

## Cytological Analysis for Human Papillomavirus DNAs in Cervical Intraepithelial Neoplasia by *In situ* Hybridization

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### ABSTRACT

Human papillomavirus (HPV) type 16 and 18 DNAs are reported to be associated with uterine cervical cancer. In order to investigate the relationship between the presence of HPV DNA and cervical intraepithelial neoplasia (CIN), we attempted the cytological detection of HPV DNAs in uterine cervical smear samples. The samples included those of severe dysplasia and carcinoma in situ (CIS). They were analysed by DNA-DNA *in situ* hybridization using biotinylated HPV DNA probes.

The results of *in situ* hybridization analysis revealed that HPV sequences were present in the nuclei of cells with koilocytotic atypia. When probed for HPV type 6, 11, 16 and 18, the nuclei of dysplastic cells and cancer cells were positive for HPV type 16 and 18 DNA. Out of 26 CIN cases, 17 contained HPV type 16 DNA and 5 contained HPV type 18 DNA.

We suggest that cytological analysis for HPV sequences by the *in situ* hybridization technique might provide a molecular diagnosis for assessment in uterine cervical intraepithelial neoplasia.

**Key words:** *In situ* hybridization, HPV, Cervical smear, CIN

Recent molecular biological studies have shown that human papillomavirus (HPV) type 16 and 18 are closely associated with uterine cervical cancer. HPV type 31, 33 and 35 are also detected frequently in squamous cell carcinoma of the uterine cervix<sup>1,4</sup>). In addition, HPV DNA becomes integrated into the cancer cells and expresses several functions<sup>15,16</sup>). Since HPV type 16 and 18 DNAs appear to correlate with uterine cervical cancer, women who are positive for HPV type 16 and/or 18 may be at increased risk of developing cervical cancer. Accordingly, effective screening is needed by which HPV type 16 and 18 DNAs can be detected in the cytological diagnosis.

Cervical intraepithelial neoplasia (CIN) is a lesion which has the potential of becoming uterine invasive carcinoma. It is diagnosed by the assessment of individual cytological atypia, the disorderliness of maturation within the epithelium, and contained cervical carcinoma in situ and dysplasia. The cytological features of CIN are koilocytotic atypia, dyskeratocyte and dyskaryocyte. Koilocytotic atypia, which was first characterized by Koss and Durfee<sup>6</sup>), shows a mild atypical nucleus with hyperchromatism and perinuclear clearing. This is one of the cytological features characteristic of HPV infection as revealed by immunocytochemistry and electron microscopic studies<sup>5,12</sup>). However, koilocytotic atypia is not al-

ways detected in cervical smears and in the tissue specimens of HPV infected cases. The rate of koilocytotic atypia is reported to be about 80% in condyloma accuminata and 20%–80% in cervical intraepithelial neoplasia (CIN)<sup>13</sup>). Meiseles et al<sup>9</sup>) and Kurman et al<sup>7</sup>) showed that the dyskeratocytes, condylomatous parabasal cells and bi-multipinucleated cells are additional cytologic features of HPV infection.

Application of the *in situ* hybridization technique with the biotinylated HPV DNA probe in the cells obtained from the cervical smear is useful not only for the analysis of the infected HPV type and its localization but also as a diagnostic criterion of CIN. The purpose of the present study was to examine the distribution of HPV type 16 and 18 DNAs as well as HPV type 6 and 11 DNAs in neoplastic cells and cells with koilocytotic atypia in cervical smear specimens by the *in situ* hybridization technique for clinical screening of HPV infection.

### MATERIALS AND METHODS

#### Samples

Cervical smear samples were taken from 31 patients examined in the Department of Gynecology and Obstetrics, School of Medicine, Hiroshima University. Of the 26 CIN cases, 6 were of severe dysplasia, 20 were of carcinoma in situ, and 5

cases were of chronic cervicitis by Papanicolaou stain and punch biopsy. Cervical biopsy samples of 3 cases of severe dysplasia and 14 cases of carcinoma in situ from the same group of patients were examined for cytological *in situ* hybridization. We also examined CaSki cells which carry about 500 copies of HPV type 16 sequence, and the sensitivity of hybridization was investigated.

