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Persistent HPV infection in postmenopausal age women

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ABSTRACT

Objective: Persistence of human papillomavirus (HPV) is associated with an increased risk of developing cervical SIL and cancer in young women. Because this association in older, postmenopausal age women has received little attention, we evaluated persistence of HPV among women in this age group. **Methods:** Women ($n=105$) ages 45-64 were examined annually for 7 years to evaluate HPV in cervical cytologic specimens. PCR, dot blot hybridization and DNA sequencing were used to detect HPV types. **Results:** The cumulative prevalence of HPV was 34%, and 24% had HPV high-risk oncogenic types which are associated with genital cancers. The most common oncogenic types were HPV-16 (72%) and HPV-31 (16%). The persistence rate of HPV infection was 16%. No specific risk factors were associated with repeat viral positivity. **Conclusion:** Postmenopausal women are infected with persistent oncogenic HPV at a substantial rate, supporting the need for continued screening in postmenopausal women to detect preneoplastic genital lesions.

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1. Introduction

Human papillomavirus (HPV) is a necessary cause for the development of cervical neoplasms and a large portion of other genital tumors [1]. In the United States, the incidence of invasive cervical cancer increases up to age 45 ($16/10^5$) where after the rate plateaus and does not drop in older ages [2]. Premenopausal women who test positive for HPV persistently over time have been shown to be at highest risk of developing preneoplastic genital disease [3-7]. Only a few studies have evaluated healthy women over age 40 to detect the prevalence or persistence of HPV infection in the lower genitals, and the frequency of HPV infection has been reported to vary widely [8-11]. Whereas in a cross-section of women and based on a single HPV assessment, Ferenczy et al. [9] detected only 1% with viral infection; in a prospective study [11], we reported a cumulative prevalence of 14% over a 2-year period. A comparison of women of all ages in Spain, Columbia and Brazil [10] showed that HPV frequency not only varied widely among countries but also among women ages 40 to 60+ living in different parts of the world (1-22%). In two of the countries, the prevalence rate of HPV was similar in the 60+ age group to that of the youngest age <25 years old.

Oral contraceptive (OCs) steroid hormones have been shown to increase the risk of cervical dysplasia and cancer in the presence of an HPV infection in younger age women [1,12]. In contrast, the risk associated with use of hormone replacement therapy (HRT) is relatively unknown. Despite recent reports of increased risk of breast cancer and other diseases, many postmenopausal women are continuing to use HRTs to deter side effects of menopause and osteoporosis. The adverse effects of using HRTs for long duration and subsequent development of cervical squamous epithelial neoplasia (SIL) are unknown and it is unclear whether their effects will mimic those found for OCs. The *in vitro* evidence indicates that there is greater HPV gene expression and cell transformation by the viral DNA in the presence of progesterones [13-15].

The previous results of this study population [8] were based on a shorter time interval, 2 years, and used a less-sensitive HPV-typing technique than employed in the current study. The aims of this investigation were to determine the prevalence of HPV and oncogenic types in postmenopausal women annually over a 7-year interval between 1990 and 1996, the prevalence and types of HPV associated with persistent viral infection and the association

between HRT regimens and detection of HPV over time.

2. Methods

2.1. Patient population

Patient characteristics and details of the study design have been described previously [16]. Briefly, women were enrolled in this ancillary study at the University of Iowa between 1989 and 1990 as part of the Postmenopausal Estrogen and Progestin Intervention (PEPI) randomized clinical trial (RCT). Participants were between ages 45 and 64 prior to randomization to treatment, not currently using hormone replacement therapy at least 6 months prior to enrollment and had experienced menopause at least 1 year before or had a hysterectomy at least 2 months prior to study entry. Those who had a hysterectomy were included because they were also at risk of condylomata and genital cancers other than the cervix and were also associated with HPV infection. None of the participants had a genital dysplasia or cancer at the time of enrollment. Women were administered a University-approved human subjects review form for this ancillary study to the PEPI randomized clinical trial (RCT) before enrollment. Participants were followed annually for HPV specimens and Pap smears at baseline and three follow-ups with a 1-year hiatus after the RCT was completed, and prior to the funding of the follow-up period with an additional three annual follow-ups for a total of one baseline and six follow-up HPV and Pap smear specimens. These specimens were collected annually between 1990 and 1993 and again between 1994 and 1996. Investigators and laboratory staff were blinded to HRT treatment group, Pap smear and risk factor results until the HPV study was completed.

