Type-Specific Persistence of High-Risk Human Papillomavirus Infections in the New Independent States of the former Soviet Union Cohort Study

Satu-Maria A. Kulmala,[1] Irena P. Shabalova,[2] Nikolay Petrovitchev,[2] Kari J. Syrjänen,[3] Ulf B. Gyllensten,[4] Bo C. Johansson,[5] Stina M. Syrjänen,[1] and the New Independent States of the former Soviet Union Cohort Study Group

[1]Department of Oral Pathology, Institute of Dentistry, and MediCity Research Laboratory, University of Turku; [2]N.N. Blokhin Cancer Research Center of Russian Academy of Medical Sciences, Moscow, Russia; [3]Department of Oncology and Radiotherapy, Turku University Hospital, Turku, Finland; [4]Rudbeck Laboratory, Department of Genetics and Pathology, University of Uppsala, Uppsala, Sweden; and [5]Department of Laboratory Medicine, Division of Clinical Virology, Karolinska University Hospital at Huddinge, Karolinska Institute, Stockholm, Sweden

Abstract

Background: **Prospective follow-up studies have recently suggested that persistent high-risk human papillomavirus (HPV) infections play a key role in the progression of CIN lesions and in the development of cervical cancer. However, data on type-specific persistence, viral integration, and the role of multiple infections are scanty.**

Materials and Methods: **A cross-sectional/cohort study was conducted between 1998 and 2002 in three New Independent States of the former Soviet Union comprising a cohort of 3,187 women, of whom 854 women were followed up for a mean of 17 months (SD, 11.6). HPV genotyping was done with real-time PCR, detecting HPV types 16, 18/45, 31, 33/52/58, 35, and 39. The integration status of HPV16 was examined by using a novel Taqman-based PCR method.**

Results: **The mean clearance time for the individual high–risk–type infection was 16.5 months (range = 0.9-34.9 months). HPV16 and HPV31 were the most persistent infections (clearance times = 18.1 and 16.2 months, respectively), whereas HPV39 infections cleared most rapidly. The mean copies per cell in HPV18/45, HPV31, HPV33/52/58, and HPV39 infections were higher in persisting HPV infections than in HPV infections that cleared, but the difference was not significant. Integration of HPV16 was not found to correlate with HPV persistence.**

Conclusions: **A large proportion of women remained high-risk HPV positive after 18 months. Coinfection with multiple HPV types, viral load, or integration status did not correlate with persistence of high-risk HPV infections.** (Cancer Epidemiol Biomarkers Prev 2007;16(1):17–22)

Introduction

Infection with high-risk human papillomavirus (HPV) is the single most important risk factor of cervical cancer and its precursors (1, 2). Both high-risk and low-risk HPV infections are known to be extremely common especially among young women. Most cervical HPV infections are cleared in about 12 months with no residual lesion in the cervix (3-5), whereas some infections persist. Persistence of high-risk HPV infections is shown to play a key role in the progression of CIN lesions and in the development of cervical cancer (6, 7). Thus, the viral risk factors for HPV persistence are currently being studied intensely.

We previously studied HPV persistence using the hybrid capture 2 test (HC2) and a Southern blot confirmed in-house PCR assay in the New Independent States of the former Soviet Union (NIS) cohort and found current smoking, high viral load with HC2, high-risk positive PCR result, and incident abnormal Papanicolaou (Pap) test to be significant predictors of persistence during follow-up period (8). Specific high-risk HPV genotypes have been suggested as one potential risk factor for persistency (6), and HPV16 has been shown to be among the most persistent types (9, 10). Some authors have associated HPV infections with multiple types with prolonged persistence in young women (11, 12), whereas others have not confirmed this (13). Infections with high viral loads, especially with HPV16, have been reported to be more likely to persist (14, 15). Despite these several studies, however, the significance of the HPV type, multiple infections, and viral load on HPV persistence are still incompletely understood.

We recently conducted a follow-up study to investigate the persistence of specific high-risk HPV types in a cohort of 854 women from three NIS. To our knowledge, this is the first study where also the role of the physical state of HPV16 on persistence is assessed.

Materials and Methods

Subjects and Study Design. The subjects of this follow-up study represent a subcohort of 854 women derived from a multicenter cross-sectional/cohort study conducted between 1998 and 2002 in three NIS. The cohort consisted of 3,187 consecutive women enrolled by six different outpatient clinics in Moscow, Novgorod (Russia), Minsk (Belarus), and Riga (Latvia). This cohort included women at different risk for HPV infections and CIN: (*a*) women being screened for cervical cancer (1,696 women), (*b*) gynecologic outpatients (768 women), and (*c*) women attending sexually transmitted disease clinics (723 women). The complete study design has been described earlier, and the current prospective cohort is fully representative of the original cohort in all clinical and epidemiologic aspects (16, 17). All women had three tests done: (*a*) Pap smear, (*b*) HC2, and (*c*) PCR with Southern blot confirmation using a probe cocktail for high-risk HPVs. Both

Received 8/1/06; revised 9/28/06; accepted 11/7/06.