### Preparation of samples

The cervical cells were placed on 50 $\mu$ g/ml poly L-lysine (Sigma)-coated slides in order to facilitate adhesion of the cells. They were fixed in cold Carnoy's fixative (60% ethanol, 30% chloroform, 10% acetic acid) and washed briefly in 95% ethanol, air dried and stored at  $-70^{\circ}\text{C}$  until use<sup>2,10</sup>. The smear samples were stained with Papanicolaou stain.

We also examined the punch biopsies obtained from the same patients which were fixed in 10% formalin fixative and embedded in paraffin.

### *In situ* hybridization

The smear slides were rehydrated in graded ethanol. They were then placed in 0.2N HCl for 15 minutes, washed in phosphate buffered saline (PBS) and incubated with 1- $\mu$ g/ml proteinase K in  $2 \times \text{SSC}$  for 15 minutes at room temperature. After rinsing in PBS, the slides were fixed in 4% paraformaldehyde for 30 minutes to crosslink nucleic acid, and acetylated with 0.25% v/v acetic anhydride to remove the background binding. Cloned HPV type 6, 11, 16 and 18 DNAs which were provided by Drs. Harold zur Hausen and Lutz Gissmann were biotin-labeled by nick translation. One  $\mu$ g of HPV DNA was incubated with biotin-UTP, DNase and DNA polymerase according to the protocol of the BluGENE<sup>TM</sup> DNA detection kit (BRL). The reaction was terminated by addition of 300mM Na<sub>2</sub>EDTA, and the labeled probe was separated by gel chromatography on a 5-ml Sephadex G-50 column. A cocktail consisting of about 1- $\mu$ g/ml HPV DNA probe, 50% deionized formamide, 10mM EDTA,  $2 \times \text{SSC}$ , 200- $\mu$ g/ml yeast tRNA and 10% dextran sulfate (w/v) was placed on the sections, and the specimens were covered with a doubly siliconized coverslip and sealed with rubber cement. The slides were heated to  $80^{\circ}\text{C}$  for 10 minutes, cooled rapidly and then incubated in a humidified chamber at  $42^{\circ}\text{C}$  (Tm-24) for 15 hours.

The rubber cement and coverslips were removed and the slides were washed twice at  $50^{\circ}\text{C}$  in  $2 \times \text{SSC}$  and  $0.2 \times \text{SSC}$  for each 10 minutes. The slides were then incubated according to the protocol provided by BluGENE<sup>TM</sup> Kit (BPL) and incubated sequentially in 1- $\mu$ g/ml alkaliphosphatase conjugated streptavidin, nitroblue tetrazolium (NBT) and 5-brom-4-chloro-3-indolyphosphate (BCIP) in Tris:HCl (pH 9.5). The slides

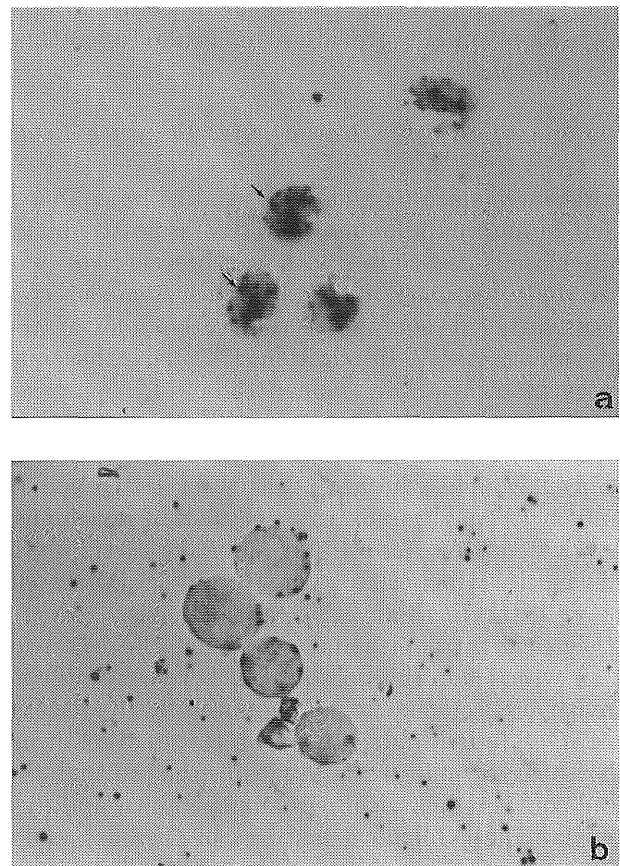
were incubated in 50mM EDTA, dehydrated through graded ethanol, stained briefly with Orange G and mounted in paramount. We examined only the intact cells with both a nucleus and cytoplasm in this study.

