concentration (vol/vol) of 0.4%, and digested with 300 μg of proteinase K per ml for 2 h at 55°C (6). After denaturation at 95°C for 10 min, lysates were stored frozen at −70°C.

Amplification procedures. In order to detect both known and novel HPV types, a PCR with consensus primers MY09 and MY11 (2) in the L1 gene was used. The L1 consensus PCR assay amplifies a 450-bp fragment from the 20 known genital types of HPV and also from untyped HPVs (2).

One microgram of extracted DNA from ovarian lesions or a lysate corresponding to 100,000 cells from cell cultures was amplified in duplicate by PCR. The amplification reaction was done in a 100-μl reaction volume containing 6.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), 50 mM KCl, 50 pmol of each primer, 100 mM each deoxynucleotide triphosphate, and 2.5 U of *Taq*

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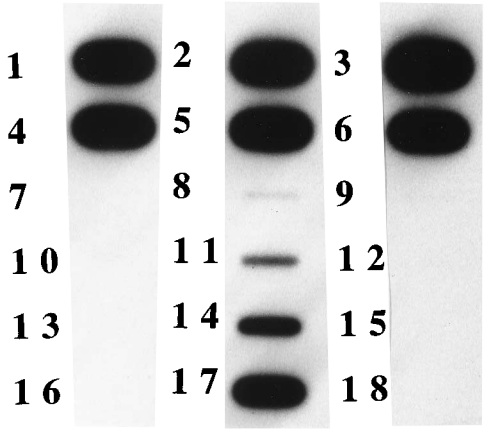

FIG. 1. PCR detection of HPV DNA in cell cultures from ovarian epithelial lesions. Cells were treated and amplified for HPV as described in Materials and Methods. Products were spotted onto filters and hybridized with a generic probe under low-stringency conditions. Positive controls (well 1, HPV33; well 2, HPV35, well 3, HPV45, well 4, HPV6/11, well 5, HPV16, well 6, HPV31) and a negative control (cells from human fibroblasts in well 8) were included. Wells 11, 14, and 17 contain 5, 50, and 500 HPV18 copies, respectively, from HeLa cell lysates. Wells 7, 9, 10, 12, 13, 15, 16, and 18 contain cultured cells from malignant ovarian epithelial lesions.

polymerase. The amplification parameters were set for 40 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. In each run, negative controls (buffer only and WI-135 fibroblast cell lysate) and positive controls of 40 HPV type 18 (HPV18) copies and HPV plasmids for types 6/11, 16, 31, 33, 35, and 45 were also included. Special procedures were maintained to avoid contamination (14). Primers for the β-globin gene RS42/RM29 (5, 20) amplifying a 536-bp fragment were used as a cellular control to monitor the integrity of DNA and the absence of inhibitors.

Amplified products were electrophoresed on a 2% agarose gel stained with ethidium bromide. Ten microliters of amplified products was also fixed on nylon membranes. These products were analyzed by dot blot hybridization (2, 8), first under nonstringent conditions with a generic probe (2, 8) which reacts with most known genital HPV types. The probe is synthesized (8) by amplification of plasmids from HPV types 16, 18, and 31 with L1 nested primers and radioactive nucleotides. The sensitivity endpoint of PCR with the generic probe reached five copies (Fig. 1) of HPV18 DNA. All controls for the various genital HPV types were positive (Fig. 1). A small amount of background reactivity was encountered with this probe because of the nonstringent hybridization conditions (Fig. 1, well 8). Filters were rehybridized with type-specific probes under stringent conditions for types 6/11, 16, 18, 31, 33, 35, and 45.

Results of detection of HPV in ovarian tissue. Samples were collected from 49 women (mean age, 58.5 ± 12.5 years; range 26 to 81 years). Twenty-four women had malignant ovarian lesions (15 cystadenocarcinomas, 3 clear-cell adenocarcinomas, 3 endometrioid adenocarcinomas, 1 borderline tumor, 1 serous cystadenocarcinoma, and 1 mucinous cystadenocarcinoma). One woman had ovarian metastasis from an endometrial cancer. Seven women had benign ovarian lesions, including four serous cystadenomas, one dermoid cyst, one lesion of endometrioma, and one fibroma. Seventeen women (including three patients with endometrial adenocarcinoma) had normal ovaries.

Thirty-nine fresh specimens, including 22 malignant ovarian lesions, 7 benign ovarian lesions, and 8 normal ovaries, were analyzed by PCR after DNA extraction. Thirty-six primary cell cultures were derived from 15 malignant ovarian lesions, 7 benign lesions, and 14 normal ovaries. Two cell cultures from ovarian malignant lesions were tested twice at different passage times. For most specimens, including 13 malignant lesions, 7 benign lesions, and 8 normal ovaries, fresh tissue and cell cultures were tested.

None of the specimens or cell lines generated a positive band for HPV on the gel after PCR (data not shown). Since HPV DNA would be present in at least one copy per cell in the cell cultures, and since ovarian epithelial cell populations accounted for >50% of the total population of 100,000 cells, this method would have detected the presence of HPV in our ovarian cultures. All samples were positive in the PCR assay for β-globin (data not shown). The dot blot assay results with the generic probe and type-specific probes remained negative for all specimens tested and cell line lysates (Fig. 1). HPV18 DNA was spiked in DNA extracted from ovarian specimens, and results were positive when this DNA was tested by the L1 consensus PCR assay.

Role of HPVs in ovarian cancers. The role of HPVs in ovarian carcinoma recently has been explored by various groups (3, 6, 10, 11, 15–18). HPV sequences have been detected in the endometrium and hypothetically could reach the epithelial surface of the ovary by ascending infection (1, 6, 12, 15) or by hematogenous spread (17). Our approach differed from those described in other published work since we tested primary cell cultures derived from various epithelial ovarian lesions and normal ovarian epithelium.

Initial results showing the presence of HPV6 in ovarian cancers (11) were not reproduced (10). Other groups until recently had failed to detect HPV DNA by PCR in ovarian lesions (3, 16, 18). However, koilocytosis, suggestive of a potential viral effect related to HPV infection, was described for up to one-third of ovarian adenocarcinomas (16). Most of these studies analyzed specimens by assays detecting only a few of the genital HPV types (10, 11, 16, 18) and could not exclude the implication of other or novel HPV types. Paraffin-embedded specimens were often analyzed (3, 18), and they yield amplified products less efficiently than do fresh specimens, because of loss of integrity of DNA. In only one work was β-globin amplified to demonstrate the integrity of extracted DNA (3).

Recently, in contrast to what we found, HPV16 and HPV18 DNAs were found by the L1 consensus PCR assay in up to 27% (3 of 11 cases) of epithelial ovarian cancer (14), reintroducing the controversy. In this work we have investigated the role of HPVs in ovarian epithelia (benign and neoplastic) by a consensus PCR assay which detects a wide range of typed and also untyped HPVs. To ensure reproducible results, fresh and cell culture lysates from the same sample were analyzed. The predominance of cells of epithelial origin strengthens results obtained with PCR combined with gel electrophoresis. The generic probe also provided negative results, while reaching high levels of sensitivity. By using sensitive techniques on fresh tissue and cell lines, as well as a large number of ovarian lesions, our study failed to identify HPV DNA in any of the ovarian epithelia. If HPV is implicated in ovarian cancer, novel types distantly related to known types must be implicated, at least in our population of patients.

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