Comparison of paired cervical scrape and tumor tissue samples for detection of human papillomaviruses in patients with cervical cancer

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Summary

Purpose of investigation: To compare the detection and distribution of HPV genotypes in paired cervical scrape samples and tumor tissue samples in patients with cervical cancer. *Methods:* Forty cervical scrape samples and 40 paired archival or fresh frozen tissue samples were collected from women with cervical cancer. Polymerase chain reaction with GP5+ and GP6+ primers was performed in all samples for HPV DNA detection. All GP5+/GP6+ negative samples were additionally tested using INNO-LiPA HPV Genotyping Extra Test. *Results:* Overall, 39/40 (97.5%) of CC samples were HPV DNA positive. HPV 16 was found in 24/40 samples, HPV 18 in 5/40 samples. A co-infection with two different HPV genotypes was identified in one cervical scrape specimen, while in tissue samples only single infections were detected. Overall agreement between paired samples was 98.75%. *Conclusion:* The present study has shown that cervical scrape samples are equally useful for HPV genotype determination as tumor tissue samples in patients with cervical cancer. They can be used as accurate clinical samples for detection of HPV genotype causing cervical cancer or for epidemiological molecular studies.

Key words: Human papillomavirus; HPV genotype; Cervical cancer; Cervical scrape; Tissue sample.

Introduction

Cervical cancer (CC) is the seventh most common cancer overall and the second most frequent cancer in women worldwide. More than 80% of CC occurs in the developing countries. In the developed countries with well established screening programs the age-standardized rate (ASR) of CC is now generally less than 14.5/100,000 women [1]. The ASR of CC in the year 2002 in Slovenia was 16.1/100,000, which is the 5t^{h} highest in Europe [2]. After the introduction of an organized national cervical cancer screening program in Slovenia in 2003, the incidence had already fallen from 21.0/100,000 to 17.6/100,000 in the year 2005 [3].

Several epidemiological, molecular and clinical studies performed in the last decade clearly showed that sexually transmitted infection with human papillomaviruses (HPV) has an important role in the development of preinvasive cervical lesions and CC [4-7]. Persistent infection with one of the 15 high-risk HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 or 82) is a necessary although not sufficient etiological factor for CC [7-9]. HPV testing has consequently become an important part of CC screening and detection algorithms in several countries. Thus, the US Food and Drug Administration (FDA) has approved a concurrent HPV and Pap smear screening of women aged 30 or more years in 2004. Additionally, several consensus guidelines recommend HPV testing in the management and triage of women with borderline cytological findings (e.g., atypical squamous cells of undetermined significance) or as a follow-up test after therapy of high-grade cervical intraepithelial lesions [10-12].

Molecular diagnostics of HPV infection can be performed in various types of samples that contain HPV infected host cells. For screening purposes, cervical scrape specimens are widely used, since the material can easily be obtained along with the material for conventional cytological testing [10]. For detection of HPV in women with preinvasive cervical lesions and CC fresh or fixated material obtained by biopsy or surgical procedure can also be used.

The distribution of HPV genotypes in cervical scrape specimens and matching biopsies in women with or without preinvasive cervical lesions has already been studied [13-18] in order to compare the detection rate of HPV and the spectrum of HPV genotypes in paired samples. The concordance between HPV genotypes detected in paired cervical scrape specimens and biopsies was between 88% and 97.5%. However, we were unable to find any study to date comparing the distribution of HPV genotypes in paired cervical scrapes and tumor tissue samples in patients with CC.

The aim of present study was to compare the distribution of HPV genotypes in paired cervical scrape samples and tumor tissue samples in patients with CC in order to assess whether cervical scrape specimens are accurate for preoperative HPV genotype determination.

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Materials and Methods

The women included in the present study were recruited in the year 2006 at the Advisory Board for Gynecological Oncology, which is held weekly at one of the three tertiary referral centers in Slovenia (the Department of Obstetrics and Gynecology at the University Medical Centre Ljubljana). Cervical scrapes were taken from every consecutive woman with CC until 40 samples were collected. Cervical scrape specimens were collected in 1 ml of Digene Specimen Transport Medium (Qiagen, Gaithersburg, MD) and stored at 4°C. DNA extraction was performed within five days. Informed consent was obtained from every included woman. The study was approved by the Medical Ethics Committee at the Ministry of Health of the Republic of Slovenia.

