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## TYPE-SPECIFIC PERSISTENCE OF HUMAN PAPILLOMAVIRUS DNA BEFORE THE DEVELOPMENT OF INVASIVE CERVICAL CANCER

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### ABSTRACT

**Background** Infection with the human papillomavirus (HPV) has been established as a cause of cervical cancer, but the association between a positive test for HPV DNA and the risk of the subsequent development of invasive cervical cancer is unknown.

**Methods** In a study of women who participated in a population-based screening program for cancer of the cervix in Sweden from 1969 to 1995, we compared the proportion of normal cervical smears (Pap smears) that were positive for HPV DNA among 118 women in whom invasive cervical cancer developed an average of 5.6 years later (range, 0.5 month to 26.2 years) with the proportion of HPV DNA-positive smears from 118 women who remained healthy during a similar length of follow-up (controls). The control women were matched for age to the women with cancer, and they had had two normal Pap smears obtained at time points that were similar to the times of the baseline smear and the diagnosis of cancer confirmed by biopsy in the women with cancer.

**Results** At base line, 35 of the women with cancer (30 percent) and 3 of the control women (3 percent) were positive for HPV DNA (odds ratio, 16.4; 95 percent confidence interval, 4.4 to 75.1). At the time of diagnosis, 80 of the 104 women with cancer for whom tissue samples were available (77 percent) and 4 of the 104 matched control women (4 percent) were positive for HPV DNA. The HPV DNA type was the same in the base-line smear and the biopsy specimen in all of the women with cancer in whom HPV DNA was detected at base line. None of the control women had the same type of HPV in both smears.

**Conclusions** A single positive finding of HPV DNA in a Pap smear confers an increased risk of future invasive cervical cancer that is positive for the same type of virus. (N Engl J Med 1999;341:1633-8.)

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**I**NFECTION with the human papillomavirus (HPV) has been established as a cause of cervical cancer.<sup>1</sup> However, most epidemiologic studies of HPV infection and cervical cancer have been conducted with the use of samples taken after the cancer has been diagnosed. Such studies provide no information on the temporal order of events and, furthermore, may be biased in their estimation of risk because the presence of the disease itself may increase the detectability of HPV. The HPV-infected tissue mass grows, and thus sampling is facilitated and amplification of the HPV genome occurs in cancer cells.

An unbiased estimate of the risk of cervical cancer associated with HPV and information on the prevalence of HPV and the duration of the stage during which HPV is continuously detectable before cancer is diagnosed can come only from prospective studies. The results of prospective studies performed to date support the concept that cervical intraepithelial neoplasia is preceded by the persistent presence of detectable HPV DNA in healthy women.<sup>2,3</sup> However, cervical intraepithelial neoplasia and carcinoma in situ are precursor lesions that may not progress and may even spontaneously regress. Prospective studies need to use the presence of invasive cancer as the end point in order for any conclusions about the cause of cervical cancer to be useful. Two small studies have found

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that the presence of HPV DNA in cytologically normal Pap smears is associated with an increased risk of future cervical cancer.<sup>4,5</sup>

Since the presence of HPV DNA in Pap smears has been associated with a very high relative risk (>50) of existing cervical intraepithelial neoplasia or cervical cancer, testing for the presence of HPV has been proposed as a complement to cytologic testing in cervical screening.<sup>6</sup> Mathematical modeling has indicated that screening for the presence of HPV could improve the cost effectiveness of cervical screening,<sup>7,8</sup> provided that the protective effect of a negative HPV test lasts longer than the protective effect of a normal Pap smear.<sup>8</sup>

We performed a population-based study of the risk of invasive cervical cancer among healthy women with normal cervical cytologic findings according to whether HPV DNA could be detected in cervical samples at base line. We chose women who were participating in a population-based cervical-cancer screening program in order to obtain population-based estimates of the risk of invasive cervical cancer among women who tested positive for HPV DNA.