2.2. Hormone replacement groups

Women in this study were randomly assigned to one of five treatment groups: (1) placebo, (2) 0.625 mg/day conjugated equine estrogen (CEE) daily, (3) 0.625 mg/day CEE daily plus cyclic medroxyprogesterone (MPA), 10 mg/daily for days 1-12 of each month, (4) 0.625 mg/day CEE daily plus MPA 2.5 mg daily or (5) 0.625 mg/day CEE daily plus cyclic micronized progesterone (MP) 200 mg/daily for days 1-12. For the purpose of this study, HRT comparisons were made between the placebo group (#1) and the estrogen only HRT group (#2)

or with the estrogen/progestin HRT groups combined (#3-5).

2.3. Pap smear and HPV specimen collection

Prior to and after randomization to placebo or an HRT, participants received Pap smear evaluations and a cervical swab to collect HPV DNA at 12-month intervals. Randomization procedures, timing of treatment and annual visit information have been described previously [16]. Unblinding of HRT treatment group did not occur until the end of the third year of the study. Only participants who remained on protocol were included in the PEPI RCT and in this 7-year ancillary study. This included 105 of the 125 women originally enrolled in the clinical trial. A single nurse practitioner for the study (D.F.) collected all specimens through the study. Women first had a Pap smear retrieved and then, using a cotton tip swab, had cells for HPV evaluation collected from the transitional zone, endocervix, ectocervix and pooled secretions in the posterior vaginal fornix. A speculum was placed, and the swab was inserted carefully through to the cervix/vagina, avoiding the perianal skin surface. This sample was prepared and frozen until tested. Pap smears were reviewed by a single SmithKline cytopathologist who was involved in the clinical trial and blinded to the HRT treatment group assignment of individuals. The Bethesda system was used to report Pap smear results. Included in the abnormal Pap smear category was atypical squamous cells of undetermined significance (ASCUS), low-grade SIL (L-SIL), high-grade SIL (H-SIL), adenocarcinoma or squamous cell carcinoma.

2.4. HPV DNA detection and typing

Description of processing the cervical cells has been described in detail elsewhere [8,11]. Briefly, after processing the cells, the HPV DNA was PCR-amplified with MY09/MY11 HPV L1 primers [17], using DNA from approximately 30,000 cells as template. An additional 3' primer that facilitates HPV-51 amplification was included [3]. Primers to amplify a 294-bp portion of the β -globin gene were used as an internal control [18]. Each set of PCR amplifications included positive and negative controls. Amplification of HPV DNA was tentatively judged positive if electrophoresis on a 1% agarose gel showed an approximately 450 bp band by staining with ethidium bromide or if a signal was detected by nylon membrane "dot blot" hybridization with [α -³²P] dATP-labeled generic HPV probes. All specimens

were B-globin-positive by gel electrophoresis for all assessments.

Automated DNA sequence analysis was used to confirm and type the HPV DNA. Amplified DNAs were purified using the Wizard PCR Preps DNA Purification Kit (Promega, Madison, WI) and sequenced on a PE Applied Biosystems automated sequencer (Perkin Elmer Cetus, Foster City, CA). When insufficient DNA was present for sequencing, HPV DNA sequences were reamplified for 25 cycles in a second PCR reaction using 1 μ l of the original PCR product and the MY09/MY11 primers or using MY09/GP5+ [19] as heminested primers. The DNA sequences were compared with those of HPV known isolates on the GenBank database using the BLAST sequence analysis program [20]. A sample was identified as a specific HPV type if it matched a type on the database by more than 90%. Several of the mucosal oncogenic, high-risk HPV (HPV-HR) types, those most commonly associated with an increased risk of cervical SIL and carcinoma and those most closely related to them, were found in this study. Sequencing also identified mucosal non-oncogenic and cutaneous HPVs and sequences matching GenBank accession numbers of as yet unnumbered types. The mucosal non-oncogenic and cutaneous HPV types are considered low-risk (HPV-LR) because of their usual association with benign lesions, or because their DNA sequence is closer genetically to these benign types than to the high-risk types. All tentatively positive samples gave interpretable sequence results. Single-strand conformational polymorphism (SSCP) analysis was performed on samples to detect multiple HPV types. This was performed in women who were detected with different HPV types during the study period. Procedures for SSCP have been described previously [11]. Samples which showed a less than 95% sequence homology with any HPV sequence on the GenBank database were considered to be a different type. The single-stranded DNA was resolved by a 6% polyacrylamide electrophoresis in buffer containing 10% glycol. Isolated bands were characterized further by DNA sequencing.