For each woman included in the present study, a formalin-fixed paraffin-embedded (FFPE) sample or fresh tissue sample was obtained at the Pathology Unit of Department of Obstetrics and Gynecology at the University Medical Centre Ljubljana, Department of Pathology at the General Hospital Celje, or Department of Pathology at the University Medical Centre Maribor. These samples were harvested by biopsy or hysterectomy. All archival samples were cut centrally at the Pathology Unit of the Department of Obstetrics and Gynecology at the University Medical Centre Ljubljana. From each sample, 3-5 tissue sections (10 μm thick) were cut and collected into tubes. The microtome blade was changed after each use. The DNA extraction was done within one hour.

Fresh tissue samples were obtained at the Department of Obstetrics and Gynecology at the University Medical Centre Ljubljana by hysterectomy. A small representative sample was taken from the tumor tissue before fixation of the whole pathological specimen in 4% buffered formalin. Samples were then kept frozen at -70°C until analysis.

Year of birth, place of residence, patient age at the time of diagnosis, histological type with differentiation grade, FIGO stage [19], and type of diagnostic and surgical procedure (when appropriate) were retrieved from the Cancer Registry of Slovenia or from medical records for each included woman.

DNA was extracted from clinical samples using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Extracted DNA was stored at -20°C until molecular analysis. DNA concentrations were estimated by spectrophotometric analysis at 260 nm using a spectrophotometer (Biophotometer, Eppedorf, Berlin, Germany). The quality of each DNA sample was verified by PCR amplification of a 268-bp fragment of beta-globin gene [20], on the real-time PCR instrument LightCycler® v1.5 (Roche Diagnostics, Mannheim, Germany) using LightCycler® FastStart DNA Master SYBR Green I kit (Roche Diagnostics) and PC04/GH20 primers. Successful and specific amplification of the beta-globin gene fragment indicated that the DNA sample was adequate for HPV DNA analysis and that no PCR inhibitors were present.

For detection of alpha-HPV, PCR amplification was performed on all samples using HotStarTaq® *Plus* DNA Polymerase kit (Qiagen) and consensus GP5+ and GP6+ primers [21], targeting approximately 150-bp fragments of the HPV L1 gene, as described previously [22]. Up to 200 ng of the extracted DNA was used for PCR in a 50 μl reaction volume.

All GP5+/GP6+ PCR-negative samples were additionally tested using the commercially available assay INNO-LiPA HPV Genotyping *Extra* Test (Innogenetics, Gent, Belgium), capable of recognizing 27 different alpha-HPV genotypes, as described previously [23].

The HPV genotypes present in CC samples were determined by direct sequencing of the GP5+/GP6+ PCR products with the same primers as those used for PCR, as described previously [24]. All samples, in which more than one HPV genotype was initially suspected from GP5+/GP6+ sequencing, were tested additionally with the INNO-LiPA HPV Genotyping *Extra* Test.

Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS) 15.0 for Windows (SPSS, Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was applied to test for a normal distribution of numeric variables. Analysis of variance, Pearson's χ^2 test and logistic regression analysis were used when appropriate. Differences were considered significant when p values were < 0.05.

Results

Forty cervical scrape specimens obtained from women with CC and 40 paired CC tissue samples (35 FFPE samples and 5 fresh frozen tissue samples) were included in the present study. Mean age of included women was 45.15 ± 11.31 years (range 22 to 77 years). Most carcinomas were squamous cell carcinomas (34/40; 85%), most carcinomas were moderately differentiated (20/40; 50%) and most carcinomas were FIGO Stage IB (30/40; 75%) (Table 1).

Table 1. — *Sample characteristics*.

	Number of cases (%)
Age at diagnosis (years) (n = 40)	
≤ 40	13 (32.5)
41 - 60	25 (62.5)
> 60	2 (5)
Histological diagnosis (n = 40)	
Squamous cell carcinoma	34 (85)
Adenocarcinoma	4 (10)
Adenosquamous carcinoma	2 (5)
Grade of differentiation (n = 40)	
Grade 1 - well differentiated	8 (20)
Grade 2 - moderately differentiated	20 (50)
Grade 3 - poorly differentiated	9 (22.5)
Not known	3 (7.5)
FIGO stage (n = 40)	
IA	4 (10)
IB	30 (75)
IIA	1 (2.5)
IIB	1 (2.5)
III	3 (7.5)
IV	1 (2.5)
Type of pathological specimen (n = 40)	
Biopsy	10 (25)
Hysterectomy	30 (75)

The 268-bp fragment of beta-globin gene was amplified successfully from all 80 samples. HPV DNA was detected using GP5+/GP6+ primers in 78/80 samples (97.5%). Two initially HPV DNA negative samples – paired samples of adenosquamous carcinoma – were additionally tested for the presence of HPV DNA using the INNO-LiPA HPV Genotyping Extra Test, but remained HPV DNA negative.