## METHODS

### Study Subjects

A population-based cervical-cancer screening program, targeted to women 25 to 59 years of age, was started in Västerbotten County in northern Sweden (population in 1995, 260,472, of whom 130,651 were women) in 1969, with women invited to participate at four-year intervals. The participation rate has been higher than 80 percent. All diagnoses based on cytologic findings in this county, both in Pap smears obtained in the organized program and in those obtained outside the program, were made at the Cytology Laboratory, Umeå University Hospital, where the smears were stored. All women with cervical cancer were treated at Umeå University Hospital, where data on clinical stage, histopathological grade, treatment, and survival were recorded and the histologic specimens were stored.

### Study Design

Eligible participants were women residing in Västerbotten County for whom at least one cytologically normal Pap smear and at least one additional smear had been stored and who had undergone no surgical treatment of the cervix. Linkage between the cytology registry and the Swedish Cancer Registry for the period from 1969 to 1995 identified 133 eligible women with invasive cervical cancer that had been diagnosed after the date of a normal smear. Four women were excluded because of incorrect data in the registry and 11 because they had noninvasive cervical neoplasia, leaving 118 women. The pathological specimens for 12 women were missing, and the tissue blocks for 2 were inadequate for analysis by the polymerase chain reaction (PCR), leaving 104 women (85 with squamous-cell carcinoma and 19 with adenocarcinoma) from whom tissue samples were available for PCR analysis. In cases in which a woman had multiple previous Pap smears with normal cytologic results, the smear obtained on the date closest to the date of the diagnosis of cancer was retrieved. The control group was made up of women in whom cervical cancer did not develop before the time of diagnosis in the corresponding women with cancer. They were matched individually to the corresponding women with cancer according to age (on the basis of the calendar year of birth), the time at which a normal Pap smear was obtained, and the time at which a normal smear was obtained after cancer had been diagnosed in the corresponding women.

The average age at which the base-line smear was obtained was 44 years (range, 19 to 74) among the women with cancer and 44 years (range, 20 to 74) among the control women. The dates of the smears of the women with cancer and those of the control women differed by one month, on average. The subsequent smears of the control women were typically obtained after the diagnosis of cancer in the corresponding women with cancer, since absence of disease in the controls for at least the same duration of follow-up was an essential component of the study design. If a control woman had several normal smears after the date of the diagnosis of cancer in the corresponding woman with cancer, the smear obtained on the date closest to the date of the diagnosis of cancer was chosen. The time of biopsy in the woman with cancer and the time the smear was obtained in the corresponding control woman differed by up to 10 months. The average age of the women with cancer at the time of diagnosis was 50 years (range, 24 to 79), and the average age of the control women at the time of the second normal smear was 50 years (range, 24 to 79).

All smears were reevaluated before laboratory analysis. Of the negative base-line smears from the 118 women who later had cancer, 53 were read as containing atypical squamous cells of unknown pathological importance or precancerous cells by at least one observer, as were the smears of 2 of the 118 control women. This grading was often equivocal and based on the examination of only a few cells. None of the smears showed any cytologic features of HPV infection.<sup>9</sup> The histologic slides of all the women with cancer were reexamined and the diagnosis confirmed. Only paraffin blocks verified to contain cancerous cells were used for DNA analysis.

Informed consent for the study of the samples was not obtained from the subjects. The archival samples were up to 35 years old, and the institutional review board of Umeå University determined that it was not feasible for us to contact the subjects and request informed consent. However, to inform women whose samples might be included in the study, we held a press conference at the beginning of the study, on May 23, 1995. The nature of the study and the fact that samples would be used without the subjects' informed consent were described. (The press conference resulted in coverage by major regional newspapers.)