2.5. Statistical analyses

The Kruskal-Wallis test was used to test for group differences at baseline in continuous variables and the Pearson Chi Square test or Fisher's Exact Test was used to test for group differences in categorized variables. Logistic regression was used to assess the association between baseline risk factors and HPV detection at baseline. To deal with the fact that each patient had repeat HPV results, the generalized estimating equation (GEE) methodol-

ogy [21-23] for longitudinal data using a logit link was used to assess the association between HPV detection over time at follow-up visits only and possible risk factors including HRT regimen, baseline HPV status and time. Within the GEE methodology, the independence “working” correlation matrix was used. Although the correlation was not specified explicitly, the intrasubject correlation was taken into account in the analyses. A total of 95% confidence intervals were calculated using the standard errors from the corresponding logistic regression or GEE models and the normal approximation. All calculations were conducted using the SAS statistical package [24].

3. Results

Prior to randomization into the PEPI clinical trial in 1990, the median age of the 105 participants was 56 years, average education was 13.4 years and almost three-quarters had a family income less than \$50,000. Most women were currently married (80%). The majority of women were 19 years of age or older at first sexual intercourse (63%) and had one male partner during their life (59%). The median age among those who had a hysterectomy prior to study enrollment (26%) was 46 years and among those with a natural menopause (74%), it was 51 years. Among the 63% who had previously taken oral contraceptives (OCs), duration averaged 58 months, whereas among the 41% who had taken HRTs prior to the study enrollment, the average was 37 months. Among prior users of hormone replacement ($n=43$), 42% used estrogen only, 56% use combination HRTs and one woman could not recall what type of hormone(s) she had used.

Table 1 shows demographic characteristics and risk factors associated with HPV detection at baseline. Results are not described by HRT treatment group because there were no significant differences across groups by risk factor including baseline HPV-positive and HPV-HR rates. Detection of HPV at baseline was significantly associated with an increase in number of sex partners (≥ 2 vs. ≤ 1 lifetime male partners; OR=2.8; 95% CI, 1.0-8.1). Risk associated with a history of an HPV-related disease, past user of OCs or current smoker was not related to an increased frequency of HPV detection in this older age group.

The frequency of HPV positivity, persistence, HPV-HR, HPV-LR and types are shown in Table 2. Over a 7-year period, more than one-third of the postmenopausal women was infected with HPV at least once. The cumulative prevalence of HPV-HR infection was 24% and included HPV-16, 18, 31, 33,

Table 1 Risk factors associated with HPV infection: baseline

Risk	$n=105$	%HPV+
<i>Age^a</i>		
46-55	46	13.0
56-65	59	20.3
<i>Education^a</i>		
≤ 12	48	16.7
> 12	57	17.5
<i>Age first intercourse</i>		
≤ 18	39	18.0
> 18	66	16.7
<i>No. of partners^b</i>		
≤ 1	63	11.1
≥ 2	42	26.2
<i>No. of pregnancies</i>		
0-3	50	18.0
4-5	35	8.6
≥ 6	20	30.0
<i>OC duration^a</i>		
0	39	17.9
1-12	25	16.0
≥ 13	41	21.1
<i>HRT^b</i>		
Never	62	19.4
Ever	43	14.0
<i>HRT duration</i>		
0	62	19.4
1-12	14	21.4
≥ 13	29	10.3
<i>Age at menopause</i>		
40-49	27	14.8
50-55	53	22.6
<i>Age at hysterectomy</i>		
30-45	13	7.7
46-55	14	14.3
<i>Smoking</i>		
Never/Ex	92	17.4
Current	13	15.4
<i>History of HPV-related disease^c</i>		
No	98	16.3
Yes	7	28.6
<i>Abnormal pap during study</i>		
No	83	15.7
Yes	22	22.7

^a Years.

^b $p < 0.05$.

^c Includes condylomata acuminata, L-SIL, H-SIL, VIN/cancer.