Grant support: INCO-Copernicus Program of the European Commission contract no. ERB IC15-CT98-0321, Emil Aaltonen Foundation, Cancer Foundations of Western Finland, Finnish Cultural Foundation (South-West Finland Funds), Government Special Foundation for Turku University Central Hospital, and Swedish Cancer Society.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Stina Syrjänen, Department of Oral Pathology, Institute of Dentistry, and Medicity Research Laboratory, Faculty of Medicine, University of Turku, Lemminkäisenkatu 2, 20520 Turku, Finland. Phone: 358-2-3338349; Fax: 358-2-3338399. E-mail: stina.syrjanen@utu.fi

doi:10.1158/1055-9965.EPI-06-0649

HPV tests were made from the same cervical scraping taken with the Digene Cervical Sampler. Women with cytologic abnormality consistent with HPV-CIN (atypical squamous cells or above) and those who were high-risk HPV positive with HC2 were referred to colposcopy and biopsy confirmation. Those women with low-grade lesions at biopsy were included in this study and subjected to prospective follow-up, whereas high-grade lesions (CIN III or higher) were promptly treated and excluded from this analysis (16, 17). The follow-up visits at 6-month intervals included colposcopy examination and Pap smear. A cytologic sample for HPV DNA testing was also collected at each follow-up visit. For this study, follow-up data from at least two (up to seven) subsequent clinical visits were available, with the mean follow-up time of 17.2 months (SD, 11.6 months; range = 1-42.9 months).

Sample Collection. The cervical samples were collected with the Digene Cervical Sampler for HC2 testing as described earlier (16-18). The same samples were then processed for DNA extraction using the high salt method of Miller et al. (19).

Pap Smears. A routine cervicovaginal Pap smear was taken from all women at every visit. The primary screening and interpretation of the smears according to conventional methods were done in the participating NIS countries. All Pap smears were also rescreened and interpreted in Finland using jointly agreed terminology (modified Pap classification). For statistical analysis, this classification was transformed to correspond to the 2001 The Bethesda System classification.

Outcome of HPV Infections. Outcome of HPV infections during the follow-up were classified using the criteria described in a series of recent articles (5, 8, 20). As an incident infection, we recorded an appearance of positive HPV test in a previously high-risk HPV-negative woman. As virus clearance, we considered all baseline HPV-positive women, who tested HPV negative during the follow-up and who remained HPV negative at their last visit. Cases who tested HPV positive subsequent to an HPV-negative test (or HPV negative between two HPV-positive tests) were considered as fluctuating infections. In calculating the cumulative clearance of individual HPV types, we used life-table techniques, as previously described (5, 20), with monthly clearance rates expressed as rates per 1,000 woman months at risk. As a persistent infection, we regarded all cases with positive HPV test at baseline and at the last visit and who did not have any HPV-negative test during the follow-up.

Detection and Quantification of High-Risk HPV by Real-time PCR. High-risk HPV detection, genotype analysis, and viral load quantification was done with a real-time PCR–based assay described recently (21). With this method, the high-risk HPV types 16, 31, 35, and 39 and members of the group 18/45 and group 33/52/58 were detected in two different reactions. Reaction 1 detected HPV types 16, 31, 18, and 45 (HPV18 and HPV45 were detected together). Reaction 2 detected HPV types 33, 35, 39, 52, and 58 (HPV33, HPV52, and HPV58 were detected together). The third parallel reaction quantified the amount of the single-copy human nuclear gene *hydroxymethylbilane synthase* (*HMBS*) to quantify the amount of cellular DNA in the sample. PCR amplification was done in a 25-μL volume containing 200 nmol/L of each primer and probe (21), 3.1 μg of bovine serum albumin (Sigma Chemical Co., St. Louis, MO), and 3 μL of DNA and either of following (*a*) Master Mix containing 1 × buffer A (Applied Biosystems, Foster City, CA); 3.5 mmol/L $MgCl_2$; 200 nmol/L each of dATP, dCTP, dCTP, and dGTP; 400 nmol/L dUTP (Pharmacia Biotech, Uppsala, Sweden); 0.625 unit AmpliTaq Gold (Applied Biosystems) or (*b*) Taqman Universal PCR Master mix (Applied Biosystems). The real-time PCR assay was done with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The amplification conditions were as described previously (21). A total of six nontemplate controls, where DNA was substituted by water, were included in each run. For the conversion of the data obtained from the ABI 7700 software to threshold cycle number, the BIOMAT version 6.0 algorithm, operating on the MATLAB program, was used because the ABI 7700 software cannot quantify three different fluorophores in the same PCR reaction. The dynamic range of the assay is 10^2 to 10^7 copies of high-risk HPV per assay (21). HPV35 detection was done only from the baseline samples.