### DNA Extraction

DNA was extracted from stored Pap smears and four 5- $\mu$ m-thick sections of each biopsy specimen, as described elsewhere.<sup>4,10,11</sup> The DNA was dissolved in 400  $\mu$ l of TRIS buffer containing 10  $\mu$ M EDTA. Knives were changed between slices, and empty paraffin blocks were placed between biopsy specimens to prevent cross-contamination. All samples were tested for integrity of DNA by PCR with the use of human ribosomal gene S14 primers<sup>10,12</sup> that produce 150-bp amplicons. Samples positive for S14 DNA but negative for HPV DNA were precipitated in alcohol, and the PCR assay was repeated.

### PCR Analysis

The HPV consensus primers MY09 and MY11<sup>13</sup> and GP5+ and GP6+<sup>14</sup> were used in a nested, single-tube PCR assay.<sup>11,15</sup> A nonnested PCR assay was also performed with the GP5+ and GP6+ primers that amplify products of 150 bp, a size similar to that generated by PCR amplification of the S14 DNA. Both PCR systems used 1.5 mM magnesium chloride and 0.4 percent bovine serum albumin in 50  $\mu$ l of buffer. The amplicons were analyzed on 2 percent agarose gels and stained with 10  $\mu$ g of ethidium bromide per milliliter.

The sensitivity of the PCR systems was determined by testing dilutions of plasmids containing HPV type 16 (HPV-16) late protein 1 (L1) (1 to 500 copies) mixed with 2  $\mu$ l of DNA extracts from 10 HPV-negative Pap smears<sup>11</sup> to check for possible inhibitors of PCR amplification.<sup>10</sup> Dilutions of DNA from the cervical-cancer cell line SiHa (1 fg to 10 pg) were also analyzed in each PCR assay simultaneously with the unknown samples.

The PCR analyses were performed on coded samples, arranged in a manner ensuring that samples from women with cancer and

samples from control women were analyzed in the same analytic runs. Blanks without DNA were included after every 24 samples. DNA was extracted from additional positive and negative controls from HPV-16–positive CaSki and HPV-negative C-33A cervical-cancer cell lines. Four analyses, each with a different volume of the sample DNA, were performed with both PCR systems. The amount of sample DNA used for the analyses ranged from 0.1 percent to 0.7 percent of the total volume.

**Sensitivity of the Assay and Quality Control**

The nested PCR assay was able to detect one copy of HPV-16 L1 plasmid when mixed with DNA extracted from HPV-negative smears.<sup>11</sup> The limit of detection for SiHa DNA in each PCR assay was consistently 1 fg or less. Titrations of plasmid or SiHa DNA produced positive reactions at concentrations above the titer for the end-point dilution, whereas titration of the extracts from the stored smears resulted in some instances in increased rates of positive tests with more diluted samples. The reason for this is not clear but may be related to the presence of inhibitors of PCR in extracts from fixed and stained Pap smears. Inhibition was less evident in the nonnested PCR analyses. In the test of the adequacy of the sample (S14 DNA PCR), 1 of 399 Pap smears and 3 of 133 biopsy specimens were negative for human ribosomal DNA.

**Typing of HPV DNA**

Primers from the E1 open reading frame (nucleotides 1768 to 1960, HPV-16) and the E7 open reading frame (nucleotides 591 to 900, HPV type 18 [HPV-18]) were used in the type-specific PCR.<sup>16</sup> For direct automated sequencing of the GP5+ and GP6+ PCR products, the amplimers were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Another round of PCR with 3.0 pmol of GP6+ primer was performed in which the fragment containing GP5+ and GP6+ was used as template DNA with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, Calif.). Sequencing was performed with the use of an ABI 310 sequencer (PE Biosystems).

**Statistical Analysis**

Odds ratios and confidence intervals were estimated on the basis of data matched for age and time of sampling by conditional logistic-regression analysis. We used the Mann–Whitney U test to analyze the differences in continuous variables between the women who were HPV-positive and those who were HPV-negative. All reported P values are two-sided.