The formalin-fixed sections obtained from punch biopsy were processed as described above after deparaphinization in xylene and rehydration in graded ethanol<sup>10</sup>.

### RESULTS

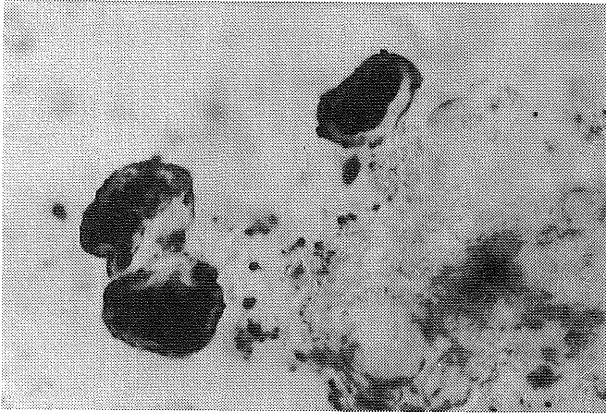
The cytological analysis for HPV type 6/11, 16 and 18 DNAs by *in situ* hybridization confirmed that normal squamous cells were negative for all types of HPV DNAs. HPV type 16 DNA was detected in the nuclei of CaSki cells as several dark spots or as a granular pattern (Fig.1a). The cells were negative for HPV type 6/11 and 18 (Fig. 1b).

The cervical smear specimens revealed that HPV type 16 was present in some of the nuclei of cells with koilocytotic atypia obtained from severe cases of dysplasia and carcinoma in situ. These nuclei showed irregular hyperchromatism

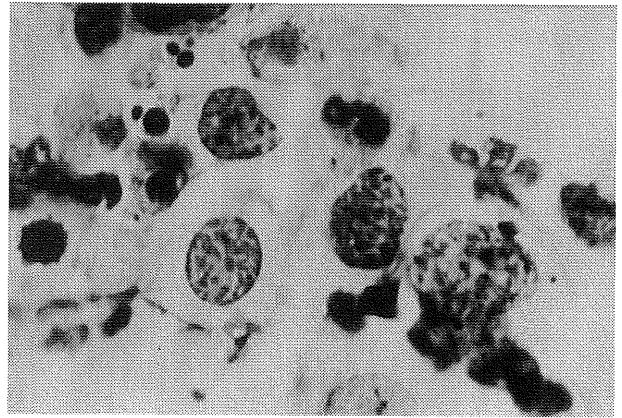


**Fig. 1a.** HPV type 16 was detected in the nuclei of CaSki cells as several dark spots or as a granular pattern (arrow).

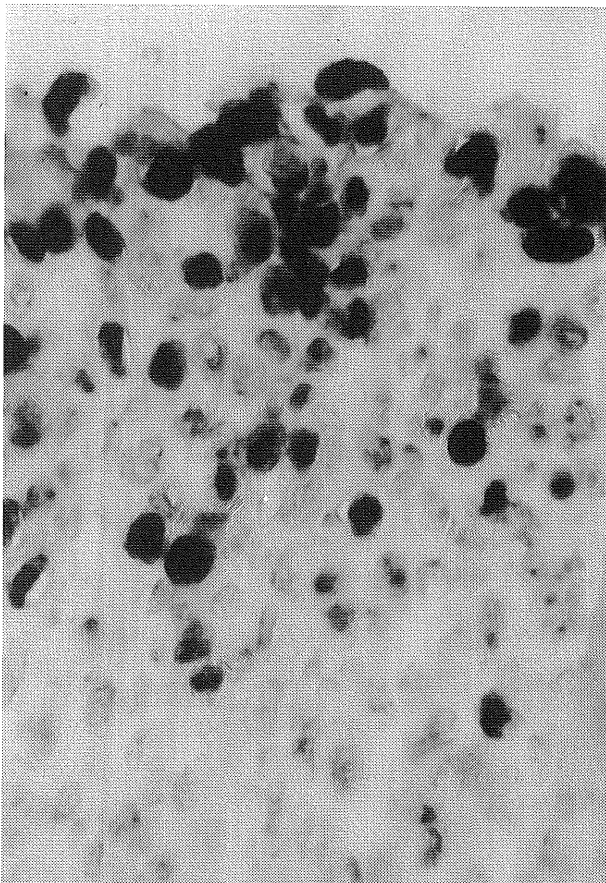
**1b.** HPV type 18 was negative. (*In situ* hybridization,  $\times 400$ ).