Integration Assay. All HPV16-positive samples at the baseline and follow-up visits were further analyzed for their physical status, using a real-time PCR method recently developed by our group (22). The amplification conditions, primers, and probes were as described earlier. PCR amplification was done in a 25-μL volume (follow-up samples) or in a 50-μL volume (baseline samples) containing 0.3 μmol/L of primers and 0.1 μmol/L of probe, Taqman Universal PCR Master mix (Applied Biosystems, Foster City), and 2 μL (follow-up samples) or 10 to 50 ng (baseline samples; 50 ng was used when possible) of target DNA from cervical swabs. Two standard curves were obtained by amplification of a dilution series of 5 million to 500 copies of a clone of HPV16 in pBR322. There was a linear relationship between the threshold cycle values plotted against the log of the copy number over the entire range of dilutions (data not shown). In each run, samples were loaded in duplicate, and a mean value of the copy numbers was used for calculations. If the duplicate values differed for >2 C_t units, the sample was rerun. If there was still a substantial difference (>2 C_t), the sample was excluded. A total of 83 E2 reactions and 51 E6 reactions were rerun, and in total, 21 samples were excluded. Four nontemplate control reaction mixtures were included in each run.

The results were recorded as copy numbers in 2 μL (follow-up) or in 50 ng (baseline) of cellular DNA. When no E2 PCR signal was detected, the HPV16 genome was interpreted as integrated. When the ratio of copies of E2 to E6 was >0.5, the physical status was interpreted to be episomal. Otherwise, the sample was interpreted to contain both episomal and integrated forms of HPV16 (mixed form). The cutoff for the E2/E6 ratio for episomal state was previously 1.0 (22, 23). For this study, a new cutoff of 0.5 was chosen based on the intra-assay deviation of calculated E2 and E6 copy numbers of the repeated measurement of dilution series of 500 to 500,000 copies of episomal HPV16 plasmid.

Statistical Analyses. Statistical analyses were done using the SPSS and STATA software packages (SPSS for Windows, version 14.01. and STATA/SE 9.2). Frequency tables for categorical variables were analyzed using the χ^2 test, with likelihood ratio or Fisher's exact test statistics. The 95% confidence interval (95% CI) were calculated where appropriate, using the exact method. Differences in the means of continuous variables were analyzed using either nonparametric tests (Mann-Whitney or Kruskal-Wallis) or ANOVA with post hoc tests (Tukey's honestly significant difference and least squares difference, least significant difference). The distribution patterns were carefully controlled for normality, and log transformations were used to correct the skewed distributions. In reporting viral loads, mean values and minimum and maximum values are indicated. Univariate survival analysis (Kaplan-Meier) was used to model the acquisition and clearance times of individual HPV types. Cox multivariate proportional hazards model was used to test the independent predictors of virus clearance, run in a backward stepwise manner with the log-likelihood ratio significance test. The assumption of proportional hazards was controlled by log-minus-log survival plots. In all tests, Ps < 0.05 were regarded statistically significant.

Table 1. Baseline data of the women participating in the follow-up study

Characteristics	Study cohort
Median age (range)	26.72 (15-76)
Patient category	854
Screening patient	435 (50.9%)
STD patient	246 (28.8%)
Gynecologic patient	173 (20.3%)
Pap smear status	850
Pap class 1	307 (35.9%)
Pap class ≥2	543 (63.6%)
Biopsy status	359
Normal	121 (33.7%)
NCIN	100 (27.9%)
CIN I	90 (25.1%)
CIN II	48 (13.4%)
HC2 result	850
HPV positive	604 (71.1%)
HPV negative	246 (28.9%)
Taqman result	812
High-risk HPV positive	394 (48.5%)
High-risk HPV negative	418 (51.5%)
High-risk HPV prevalence	812
HPV16	134 (16.5%)
HPV18/45	51 (6.3%)
HPV31	39 (4.8%)
HPV33/52/58	50 (6.2%)
HPV35	1 (0.1%)
HPV39	15 (1.8%)
Multiple type infections	104 (12.8%)
Smoking status	780
Regular smoker	214 (27.4%)
Ex-smoker	104 (13.3%)
Nonsmoker	462 (59.2%)
Sexual partners' smoking status	744
Smoker	438 (58.9%)
Nonsmoker	306 (41.1%)
Pregnancy history	791
Yes	553 (69.9%)
No	238 (30.1%)
No. deliveries	783
0	370 (47.3%)
1	250 (31.9%)
2	141 (18.0%)
≥3	22 (2.8%)
Abortion history	760
Yes	403 (47.2%)
No	357 (41.8%)
Onset of sexual activity, median (range)	18.0 (13-35)
Partners during past 2 y, median (range)	1.0 (0-30)
Mode of contraception	762
No contraception	346 (45.4%)
Hormonal	130 (17.1%)
Condom	204 (26.8%)
IUD	53 (7.0%)
Sterilization	2 (0.3%)
Other	27 (3.5%)
Venereal disease history	778
Yes	126 (16.2%)
No	652 (83.8%)
Casual sexual partners	764
Yes	122 (16.0%)
No	642 (84.0%)

Abbreviations: STD, sexually transmitted disease; IUD, intrauterine device.