39, 45, 51 and 58. Sequencing also identified 19% with HPV-LR types: HPV-6, 11, 38, 44, 53, 61, 62 and sequences matching GenBank accession num-

Table 2 Cumulative HPV prevalence and types

HPV status ^a	n=105	Percentage (%)
HPV+ ^b	36	34.3
HPV persistence ^c	17	16.2
HPV+ by time period		
BL	18	17.1
FU 1	12	11.9
FU 2	11	10.9
FU 3	12	12.2
FU 4	10	14.3
FU 5	10	16.4
FU 6	5	8.3
HPV-HR		
16	25	23.8
Other ^d	18	17.1
HPV-LR		
62	10	9.5
Other ^e	20	19.0
62	6	5.7
Other ^e	17	16.2

^a 4 with double/multiple infections; 12 with different HPV types during interval.
^b Ever positive, any time interval.
^c HPV+ >1 interval.
^d Includes HPV-18, 31, 33, 39, 45, 51, 58.
^e Includes HPV-6, 11, 38, 44, 53, 61, AF042004, U01532, U12480, U12490.

bers AF042004, U01532, U12480 and U12490. The annual prevalence of HPV detection ranged between 8% and 17% (Table 2) and between 3% and 11% for those with HPV-HR types. There was no trend toward an increase or decrease prevalence rate over time. Among women ever detected with HPV, 44% were detected with oncogenic types, 31% with HPV-LR types and 25% with both high- and low-risk HPV types. The most common HPV type was HPV-16 which accounted for 50% of the infections, followed by HPV-62 (17%) a low-risk type. Four women were detected with multiple types. There was no difference in the frequency of HPV-HR detection in women with (23%) and without (26%) a hysterectomy.

Persistent infection, defined as HPV-positive at two or more assessments at any visit during the 7-year interval, occurred in 16% of the participants (Table 2) or 47% of the infected women. A total of 41% had persistence infection with HR types. Only 2% (n=2) tested positive at every visit and both women were infected with LR and HR types at different time intervals. Another 18% of the study group had a transient infection of HPV detection at a single period. Those with persistent infection had a higher risk of reporting an abnormal cytology: OR=2.3 (0.6-8.6). There were no statistically significant differences in the frequency of other risk factors shown in Table 1 in the persistently HPV-HR women, compared to those who were persistently HPV-negative at all time intervals. Persistent HR cases also had a history of using oral contraceptives longer than

those without infection (80 months versus 38 months) but the difference was not statistically significant.

The probability of being detected with the virus over time at follow-up visits after adjusting for other significant risk factors, prior hysterectomy and number of pregnancies, showed that baseline HPV status was a significant factor (p=0.02) for predicting subsequent HPV infection. The odds of having a subsequent positive HPV result among women initially detected with HPV were three times greater compared to those who were initially HPV negative (OR=3.0; CI, 1.2-7.7). The odds did not change even if a prior history of an HPV-related disease was included in the model.

During the 7-year interval, 23% (n=22) of women had an abnormal Pap smear at least once: 16 ASCUS, 5 L-SIL and 1 H-SIL (Table 1). Among these women, all those who harbored the virus (36.4%) had only HR types: 4 were diagnosed with ASCUS, 3 with L-SIL, and 1 with H-SIL. All other women with ASCUS or L- SIL were HPV-negative at all time periods. Among those who had exclusively normal Pap smears throughout the study (79%), 7% of those had persistently HPV-HR types and another 5% had persistently HPV-LR types.

Fig. 1 shows the cumulative prevalence of HPV by HRT treatment groups. The HPV-positivity rate in nonusers of HRTs was 23% compared to 30% among users of estrogen-only HRTs and 40% in users of estrogen/progestin HRTs. Despite the increase in frequency of HPV positivity across HRT groups, the difference between HRT nonuser and user groups

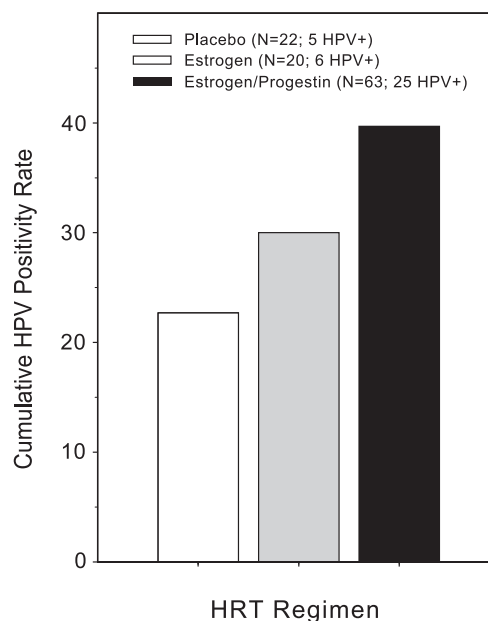


Figure 1 Cumulative prevalence of HPV by HRT regimen.

was not statistically significant, even after adjusting for number of pregnancies, prior hysterectomy or a prior history of an HPV-related disease, which were shown to be associated with an increased risk of HPV positivity (data not shown).