**RESULTS**

Three of the 118 control women (3 percent) had detectable HPV DNA in the Pap smears obtained at base line, as compared with 35 of the 118 women who later had cancer (30 percent). The odds ratio for cervical cancer among the women who were HPV-positive was 16.4 (95 percent confidence interval, 4.4 to 75.1), and an increased odds ratio was detected for up to six years before the diagnosis of cancer (Table 1).

HPV DNA could be amplified in the biopsy specimens of 80 of the 104 women with cancer for whom tissue samples were available (77 percent). Nine of the 19 adenocarcinomas (47 percent) and 71 of the 85 squamous-cell carcinomas (84 percent) were HPV DNA–positive. HPV DNA was detected in both the base-line smears and the corresponding biopsy specimens of 27 women. Only 1 of the 104 control women had HPV DNA in both smears (Table 2).

Most type-specific PCR results for HPV-16 (E1 open reading frame) and HPV-18 (E7 open reading

**TABLE 1. ODDS RATIOS FOR INVASIVE CERVICAL CANCER ASSOCIATED WITH THE PRESENCE OF HUMAN PAPILLOMAVIRUS (HPV) DNA IN CYTOLOGICALLY NORMAL PAP SMEARS OF HEALTHY WOMEN.**

LAG TIME*	WOMEN WITH CANCER	CONTROL WOMEN	ODDS RATIO (95 PERCENT CI)†
	no. HPV-positive/total no.		
<3 yr	18/48	1/48	22.5 (3.4–969)
3–6 yr	13/35	1/35	16.6 (2.3–736)
>6 yr	4/35	1/35	4.0 (0.4–196)
Total	35/118	3/118	16.4 (4.4–75.1)

\*Lag time denotes the time between the date of the base-line smear and the date of the diagnostic biopsy.

†Odds ratios were calculated by conditional logistic-regression analysis (with matching for age and the times at which samples were obtained). CI denotes confidence interval. Odds ratios indicate the relative risk of cervical cancer among women with HPV DNA–positive base-line Pap smears, as compared with the risk among those with HPV DNA–negative smears.

**TABLE 2. ODDS RATIOS FOR CERVICAL CANCER ASSOCIATED WITH THE PRESENCE OF HUMAN PAPILLOMAVIRUS (HPV) DNA IN THE PAP SMEARS OF THE STUDY PARTICIPANTS.\***

HPV DNA STATUS OF BASE-LINE SMEAR	HPV DNA STATUS OF DIAGNOSTIC BIOPSY SPECIMEN OR SMEAR†	WOMEN WITH CANCER	CONTROL WOMEN	ODDS RATIO (95% CI)‡
		no.		
Negative	Negative	20	98	1.0
Positive	Negative	4	2	15.0 (0.8–1541)
Negative	Positive	53	3	122.6 (19.3–5188)
Positive	Positive	27	1	213.4 (18.1–16,000)
Total		104	104	

\*HPV DNA status was determined on the basis of the results of PCR with a general primer and HPV typing.

†Diagnostic biopsy specimens were obtained from the women with cancer, and the Pap smears were obtained from the control women after the time of diagnosis in the corresponding women with cancer.

‡Odds ratios were calculated by conditional logistic-regression analysis (with matching for age and the times at which the samples were obtained and sampling dates). CI denotes confidence interval. Women whose smears or biopsy specimens were found to be negative at both times constituted the reference category.

frame) were confirmed by sequencing the PCR products of the L1 open reading frame. For one woman (Patient 22 in Table 3), type-specific PCR indicated that HPV-16 was present in her base-line smear and HPV-18 in her biopsy specimen, but sequencing revealed HPV type 31 (HPV-31) in both samples. In the 23 women with cancer for whom typing could

**TABLE 3.** TYPE-SPECIFIC PERSISTENCE OF HUMAN PAPILLOMAVIRUS (HPV) IN NORMAL BASE-LINE PAP SMEARS AND SUBSEQUENT BIOPSY SPECIMENS OF INVASIVE CERVICAL CANCER OR FOLLOW-UP PAP SMEAR.\*