Results

Characteristics of the Patients. The characteristics of the study population at baseline are summarized in Table 1. Altogether, 735 women who had an atypical squamous cells of undetermined significance or above lesion in the Pap test and/or were tested high-risk HPV positive by HC2 assay and 119 women who were both Pap test negative and high-risk HPV negative were enrolled in this follow-up study. Of these women, 435 were from the screening group, 246 were from the sexually transmitted disease group, and 173 were gynecologic outpatients. Four of the baseline samples were technically unsuitable for analysis, leaving 850 participants to the follow-up study. Altogether, 2,302 cervical specimens from the baseline and follow-up visits were tested by real-time PCR method. Of these specimens, 38 were *HMBS* gene and HPV negative. The median number of visits per woman was 3.0 (SD, 1.4; range, 2-7), and the median duration of follow-up was 16.6 months (SD, 11.6; range, 1-42.9). The detection rate of high-risk HPV at baseline was 48.5% with real-time PCR and 71.1% by HC2 assay. HPV16 was the most prevalent type, followed by HPV18/45 and HPV33/52/58 (HPV33 group). Multiple infections were detected in 12.8% of the samples.

Type-Specific Outcome. The HPV-type specific outcomes during the follow-up are shown in Table 2. Altogether, 246 women remained high-risk HPV negative throughout the follow-up time, whereas 99 (11.6%) acquired a new infection. In a total of 169 (19.8%) women, baseline type-specific infections persisted, whereas 116 (13.6%) patients cleared their type-specific high-risk HPV infections.

Type-Specific Clearance. The mean time to clear an HPV infection with specific type was 16.5 months (median, 15.3; range, 0.9-34.9 months). Type-specific clearance times with specific HPV types are shown in Table 3. The clearance rate was highest (40%) for HPV33 group and lowest for HPV39 (13.3%). HPV16 and HPV31 infections were the two types with the longest clearance times (18.1 and 16.2 months, respectively), but the differences in type-specific clearance were not statistically significant. HPV infections with multiple types cleared more slowly at the beginning of the follow-up period compared with single-type infections in Kaplan-Meier test, but the difference did not reach statistical significance ($P = 0.148$). The clearance rates per 1,000 woman months at risk for individual HPV types are summarized in Table 4. Baseline HPV-positive women with cytologic changes in Pap smear cleared their type-specific infections more rapidly compared with those with no changes in their Pap test at baseline ($P = 0.011$). The type-specific clearance time was longest in the sexually transmitted disease patients (18.3 months) and equal in the gynecologic patients and the screening patients (15.5 months; $P = 0.393$). There was no difference in the clearance of HPV infection in women younger than 35 years and older than 35 years ($P = 0.459$), but women at the 41- to 45-year age group had longer-lasting infections than women below 20 years of age [clearance times, 21.6 months (95% CI, 16.1-27.2) and 13.2 months (95% CI, 9.3-17.2), respectively; $P = 0.05$].

We also tested the independent predictors of virus clearance by using the Cox multivariate regression model. When the HPV types (as block) were entered into the model, the only significant ($P = 0.008$) predictors of clearance was HPV33/52/58, with odds ratio of 2.11 (95% CI, 1.21-3.66), and HPV16, with odds ratio of 1.73 (95% CI, 1.02-2.79; $P = 0.040$). This significant independent predictive value of

Table 2. High-risk HPV type-specific outcome during the follow-up

Outcome	*n* (%)
Always negative	246 (28.9)
New infection	99 (11.6)
Persistent infection	169 (19.8)
Infection cleared	116 (13.6)
One positive Taqman test	52 (6.1)
Fluctuator	26 (3.1)
Type changed (single)	59 (6.9)
Type changed (multiple)	35 (4.1)
New infection persisted	27 (3.2)
New infection cleared	23 (2.7)
Total	852 (100.0)

Table 3. Clearance times for individual types

HPV type	No. cases	No. cleared (%)	Clearance time (mo)				
			Median	Interquartile range	Mean (SD)	Range	95% CI
HPV16	134	39 (29.1)	16.9	10.0; 16.9; 29.0	18.1 (10.3)	1.1-34.2	14.8-21.5
HPV18/45	51	15 (29.4)	12.0	8.8; 12.0; 21.9	14.5 (8.7)	1.4-32.1	9.7-19.3
HPV31	39	12 (28.2)	14.8	4.6; 14.8; 28.2	16.2 (10.7)	2.0-32.0	9.4-23.0
HPV33/52/58	50	21 (40.0)	8.2	3.3; 8.2; 27.9	14.7 (12.2)	1.0-35.0	9.1-20.2
HPV39	15	2 (13.3)	12.4	9.5; 12.4; 15.4	12.4 (4.1)	9.5-15.4	−24.8 to 49.7
Multiple	104	27 (24.0)	19.6	9.4; 19.9; 28.0	17.1 (11.1)	1.2-34.4	12.7-21.5
Total	394						

HPV33 group and HPV16 was not confounded by the baseline Pap smear (another predictor in univariate analysis), but all three remained in the multivariate model, with odds ratio of 1.55 (95% CI, 1.02-2.36) for abnormal Pap ($P = 0.037$). These values did not change any further in the model, where patient category and (*a*) age or (*b*) age group or (*c*) variable below/above 35 years (alternatively) were entered in the model.