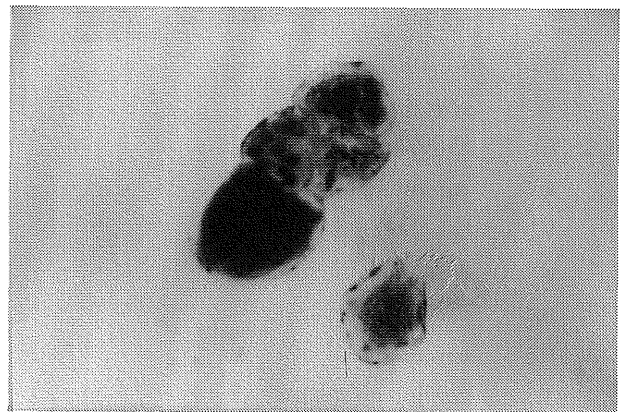
**Fig. 2.** The nuclei of dysplastic cells showing HPV type 16 DNA sequences as a uniform pattern. (*In situ* hybridization,  $\times 400$ ).



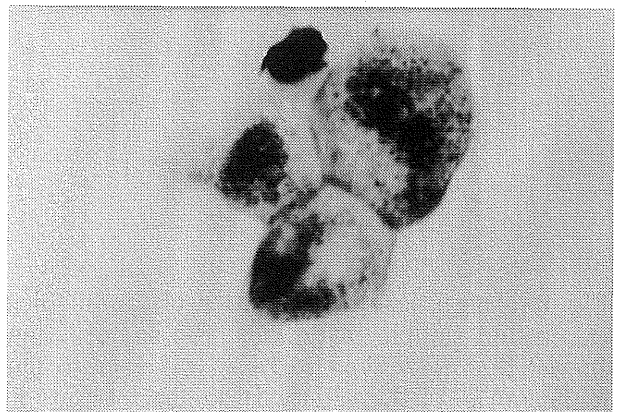
**Fig. 4.** Nuclei showing large irregular hyperchromatism, variation in size and narrow cytoplasm in carcinoma in situ case. (Papanicolaou stain,  $\times 400$ ).



**Fig. 3.** HPV type 16 DNA was detected in the nuclei of koilocytes and dysplastic cells of severe dysplasia. (*In situ* hybridization,  $\times 200$ ).



**Fig. 5.** HPV type 16 DNA was positive in the nuclei of cancer cells in carcinoma in situ case. (*In situ* hybridization,  $\times 400$ ).



**Fig. 6.** HPV type 18 DNA was positive as a granular pattern in carcinoma in situ case. (*In situ* hybridization,  $\times 200$ ).

and were stained uniformly purple by NBT and BCIP. The dysplastic cells obtained from severe cases of dysplasia of the uterine cervix exhibited enlargement and hyperchromatism when examined cytologically. They also sometimes showed pyknosis and elongation of the nucleus and there was not so much of an increase in the

nuclear-cytoplasmic ratio. HPV type 16 was detected in some of the nuclei of dysplastic cells in smear samples taken from severe dysplasia cases

**Table 1.** Detection of HPV DNAs in uterine cervical smear by *in situ* hybridization

Histology	Cases	No.	No.	No.	No.
		HPV 16 (%)	HPV 18 (%)	HPV 6/11 (%)	negative
severe dysplasia	6	6 (100)	0 (0)	1* (16.9)	0
carcinoma in situ	20	11** (55.0)	5** (25.0)	0 (0)	6
Total	26	17 (65.4)	5 (19.2)	1 (3.8)	6

\*: One case of severe dysplasia was positive for HPV type 6/11 and 16 DNA under stringent conditions.

\*\* : Two cases of carcinoma in situ contained HPV type 16 and 18 DNA under stringent conditions.