Table 2. — *HPV genotypes in paired cervical scrape and tumor tissue samples in patients with cervical cancer.*

HPV genotype	Cervical scrape	Tissue sample	Total percentage (%)
16	24	24	60
18	4	5	11.25
18+51	1	0	1.25
31	4	4	10
33	3	3	7.5
51	2	2	5
45	1	1	2.5
HPV negative	1	1	2.5
Total	40	40	100

Overall, 78/80 CC samples were HPV positive (97.5%). HPV 16 was a predominating HPV genotype, followed by HPV 18 (Table 2). Direct sequencing of GP5+/GP6+ PCR products revealed the presence of a single HPV genotype in 77/78 HPV DNA positive samples (98.7%). In one sample, a co-infection with at least two different HPV genotypes was detected and was verified additionally by INNO-LiPA HPV Genotyping *Extra* Test. The distribution of HPV genotypes in paired CC samples is shown in Table 2.

There was a 100% agreement between 38 pairs of samples where a single HPV genotype was detected. In one cervical scrape sample, HPV 51 was found along with HPV 18, which was the only HPV genotype found in paired FFPE sample (also confirmed by the INNO-LiPA HPV Genotyping *Extra* Test) (Table 2). Since both genotypes belong to the high-risk group, each was given a relative proportion of 0.5 in this case; overall agreement was thus 98.75%.

Discussion

The aim of the present study was to investigate whether HPV genotypes found in cervical scrape specimens of patients with CC are consistent with HPV genotypes found in tumor tissue samples from the same patients.

Studies comparing the distribution of HPV genotypes in paired cervical scrape and biopsy specimens have already been performed using in situ hybridization [13, 14], antigen detection [15] and PCR [16-18], but none of them have focused on patients with CC. Therefore, it remained unclear how well cervical scrape samples obtained from patients with CC represented the actual HPV status of the patient's cervical region and to what extent they included HPVs from other cervical regions, if they are present. Cervical scrape samples in the present study were taken by gynecologists from visible tumor on the cervix or from the part that was suspicious; adjacent parts of the cervix might also have been scraped. Tissue samples were obtained from visible tumor tissue or from FFPE blocks in which tumor tissue was present according to the pathologist's report. In the present study, we found a 98.75% agreement between HPV genotypes present in cervical scrape samples and paired tissue samples, which is better than in previous studies [17, 18] investigating paired samples of women with low- and high-grade cervical lesions.

It has already been reported [17, 18] that infections with multiple HPV genotypes are more frequently detected in cervical scrapes or exfoliated cell samples than in tissue samples. Similarly, in the present study, we did not find any multiple HPV infections in CC tissue samples and only one CC scrape sample contained more than one HPV genotype. We believe that the additional HPV genotype (HPV 51) found in one cervical scrape specimen represents transitional infection and is not involved in the etiology of CC, as only HPV 18 was found in a paired tumor tissue sample from the same patient. Alternatively, an additional HPV genotype in a cervical scrape specimen may stem from parts of the cervix surrounding the tumor. In previous studies, the prevalence of multiple HPV infections in women with CC ranged from 7.83% [25] to 22% [26]. The 1.3% prevalence of multiple HPV infections determined in the present study might be to some extent attributed to direct sequencing of HPV PCR products, which is known to detect mainly the predominant HPV genotype present in a particular sample [23, 27].

The HPV genotype distribution in CC on a worldwide perspective has already been published [25, 28]. HPV 16, 18, 45, 31, 33, 52, 58 and 35 were identified as eight predominate genotypes in decreasing order of frequency in the first meta-analysis [25], while HPV 16, 18, 33, 45, 31, 58, 52 and 35 were found as predominate genotypes in the most recent meta-analysis update [28]. Interestingly, even on a relatively small number of CC samples, we found a similar distribution of HPV genotypes in similar order of frequency. The only exception was HPV 51, which ranked as 12th in a previous report [25], and was in the present study found in three CC cases (7.5%). HPV 16 and HPV 18 were responsible for 71.9% of CC in the present study, which is also in accordance with the published data [25, 28].

In conclusion, the present study has shown that cervical scrape samples are equally useful in HPV genotype determination as tumor tissue samples in women with CC. Since cervical scrape samples are relatively easy to obtain, they can be used as accurate preoperative clinical samples for exact determination of HPV genotype causing cervical cancer or for epidemiological molecular studies.

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