SUBJECT No.	BASE-LINE PAP SMEAR†			HISTOLOGIC DIAGNOSIS	DIAGNOSTIC BIOPSY SPECIMEN		
	HPV STATUS BY GP PCR	HPV TYPE-BY TYPE-SPECIFIC PCR	HPV TYPE BY DNA SEQUENCING		HPV STATUS BY GP PCR	HPV TYPE-BY TYPE-SPECIFIC PCR	HPV TYPE BY DNA SEQUENCING
With cancer							
1	+	18	18	Poorly differentiated SCC	+	18	18
2	+	–	Inconclusive	Poorly differentiated SCC	+	16, 18	18
3	+	16, 18	16	Poorly differentiated SCC	+	16	16
4	+	16	16	Poorly differentiated SCC	+	16	16
5	+	16	16	Moderately differentiated SCC	+	16	16
6	+	16	16	Moderately differentiated SCC	+	16	16
7	+	16	16	Moderately differentiated SCC	+	16	16
8	+	16	16	Moderately differentiated SCC	+	16	16
9	+	16	16	Moderately differentiated SCC	+	–	16
10	+	16	16	Moderately differentiated SCC	+	16	16
11	+	16	16	Moderately differentiated SCC	+	16	16
12	+	16	16	Moderately differentiated SCC	+	16	16
13	+	16	16	Moderately differentiated SCC	+	16	16
14	+	16	16	Moderately differentiated SCC	+	16	16
15	+	16	16	Moderately differentiated SCC	+	16	16
16	+	16	Inconclusive	Moderately differentiated SCC	+	–	31
17	+	16, 18	Inconclusive	Moderately differentiated SCC	+	16	16
18	+	–	33	Moderately differentiated SCC	+	–	33
19	+	–	Inconclusive	Moderately differentiated SCC	+	16	16
20	+	–	73	Moderately differentiated SCC	+	–	73
21	+	16	16	Highly differentiated SCC	+	16	16
22	+	16	31	Highly differentiated SCC	+	18	31
23	+	–	18	Moderately differentiated AC	+	18	18
24	+	–	18	Moderately differentiated AC	+	–	18
25	+	16	16	Moderately differentiated AC	+	16	16
26	+	16	16	Moderately differentiated AC	+	16	16
27	+	18	18	Highly differentiated AC	+	18	18
Control							
1‡	+	–	33	Not applicable	+	18	ND

\*GP PCR denotes general-primer polymerase chain reaction, SCC squamous-cell carcinoma, AC adenocarcinoma, and ND not done.

†The mean storage time of the base-line smears was 12 years (range, 5 to 28); the mean time between the date of the base-line smear and the date of the diagnosis of cancer was 4 years.

‡This was the only control woman who was HPV-positive at both times. In her case, there was no biopsy specimen, and a second Pap smear was analyzed.

be performed conclusively, the type of HPV DNA was the same in both the base-line smear and the subsequent biopsy specimen of the cancer (Table 3). None of the control women were positive for the same HPV type in both the base-line smear and the subsequent smear. The odds ratio for cervical cancer associated with type-specific persistence of HPV was 58.7 (95 percent confidence interval, 10.2 to ∞). Among the 104 women with cancer for whom tissue samples were available, 80 were positive for HPV DNA. HPV-16 was detected in the cancers of 43 percent of the 104 women (45 women), HPV-18 in 21 percent (22 women), HPV-31 in 3 percent (3 women), and HPV type 33 (HPV-33) in 6 percent (6 women). Both HPV-16 and HPV-18 were detected by type-specific PCR in the biopsy specimens of four women with cancer. A novel type (HPV type 73 [HPV-73])

was detected in both the base-line smear and the subsequent biopsy specimen in one woman with cancer. The results of the analysis of the samples from three women were inconclusive.