Duration of Type-Specific Infection. The mean duration of specific high-risk HPV infection (with persisting, cleared, and incident infections included) was 12.9 months (median, 11.1; range, 0-38.6). When persistent and cleared infections as well as type changers and fluctuators were included, the mean duration was longer (i.e., 14.6 months; median, 13.4; range, 0-41.3). The specific duration of infections with different HPV types is shown in Table 5. HPV16 infections had the longest duration (16.1 months), followed by HPV18/45.

Type-Specific Viral Loads in Persistent and Cleared Infections. Type-specific normalized viral loads (i.e., copies per cell) at baseline were compared among cleared and persisting HPV infections. The mean viral loads in women with persistent infections were 55.1 (range, 0.0001-1004.8) copies per cell for HPV16, 99.6 (range, 0.0001-3668.0) copies per cell for HPV18/45, 841.3 (range, 0.0001-34535.0) copies per cell for HPV31, 828.5 (range, 0.01-32711.0) copies per cell for HPV33 group, and 825.2 (range, 0.15-4170.4) copies per cell for HPV39. The mean viral loads in cleared infections were 78.8 (range, 0.0001-2037.3) copies per cell for HPV16, 121.4 (range, 0.0001-2834.9) copies per cell for HPV18/45, 4.1 (range, 0.0001-31.4) copies per cell for HPV31, 143.5 (range, 0.0001-2219.6) copies per cell for HPV33 group, and 99.3 (range, 0.06-612.2) copies per cell for HPV39. These differences did not reach statistical significance, even when log-transformed values were used.

Integration of HPV16. A total of 816 HPV16-positive samples from the baseline and follow-up visits were further analyzed for their physical status by real-time PCR–based integration assay. The physical state of HPV16 is related to different type-specific outcomes in Table 6. Purely integrated form was equally frequent in persistent and cleared infections (4.1%), but mixed form was more frequent in cleared infections. Among the women with persistent Pap smear abnormalities, HPV16 was episomal in 40.5% (30 of 74), integrated in 9.5% (7 of 74), and mixed in 50.0% (37 of 74). Among women who cleared their Pap smear abnormalities, HPV16 was episomal in 48.6% (18 of 37) and mixed in 51.4% (19 of 37). To show the ''sensitivity'' of integration status to predict the different type-specific HPV outcomes, we also calculated the receiver operating characteristic analysis for the different outcomes using integration status dichotomized (yes or no). The areas under the receiver operating characteristic curve are shown in Table 6, and vary from 0.3 up to 0.560. The difference between the areas under the receiver operating characteristic curves is almost significant ($P = 0.078$).

To test the eventual integration load cutoffs that would yield the best balance between sensitivity and specificity, we also calculated receiver operating characteristic curves for all different HPV outcomes listed in Table 6. The areas under the receiver operating characteristic curve varied around 0.5 (the value obtained by chance), and none of the curves was even close to statistically significant. This precludes the pick-up of any meaningful integration load cutoffs that would be of any practical value as sensitivity-specificity indicators.

Discussion

The present study was designed to investigate the influence of type specificity, coinfection, and viral load as well as physical state of HPV16 infection on persistence of high-risk HPV infections in a cohort of women followed-up as part of the NIS cohort study (16, 17). In this analysis, we calculated the duration of the type-specific infection from the first positive sample to the first (same type) negative sample using the sample collection times. Clearance time was the time to clear the infection detected at baseline. Both these times must be considered as estimates because it is not known how long a woman has been infected before the first positive test (at enrollment), or the exact time of virus disappearance during the follow-up. We defined persistence as an HPV infection that remained positive for the same HPV type throughout the follow-up. The possibility cannot be excluded that some of these infections here defined as persistent are eventually cleared or represent a reinfection with the same HPV type. This is, however, unlikely as reinfections with the same HPV type have been shown to be rare (24). We have also previously

Table 4. Clearance rates for individual HPV types

HPV type	Clearance rate per 1,000 WMR	95% CI
All cases*		
HPV16 ($n = 196$)	16.52 (57/3,450)	12.3-20.8
HPV18/45 ($n = 108$)	16.00 (28/1,749)	10.1-21.9
HPV31 ($n = 85$)	15.54 (20/1,287)	8.8-22.3
HPV33/52/58 ($n = 98$)	21.01 (29/1,380)	13.3-28.6
HPV39 ($n = 37$)	10.83 (7/646)	2.8-18.8
	† $P = 0.704$	
Single infections		
HPV16 ($n = 134$)	15.57 (39/2,504)	10.7-20.4
HPV18/45 ($n = 51$)	16.77 (15/894)	8.3-25.2
HPV31 ($n = 39$)	16.54 (11/665)	6.8-26.2
HPV33/52/58 ($n = 50$)	23.89 (20/837)	13.5-34.2
HPV39 ($n = 15$)	6.96 (2/278)	2.6-16.5
	† $P = 0.398$	
MI ($n = 104$)	16.16 (25/1,547)	9.8-22.4
Total	16.37 (116/7,085) ‡	13.4-19.3

Abbreviations: WMR, woman months at risk; MI, multiple infections.
*HPV type as single or as part of multiple infection.
†Fisher's exact test.
‡Mean clearance rate: 116 events during 7,085 WMR (all baseline HPV-positive women).