**Table 2.** Correlation of cytological and histological detection for HPV DNAs

Case	Histology	HPV 16		HPV 18		HPV 6/11	
		smear	tissue	smear	tissue	smear	tissue
1.	severe dysplasia	+	+	-	-	-	-
2.	severe dysplasia	+	+	-	-	-	-
3.	severe dysplasia	+	+	-	-	-	-
4.	severe dysplasia	+	+	-	-	-	-
5.	carcinoma in situ	-	-	+	+	-	-
6.	carcinoma in situ	+	+	-	-	-	-
7.	carcinoma in situ	+	+	-	-	-	-
8.	carcinoma in situ	+	+	-	-	-	-
9.	carcinoma in situ	-	-	+	+	-	-
10.	carcinoma in situ	+	+	+	+	-	-
11.	carcinoma in situ	+	-	+	+	-	-
12.	carcinoma in situ	-	-	-	+	-	-
13.	carcinoma in situ	-	-	+	+	-	-
14.	carcinoma in situ	-	-	-	-	-	-
15.	carcinoma in situ	-	+	-	-	-	-
16.	carcinoma in situ	+	+	-	-	-	-
17.	carcinoma in situ	-	+	-	-	-	-
18.	carcinoma in situ	+	+	-	-	-	-
Total		11/18	12/18	5/18	6/18	0/18	0/18
Detection rate		(61.1)	(66.7)	(27.8)	(33.3)	(0)	(0)

(Fig. 2). The *in situ* hybridization of the histological section sample taken from the same patient showed that HPV type 16 DNA was also present in the nuclei of the cells with koilocytotic atypia and dysplastic cells (Fig. 3).

The cancer cells obtained from carcinoma in situ cases showed a predominance of round cells with a large nucleus and a narrow cytoplasm. The nuclei revealed an abnormal distribution of chromatin, irregular outlines, variation in size and often hyperchromatism (Fig. 4). HPV type 16 DNA was detected in the nuclei of cancer cells as a granular or uniform pattern by *in situ* hybrid-

ization under the high-stringent condition (Fig. 5). HPV type 18 DNA, as well as type 16 DNA, was detected in the nuclei of cancer cells obtained from some carcinoma in situ cases (Fig. 6). HPV type 6/11 DNA was detected in the nuclei of dysplastic cells of one severe dysplasia case (Table 1).

The rate of detection of HPV DNA by *in situ* hybridization found in this study is shown in detail in Table 1. HPV type 16 DNA was detected in all 6 cases of severe dysplasia and 11 (55.0%) of the 20 carcinoma in situ cases. HPV type 18 DNA was detected in 5 (25%) of the 20 carcino-

ma *in situ* cases. However, none of the severe dysplasia cases was positive for HPV type 18 DNA in this study. Two carcinoma *in situ* cases contained both HPV type 16 and 18 DNA (Table 1). One severe dysplasia case was positive for both HPV type 6/11 and 16 DNA by *in situ* hybridization.

Table 2 shows the correlation between cytological and histological detection of HPV sequences by *in situ* hybridization. All 4 severe dysplasia cases were positive for HPV type 16 DNA in both cytological and histological specimens. Nine of the 15 carcinoma *in situ* cases contained HPV type 16 DNA in cytological and/or histological specimens, and 6 of the 15 carcinoma *in situ* cases were positive for HPV 18 DNA in cytological and/or histological specimens. Correlation of the total cytological and histological detection rate of HPV DNAs showed that HPV type 16 was present in 11 cases (61.1%) of the cytological specimens and in 12 cases (66.7%) of the histological specimens. The positive rate for HPV type 18 DNA was 27.8% in smear specimens and 33.3% in tissue specimens. In this 18 case study, HPV type 6/11 DNAs were negative for cytological and histological *in situ* hybridization.

## DISCUSSION

Koilocytotic atypia, dyskeratocyte, condylomatous parabasal cells, bi-multinucleated cells, nuclear wrinkling and the presence of epithelial spikes are well known features of HPV infection in Papanicolaou staining<sup>6,7,9</sup>. In addition, HPV capsid antigen and HPV-like particles are observed in the nuclei of koilocytotic atypia by immunocytochemistry and electron microscopy<sup>5,12</sup>. On the other hand, the rate of koilocytotic atypia decreased steadily from 50% to 20% in CIN as the grade of CIN became higher. The positive rate of immunocytochemistry for HPV capsid antigen and HPV-like particles declined in the CIN of higher grades and HPV antigen or HPV particles have not been found in invasive cervical cancer<sup>13</sup>.