The median storage time (the interval between the time the smear was obtained and the time of DNA extraction) was 12 years for the samples that were positive for HPV DNA and 16 years for the samples that were negative (P=0.02). The median time between the date of the base-line smear and the date of the diagnosis of the cancer was three years for the HPV DNA-positive samples and five years for the HPV DNA-negative samples (P=0.05). Among the women who were HPV-negative, the median age at the time of the base-line smear was 46 years, and among the women who were HPV-positive, it was 39 years (P=0.02). There was no correlation between posi-

tivity for HPV DNA and the clinical characteristics we studied, such as disease stage or survival (data not shown).

### DISCUSSION

In a prospective, population-based study, we found that a test for HPV DNA can predict the risk of cervical cancer among women with normal Pap smears. We do not know whether the cancer in the HPV-negative women developed without a preclinical stage of continuously detectable HPV infection or whether the fact that stored rather than fresh samples were used resulted in an underestimation of the proportion of women who were HPV-positive before cervical cancer developed. The fact that only 77 percent of the biopsy specimens were HPV-positive and that there was a small tendency for HPV positivity to increase with a shorter duration of storage time suggests that there was some underestimation. However, misclassification due to the suboptimal sensitivity of DNA analysis of stored specimens is not likely to have affected the estimate of risk substantially, because cancer-free samples from both the women who later had cancer and the controls were analyzed. Case-control studies conducted with samples obtained after the diagnosis of cancer may be subject to differential misclassification, which may result in unpredictable biases because the presence of the cancer makes HPV easier to detect.

Previous studies in which stored cervical smears and biopsy specimens were used found that HPV was more commonly detected in stored smears containing cervical intraepithelial neoplasia than in biopsy specimens,<sup>11</sup> and the results of the S14 PCR analysis in our study suggested that DNA is preserved at least as well in smears as in biopsy specimens, if not better. Our results indicate that the increased prevalence of HPV in diagnostic biopsy specimens is not related to the type of archival specimen used but is most likely a result of increased detectability caused by the cancer. Although most smears were consistently positive for HPV at all dilutions tested, some smears had different "windows" of HPV detectability. Rigorous quality control, including sample titration, repeated analysis, and the inclusion of a sensitivity panel in each analysis, is crucial for the interpretation of molecular epidemiologic investigations.

The base-line Pap smears of several of the women in the study, on careful reevaluation, were found to have cytologic abnormalities that had originally been missed. This was a population-based study, in which all the participants were at risk for cervical cancer during follow-up. Thus, women who had undergone surgical treatment for diseases of the cervix because of diagnosed cytologic abnormalities were excluded. However, women with falsely negative cytologic results never underwent any treatment and were therefore at risk for cervical cancer. The exclusion of these

women from the study would have made the results difficult to interpret, since it would have redefined the study population by using additional tests that are related to both the exposure and the outcome. We believe that the study of women at risk in a defined population provides the most valid and interpretable estimates of risk.

An increased risk of cervical cancer in women with HPV DNA-positive smears was also evident three to six years before diagnosis. Younger age and a shorter time between the base-line Pap smear and the diagnosis of cancer were associated with increased detectability of DNA. It is possible that HPV infection may be more readily detectable at times closer to the primary infection and at the stage at which the cellular abnormalities start to occur. It has been suggested that screening be performed only for women more than 35 years of age.<sup>2,6,8</sup> The mean age of the women in this study was 44 years at base line.

In conclusion, the presence of HPV DNA in cytologically normal Pap smears was associated with an increased risk of invasive cervical cancer, although only a small number of women with cancer were positive for HPV before the diagnosis of cancer. With the exception of one woman with a novel HPV type (HPV-73), only high-risk HPV types were present (HPV-16, HPV-18, HPV-31, and HPV-33), and there was a strong concordance between the type of HPV found in the base-line smear and that found in the biopsy specimen of the invasive cancer, further supporting the hypothesis of viral persistence in the development of cervical cancer.

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