Table 5. Type-specific duration of HPV infections

HPV type*	No. cases	No. included †	Type-specific duration (mo)				
			Median	Interquartile range	Mean (SD)	Range	95% CI
HPV16	134	116	14.0	7.4; 14.0; 25.1	16.1 (10.4)	1.0-38.0	14.2-18.0
HPV18/45	51	47	12.0	7.2; 12.0; 24.3	15.0 (10.4)	1.4-36.3	11.9-18.0
HPV31	39	36	13.7	6.5; 13.7; 20.5	14.2 (8.7)	2.0-31.9	11.4-17.2
HPV33/52/58	50	49	15.1	2.8; 15.1; 24.3	14.6 (11.2)	1.0-34.9	11.4-17.8
HPV39	15	9	7.0	3.4; 7.0; 30.0	13.8 (13.1)	1.4-32.7	3.7-23.8
Multiple	104	97	12.4	5.1; 12.4; 19.7	13.2 (9.3)	1.0-34.4	11.3-15.0

*HPV35 excluded.
† Data from persistent infections, cleared infections, type changers, and fluctuators are included.

shown that reinfection with the same HPV type is quite unlikely, based on the sequence data on HPV16 LCR (25).

For the high-risk HPV detection and quantification of the viral load, we used the real-time PCR–based assay described in detail earlier (21). The dynamic range per assay is from 10^2 to 10^7 HPV copies. Identification and viral load estimation of different HPV types in a multiple infection are reliable as long as the other types represent at least 1% to 10% of the amount of the main HPV type (21). Although the HPV genotypes included in our real-time PCR assay are among the most frequent high-risk HPV types, the relatively small number of individual HPV types included in the assay can be considered as a limitation. Despite the high sensitivity of this assay, a possibility always exists that some persistent infections might have been misinterpreted as cleared infections or fluctuators, if the viral loads have been lower than the threshold of detection.

The baseline HPV positivity detected by HC2 was higher than the rate detected by real-time PCR. Undoubtedly, this is in part due to the wider range of high-risk HPV types included in the HC2 assay. Other possible reasons might be the higher threshold of detection for the real-time PCR assay, mutations in the primer locations or inhibitory agents to the PCR but also false negatives due to the assay as such or the quality/quantity of the double-stranded DNA. DNA extraction was done from the samples collected into the transport media, which is alcalic and starts the denaturation of DNA. Thus, part of the double-stranded DNA might be lost during the DNA extraction. Interestingly, 1.6% (13 of 816) of the samples shown as HPV16 positive by real-time PCR were found negative by our integration assay. This might be due to (*a*) cross-reactions or (*b*) mutations in the integration assay primer sites.

While comparing the duration of infections between published studies, different designs must be considered. Some authors have analyzed clearance times among women with incident HPV infections (9, 11). In our study and in the studies of Franco et al. (4) and Giuliano et al. (26), the clearance time was calculated for infections present at baseline. We also calculated duration of infections separately by including only persistent infections, or those being type changers and fluctuators.

Franco et al. (4) reported loss of initial type-specific positivity for oncogenic HPV types to be only 8.9 months, which is markedly shorter than in our series (mean clearance time, 16.5 months). Similarly, in the study of young women by Ho et al. (11), the median duration of type-specific infection was 8 months. One explanation for these differences could be the older age of the subjects in our study, although in agreement with other studies (4, 14), age was not shown to directly influence on the type-specific clearance rates in our series. On the other hand, in a recent study by Richardson et al., the mean duration of incident type-specific high-risk HPV infection was 16.3 months, which was similar to our results (16.5 months; ref. 9). In addition, others have reported median durations of HPV infections longer than 1 year (12). In our study, the most persistent high-risk HPV types were HPV16 and HPV31, which is in alignment with other studies (9). However, in the multivariate analysis, HPV16 was found to be an independent predictor of virus clearance. This can be explained by the high prevalence of HPV16 in our series, of which most are cleared, or in the HPV16 group, there might be a subgroup clearing their infection faster, and another subgroup clearing their infection more slowly. We also found that the clearance time was shortest for HPV39 infections, which is in agreement with results of Ho et al. (11).

Recent studies on the effect of multiple type infections on HPV persistence have resulted in confounding findings. Although some studies have questioned the correlation between persistence and coinfection with multiple HPV types (10, 13), there are others that associate multiple-type infections with prolonged duration (11, 12). In our series, multiple infections had the second longest clearance time after HPV16, but the clearance rate per 1,000 woman months at risk was similar to the mean clearance rate (Table 4).