Recently it became possible to detect HPV DNAs in uterine cervical cancer by molecular hybridization. Our DNA-DNA *in situ* hybridization technique with biotinylated DNA probes is a useful method for detection and determination of the HPV type and the localization of HPV sequences in the cervical smear and tissue specimens. The technique is simple as compared with examination by immunocytochemistry and electron microscopy. Biotinylated probes have become a more convenient method than radioactive labeling for their safety, stability and accuracy of detection<sup>8</sup>. Biotinylated probes bind streptavidin strongly and can detect as low as 10 to 30 copies of HPV sequences<sup>18</sup>. Neumann et al<sup>11</sup>) concluded that application of biotinylated DNA probes was pos-

sible without any loss of sensitivity as compared to radioactive probes. With this probe, we are able to detect HPV type 16 in the nuclei of koilocytotic atypia in cervical smear specimens obtained from severe dysplasia and carcinoma *in situ* cases<sup>2,10</sup>. In the present study, HPV type 16 DNA was also detected in the nuclei of dysplastic cells obtained from severe dysplasia cases. HPV type 18 DNA as well as type 16 DNA were detected in the nuclei of cervical cancer cells without koilocytotic atypia in the cervical smear specimens obtained from carcinoma *in situ* cases. The reason why HPV positive and negative cells were present in the same samples is thought to depend upon the copy number of HPV.

The staining pattern of CaSki cells is granular. We suggest that HPV sequences are clustered in the nuclei for cells carrying as many as 500 copies of HPV type 16. The uniform pattern might mean that the copy number of HPV is much higher and that HPV exists as an episomal form. To explain results in which granular or uniform patterns of HPV were present in the same samples, we would suggest that the infected form of HPV might be different in each cell.

The koilocytotic atypia cases which showed positive for HPV type 16 DNA in the present study might be correlate with cervical cancer and should be followed up as a high risk group for developing malignant disease. More interestingly the present results showed the presence of HPV type 16 or 18 DNA in the nuclei of cervical dysplastic cells and cancer cells exhibiting no koilocytotic change. Since the positive rate of HPV particles or antigens decreased as the grade of CIN became higher<sup>13</sup>, the *in situ* hybridization of HPV DNA in the cervical smear would facilitate the detection of HPV type 16 and 18 not only in cells of koilocytotic atypia but also in cervical dysplastic cells and cancer cells. In the analysis of histological grading, the detection rate of HPV type 16 DNA was lower for carcinoma *in situ* than for severe dysplasia. From this result, we assumed that the copy number of HPV DNA tended to decrease as the grade of CIN became higher.

The application of *in situ* hybridization for detecting HPV sequences in cytological diagnosis is valuable for the screening and prospective follow-up examination of women infected with HPV. Wagner et al<sup>17</sup>) showed that 68% of CIN grade III cases were positive for type 16 and/or 18 DNA by a filter *in situ* hybridization technique in which cervical smear cells were placed onto a nitrocellulose membrane, denatured, neutralized and hybridized *in situ* with radiosensitive labeled HPV DNA probes. Schneider et al<sup>14</sup>) showed that 56 (68%) of 82 cytologically positive cases contained HPV sequences, 48% HPV type 16/18 and 19% HPV 16/18 type in combination with HPV type



6/11 by filter *in situ* hybridization. Our results showed 65.4% (17/26) of cervical intraepithelial neoplasia cases contained HPV type 16 DNA and 19.2% (5/26) contained HPV type 18 DNA (Table 1). These results suggest that HPV type 16 and 18 in abnormal cervical smear specimens show a higher risk of developing a malignant disease in the patient than any other types of HPV. On the other hand, a recent study showed approximately 10% of patients with normal smears were positive for HPV DNA<sup>3)</sup>, which were supposed to be latent HPV infection cases.

We suggest that cytological detection of HPV DNA by *in situ* hybridization is valuable for HPV DNAs diagnosis and the prospective follow-up examination of early uterine cervical neoplasia.