4. Discussion

This study found that the prevalence rate of HPV DNA in the cervix or vagina of postmenopausal women was high. Over a third of the women were detected with the virus at least once during a 7-year period and two-thirds of those were detected with oncogenic types. This finding is contrary to the long-standing belief that only young women, particularly those <age 25, have high rates of HPV infection and that rates are much lower in middle and older age [9,25]. This high rate of HPV detection also is surprising because our study group would appear to be at low risk of HPV infection based on their lack of risk factors for HPV including an average of only one lifetime male sex partner. Previous studies have included few if any older women, generally defined as over age 40, have focused on higher-risk older populations, and have rarely followed-up women in this age group at multiple time periods to examine viral persistence [10]. In contrast, our investigation included women ranging between the ages of 45 and 64 at baseline to ages 52 and 71 at the final follow-up period.

Two issues considered of major importance: persistence of HPV-HR types and the relationship between short- and long-term persistence [4,26], which were also found to be relatively stable in our study, between 7% and 10%. In younger women, it has been shown that persistent viral detection represents a more accurate measure of risk for development of cervical neoplasia than do tests taken at a single point in time [3,4,27]. Like younger aged women, the cumulative prevalence of HPV among postmenopausal women was much higher than at individual time points. The known association between detection of HPV oncogenic types in women with an abnormal Pap smear was supported by our data with only HPV-HR types detected among those with an abnormal cytology, whereas those who had normal Pap smears throughout the study had a much lower rate of HR type persistence, 36% versus 7%. A small percentage of women in our investigation were persistently positive for only LR types (4%). None of these women was identified with an abnormal Pap smear. In contrast, in a primarily premenopausal age group, women detected with low-risk types were

at greater risk of being diagnosed with an abnormal Pap smear [3,4]. However, these investigations retested women over shorter time intervals and may have missed some of the intermittent oncogenic types that we detected over a longer interval.

We recognize that the lack of HRT associated findings of increased HPV detection in this investigation may be due to our small sample size. Yet we believe that our findings, performed in a tightly controlled randomized clinical trial, are sufficiently intriguing and compelling that larger studies should investigate these HPV-related issues among older women. HPV replication in vitro increases in the presence of steroid hormones and glucocorticoids [15,28]. Steroid hormone exposure potentiates HPV-induced neoplastic transformation of cells in culture [15,28]. These molecular experiments help to explain the known association between use of OCs and increased risk of HPV-related cervical cancer. Our in vivo findings of HRT steroids showed a slight increased frequency of HPV detection between nonusers of HRTs compared to women who took estrogen only or in combination with progestin, and possibly is suggesting that these hormones up-regulate the HPV gene. In a previous, larger study that examined HPV over two periods, we showed a significant association between HPV detection and duration in estrogen/progestin HRT use (OR=1.8, 1.1-3.1) compared to never users. Further clarification is needed to determine whether women using HRTs will have a higher risk of developing cervical and other gynecologic cancers.

The misconception that HPV prevalence is low in the cervix of older women may be detrimental to their health because many women after their childbearing years no longer have Pap smears or routine gynecologic examinations. Coupled with the relatively high frequency of HPV-HR detected over this 7-year study in a group of women at low risk of HPV-related genital cancers, this study suggests that being detected with HPV is a significant predictor of subsequent HPV positivity and may be predictive of the development of preneoplastic lesions as it is in younger women. The fact that the incidence of cervical neoplasia does not decrease with increasing older age also supports the argument that, because HPV is a necessary cause of this cancer and because HPV-positivity predicted subsequent infection, testing older women for the virus may be a cost-effective method of disease prevention. Thus, the continual need for Pap smears and pelvic exams in menopausal age women need to be emphasized to patients and providers.

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