In a recent study using a real-time PCR method, high HPV16 viral load was associated with a decreased chance of viral clearance (15). Dalstein et al. (14) studied viral loads with a semiquantitative HC2 test and concluded that high viral load (>10 pg/mL) infections had a slower clearance rate compared with infections with lower viral loads. We confirmed this with

Table 6. Physical state of HPV16 as related to type-specific outcomes

Type-specific outcome	Episomal (%)	Integrated (%)	Mixed* (%)	Total	Area under ROC curve (95% CI) †
Persistent infection	40 (54.1)	3 (4.1)	31 (41.9)	74 (100.0)	0.390 (0.316-0.463)
Infection cleared	16 (32.7)	2 (4.1)	31 (63.3)	49 (100.0)	0.562 (0.482-0.642)
One positive real-time PCR test	3 (15.8)	3 (15.8)	13 (68.4)	19 (100.0)	NA
Fluctuator	3 (75.0)	0 (0.0)	1 (25.0)	4 (100.0)	0.329 (0.081-0.576)
Type changed (single)	5 (31.3)	2 (12.5)	9 (56.3)	16 (100.0)	0.557 (0.433-0.680)
Type changed (multiple)	4 (44.4)	1 (11.1)	4 (44.4)	9 (100.0)	0.485 (0.308-0.661)
Total	71	11	89	171	
			Comparison of areas under ROC curves		$P = 0.078$ ‡

Abbreviations: ROC, receiving operating curve; NA, not applicable.
*Episomal and integrated form.
† Calculated using the dichotomous variable integration (yes/no).
‡ Calculated using STATA/SE software (ROC curve options).

the HC2 test (8), but the real-time PCR detected viral load did not seem to influence HPV persistence in the present study. This reflects the differences between these two techniques and emphasizes the fact that at least, HC2 assay can be considered only semiquantitatively, as recently discussed (18).

Integration of HPV16 into the host genome has been proposed as a potential marker of progressive cervical disease (27). Because the distinction of transient infections from those that will persist is difficult, we analyzed whether the physical status of HPV16 would be different in these two. Integration of HPV16 was found to be equal (4.1%) in persistent and cleared infections. Interestingly, however, no pure integrated HPV16 form was found in women who cleared their Pap smear abnormalities as contrasted to women with persistent Pap smear abnormalities, where HPV16 was often integrated. This might implicate that HPV integration predisposes cervical lesions (abnormal Pap) to persist. However, integration of HPV16 was not found to correlate with persistence of HPV infection per se. This might be due to an undetectably low copy number of the integrated form in several persistent lesions, or it might be that cells containing integrated HPV are also destroyed by the host defense mechanisms. The HPV16-positive samples taken during the follow-up were also analyzed for HPV16 integration, to determine whether or not the integration status changed during the follow-up and, if so, to which direction (episomal to integrated, mixed to integrated, etc.). There seem to be changes to all directions among the three physical states (integrated, mixed, and episomal), but even these changes did not correlate to any of the viral events analyzed. However, the follow-up time in our study was probably too short to draw definite conclusions because the final selection of the cell populations for malignant progression is a long-lasting process (22).

To conclude, a large proportion of women remained positive for the same high-risk HPV type after 17 months of mean follow-up. Clearance time was longest for HPV16, which also showed the longest duration of infection, although the differences to other high-risk HPV types were not statistically significant. Coinfection with multiple HPV types, viral load, or integration status of HPV16 was not found to correlate with persistence/clearance of high-risk HPV infections.

Appendix A. The NIS Cohort Study Group

The members of the NIS Cohort Study Group are V. Kozachenko, J. Podistov, O. Ivanchenko, S. Zakharenko, R. Nerovjna, L. Kljukina, M. Branovskaja, V. Grunberga, A. Juschenko, T. Zakharova, J. Pajanidi, G. Chemeris, L. Sozaeva, E. Lipova, I. Tsidaeva, A. Pshepurko, O. Erokhina, M. Nikitina, A. Grunberg, M. Cintorino, R. Santopietro, and P. Tosi.

Acknowledgments

We thank Digene Europe (London, United Kingdom) for providing the hybrid capture analyzer, samplers, and test kits; Quantovir AB (Uppsala, Sweden) for providing the reagents for determining part of the HPV titers; and Inger Gustavsson, Kaisa Kurvinen, Tatjana Peskova, and Niina Wahlroos for their skillful technical assistance.