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#### REFERENCES

1. Boshart, M., Gissmann, L., Ikenberg, H., Heinheinz, A., Scheurlen, W. and zur Hausen, H. 1987. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO. J.* **3**: 1151-1157.
2. Crum, C.P., Nagai, N., Levine, R.U. and Silverstein, S. 1986. In situ hybridization analysis of HPV 16 DNA sequences in early neoplasia. *Am. J. Pathol.* **123**: 174-182.
3. de Villiers, E.M., Wagner, D., Schneider, A., Wesch, H., Miklaw, H., Wahrendorf, J., Papendick, U. and zur Hausen, H. 1987. Human papillomavirus infections in women with and without abnormal cervical cytology. *The Lancet* **26**: 703-705.
4. Dürst, M., Gissmann, L., Ikenberg, H. and zur Hausen, H. 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc. Natl. Acad. Sci. U.S.A.* **80**: 3812-3815.
5. Ferenczy, A., Braum, L. and Shah, K. 1981. Human papillomavirus (HPV) in condylomatous lesion of cervix. : A comparative ultrastructural and immunohistochemical study. *Am. J. Surg. Pathol.* **5**: 661-669.
6. Koss, L.G. and Durfee, G.R. 1956. Unusual patterns of squamous epithelium of the uterine cervix. : Cytologic and pathologic study of koilocytotic atypia. *Ann. NY. Acad. Sci.* **63**: 1245-1361.
7. Kurman, R.J., Jensen, A.B. and Lancaster, W.D. 1983. Papillomavirus infection of the cervix II. Relationship to intraepithelial neoplasia based on the presence of specific viral structural proteins. *Am. J. Surg. Pathol.* **7**: 39-52.
8. Leary, J.J., Brigati, D.J. and Ward, D.C. 1983. Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose. *Bio-blots Proc. Natl. Acad. Sci. U.S.A.* **80**: 4045-4049.
9. Meiseles, A., Morin, C. and Casas-Cordero, M. 1982. human papillomavirus infection of uterine cervix. *Int. J. Gynecol. Pathol.* **1**: 75-94.
10. Nagai, N., Nuovo, G., Friedman, D. and Crum, C.P. 1987. Detection of papillomavirus nucleic acids in genital precancers with the *in situ* hybridization technique. *Int. J. Gynecol. Pathol.* **6**: 366-379.
11. Neumann, R., Heiles, B., Zippel, C., Eggers, H.J., Zippel, H.H., Holzmann, L. and Schulz, K. D. 1986. Use of biotinylated DNA probes in screening cells obtained from cervical swabs for human papillomavirus DNA sequences. *Acta Cytologica.* **30**: 603-607.
12. Reid, R., Lavery, C.R., Coppleson, M., Isarangkaj, W. and Hills, E. 1980. Noncondylomatous cervical wart virus infection. *Obstet. Gynecol.* **55**: 476-483.
13. Sato, S., Okagaki, T., Clark, B.A., Twiggo, L.B., Fukushima, M., Ostrow, R. S. and Faranz, A. J. 1986. Sensitivity of koilocytosis, immunocytochemistry and electron microscopy as composed to DNA hybridization in detecting human papillomavirus in cervical and vaginal condyloma and intraepithelial neoplasia. *Int. J. Gynecol. Pathol.* **5**: 297-307.
14. Schneider, A., Kraus, H., Schuhmann, R. and Gissmann, L. 1985. Papillomavirus infection of the lower genital tract : Detection of viral DNA in gynecological swabs. *Int. J. Cancer* **35**: 443-448.
15. Swarz, E., Schneider-Gädicke, A., Roggenbuck, B., Mayer, W., Gissmann, L. and zur Hausen, H. 1986. Expression of human papillomavirus DNA in cervical cell lines. *Banbury Rep.* **21**: 281-290.
16. Seedorf, K., Olterdorf, T., Krammer, G. and Röwekamp, W. 1987. Identification of early proteins of the human papillomavirus type 16 (HPV 16) and type 18 (HPV 18) in cervical carcinoma cells. *EMBO. J.* **6**: 139-144.
17. Wagner, D., Ikenberg, H., Boehem, N. and Gissmann, L. 1984. Identification of human papillomavirus in cervical swabs by deoxyribonucleic acid *in situ* hybridization. *Obstet. & Gynecol.* **64**: 767-772.
18. Walboomers, J.M.M., Melchers, W.J.G., Mullink, H., Meijer, C.J.L.M., Struyk, A., Quint, W.G.J., Noordaa, J. and Schogget, J. 1988. Sensitivity of *in situ* detection with biotinylated probes of human papillomavirus type 16 DNA in frozen tissue sections of sequences cell carcinoma of the cancer. *Am. J. Pathol.* **131**: 587-594.