References

1. Walboomers JM, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999; 189:12–9.
2. zur Hausen H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. J Natl Cancer Inst 2000;92: 690–8.
3. Nobbenhuis M, Helmerhorst T, van den Brule A, et al. Cytological regression and clearance of high-risk human papillomavirus in women with an abnormal cervical smear. Lancet 2001;358:1782–3.
4. Franco EL, Villa LL, Sobrinho JP, et al. Epidemiology of acquisition and clearance of cervical human papillomavirus infection in women from a high-risk area for cervical cancer. J Infect Dis 1999;180:1415–23.
5. Syrjänen S, Shabalova I, Petrovichev N, et al. Clearance of high-risk human papillomavirus (HPV) DNA and PAP smear abnormalities in a cohort of women subjected to HPV screening in the New Independent States of the former Soviet Union (the NIS cohort study). Eur J Obstet Gynecol Reprod Biol 2005;119:219–27.
6. Kjaer S, van den Brule A, Paull G, et al. Type specific persistence of high risk human papillomavirus (HPV) as indicator of high grade cervical squamous intraepithelial lesions in young women: population based prospective follow up study. BMJ 2002;325:572.
7. Ylitalo N, Josefsson A, Melbye M, et al. A prospective study showing long-term infection with human papillomavirus 16 before the development on cervical carcinoma *in situ*. Cancer Res 2000;60:6062–32.
8. Syrjänen S, Shabalova I, Petrovichev N, et al. Factors predicting persistence of high-risk human papillomavirus (HPV) infections in women prospectively followed-up in three New Independent States (NIS) of the former Soviet Union. Eur J Gynaecol Oncol 2005b;26:491–8.
9. Richardson H, Kelsall G, Tellier P, et al. The natural history, of type-specific human papillomavirus infection in female university students. Cancer Epidemiol Biomarkers Prev 2003;12:485–90.
10. Liaw KL, Hildeshaeim A, Burk RD, et al. A prospective study of human papillomavirus (HPV) type 16 DNA detection by polymerase chain reaction and its association with acquisition and persistence of other HPV types. J Infect Dis 2001;183:8–15.
11. Ho GY, Bierman R, Beardsley L, Chang CJ, Burk RD. Natural history of cervicovaginal papillomavirus infection in young women. N Engl J Med 1998;338:423–8.
12. Woodman CB, Collins S, Winter H, et al. Natural history of cervical human papillomavirus infection in young women: a longitudinal cohort study. Lancet 2001;357:1831–6.
13. Rousseau MC, Pereira JS, Prado JC, Villa LL, Rohan TE, Franco EL. Cervical coinfection with human papillomavirus (HPV) types as a predictor of acquisition and persistence of HPV infection. J Infect Dis 2001;184:1508–17.
14. Dalstein V, Rietmuller D, Pretet JL, et al. Persistence and load of high-risk HPV are predictors for development of high- grade cervical lesions: a longitudinal French cohort study. Int J Cancer 2003;106:396–403.
15. van Duin M, Snijders PJ, Schrijnemakers HF, et al. Human papillomavirus 16 load in normal and abnormal cervical scrapes: an indicator of CIN II/III and viral clearance. Int J Cancer 2002;98:590–5.
16. Syrjänen S, Shabalova I, Petrovichev N, et al. Human papillomavirus testing and conventional pap smear cytology as optional screening tools of women at different risks for cervical cancer in the countries of the former Soviet Union. J Lower Genital Tract Dis 2002;6:97–110.
17. Syrjänen S, Shabalova I, Petrovichev N, et al. Sexual habits and human papillomavirus (HPV) infections among women in three New Independent States of the former Soviet Union. Sex Transm Dis 2003;30:680–4.
18. Kulmala SM, Syrjänen S, Shabalova I, et al. Human papillomavirus testing with the hybrid capture 2 assay and PCR as screening tools. J Clin Microbiol 2004;42:2470–5.
19. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988; 16:1215.
20. Syrjänen S, Shabalova I, Petrovichev N, et al. Acquisition of high-risk human papillomavirus infections and pap smear abnormalities among women in the New Independent States of the former Soviet Union. J Clin Microbiol 2004;42:505–11.
21. Moberg M, Gustavsson I, Gyllensten U. Real-time PCR-based system for quantification of human papillomavirus types associated with high risk of cervical cancer. J Clin Microbiol 2003;41:3221–8.
22. Peitsaro P, Johansson B, Syrjänen S. Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique. J Clin Microbiol 2002;40:886–91.
23. Kulmala SM, Syrjänen SM, Gyllensten U, et al. Early integration of high copy HPV16 detectable in women with normal and low grade cervical cytology and histology. J Clin Pathol 2006;59:513–7.
24. Xi LF, Demers GW, Koutsky LA, et al. Analysis of human papillomavirus type 16 variants indicates establishment of persistent infection. J Infect Dis 1995;172:747–55.
25. Kurvinen K, Yliskoski M, Saarikoski S, Syrjänen K, Syrjänen S. Variants of the long control region of human papillomavirus type 16. Eur J Cancer 2000; 36:1402–10.
26. Giuliano A, Harris R, Sedjo RL, et al. Incidence, prevalence, and clearance of type-specific human papillomavirus infections: the young women's health study. J Infect Dis 2002;186:462–9.
27. Kalantari M, Karlsen F, Kristensen G, Holm R, Hagmar B, Johansson B. Disruption of the E1 and E2 reading frames of HPV 16 in cervical carcinoma is associated with poor prognosis. Int J Gynecol Pathol 1998;17:146–53.

Type-Specific Persistence of High-Risk Human Papillomavirus Infections in the New Independent States of the former Soviet Union Cohort Study

Satu-Maria A. Kulmala, Irena P. Shabalova, Nikolay Petrovitchev, et al.

Cancer Epidemiol Biomarkers Prev 2007;16:17-22.