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Comparison of the sensitivity and specificity of real-time PCR and *in situ* hybridization in HPV16 and 18 detection in archival cervical cancer specimens

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Abstract: The aim of this study was to analyze the correlation between real-time PCR (RT-PCR) treated as a reference method and *in situ* hybridization with tyramide amplification system (ISH-TSA) in the detection of HPV16 and 18 infection and the assessment of viral genome status. The study was performed on cervical cancer biopsies fixed in 10% neutral buffered formalin and embedded in paraffin obtained from 85 women. TaqMan-based 5'exonuclease RT-PCR with type-specific primers was used to assess HPV16 and 18 infections and genome status. Viral infection and genome status was also assessed by ISH-TSA. RT-PCR revealed 76 (89.4%), and ISH-TSA 81 (95.3%) cancers with HPV16 and 18 infections. The ISH-TSA sensitivity and specificity were: 96.1% and 11.1% compared to RT-PCR. The difference between these techniques in HPV detection was significant (p = 0.000). Among 76 HPV16/18 positive cancers in RT-PCR, there were 30 (39.5%) with integrated and 46 (60.5%) with mixed viral genome form. According to ISH-TSA, there were 39 (51.3%) samples with integrated and 37 with mixed form (48.7%). The sensitivity and specificity of ISH-TSA in genome status assessment were 70.0% and 60.9%, respectively. The difference between RT-PCR and ISH-TSA in genome state detection was not statistically significant (p = 0.391). These results suggest that ISH-TSA shows insufficient specificity in HPV detection for use in clinical practice. However, this assay could be applied for viral genome status assessment. *(Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 2, 239–247)*

Key words: HPV16 and 18, RT-PCR, *in situ* hybridization

Introduction

Human papillomaviruses (HPV) are small double-stranded DNA viruses that infect the human epithelium. Over 100 HPV genotypes have been isolated to date, and 15 of them, classified as high-risk HPV (HR-HPV), have been found to be associated with the development of high-grade cervical intraepithelial neoplasia or cervical cancer [1]. The commonest oncogenic HPV types in cervical cancer are HPV16 and 18 [2, 3]. Persistent HR-HPV infection and viral genome integration into the host genome are considered to be key events in the progression to invasive cancer. Integration causes disruption of viral *E2* gene — a negative regulator of the *E6* and *E7* genes promoter [4]. This process leads to increased expression of E6 and E7 viral oncoproteins which target the *P53* and *Rb* tumor suppressor genes, resulting in loss of cell-cycle control.

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Accurate HPV detection, genotyping and genome state evaluation is becoming increasingly important, because of: (1) cervical cancers screening programs; (2) investigating the efficacy of HPV vaccines; (3) HPV infection worldwide evaluation in epidemiological studies; (4) the role of HPV infection during diagnosis of different malignancies and the prognostic and predictive potential of HPV infection for different treatment types, particularly for the introduction of type-specific antiviral therapies or monovalent vaccines [5].

A variety of diagnostic methods of differing sensitivity and specificity have been developed to detect HPV in cervical scrapings and biopsy material [6, 7]. Since HPV cannot be cultured efficiently and the clinical performance of serological assays is poor, the diagnosis of HPV infection is almost entirely based on molecular tools. Currently, RT-PCR using both consensus and type-specific primer systems offers extreme sensitivity and can be performed on smears as well as on paraffin-embedded specimens [8–13]. Since HPV integration disrupts the *E2* gene, there is also the possibility of virus genome status assessment, based on *E2* gene copy number to E6 gene copy number ratio (*E2*/*E6*) [14, 15]. However, the major disadvantage of this technique is the lack of the possibility of histological tissue evaluation. RT-PCR also requires a high-tech laboratory infrastructure and is associated with a risk of contamination.

In situ hybridization (ISH) is a successful method for the localization of specific viral DNA inside an individual cell with preservation of tissue morphology, thus allowing simultaneous assessment of the morphological tissue alterations [7]. It is applicable to formalin fixed paraffin-embedded tissues [16–19]. However, the value of conventional ISH is limited by its detection sensitivity of about 20 HPV copies per cell [16]. The sensitivity of ISH has been greatly improved by the application of tyramide signal amplification (TSA) [17, 18]. In this system, after an initial binding of streptavidin-peroxidase (POD) to the biotinylated probe, POD catalyzes the oxidation of biotyl tyramide which immediately forms covalent bonds with aromatic groups in the specimen. This reaction deposits a large number of biotin at the site of hybridization, which is used to capture more streptavidin-POD. ISH-TSA allows detection of 1–2 copies of HPV DNA per cell [17, 18]. It is also generally accepted that ISH-TSA is capable of distinguishing the physical state of a viral genome on the basis of hybridization signal type [17]. Punctuated signals represent an integrated viral genome, diffuse signals indicate an episomal form, and the coexistence of both signals represents a mixed type viral genome [19, 20].

To the best of our knowledge, there has been only one paper considering the comparison between TaqMan-based 5’exonuclease RT-PCR with type-specific primers and ISH-TSA in the sensitivity of detection of HR-HPV infection in the group of paraffin-embedded cervical cancer specimens. Birner et al. [21] found a significant correlation between these two methods in a group of 86 paraffin-embedded cervical cancer samples. However, they did not compare the sensitivity of RT-PCR and ISH-TSA in the assessment of viral genome state. In turn, Fujii et al. [22] have shown in cervical neoplasia a strong concordance between RT-PCR and ISH-TSA according to the frequency of HPV16 integration. Therefore, large scale clinical studies are still required to validate the ISH-TSA method [7]. The aim of this study was to compare, for the first time in the same group of paraffin-embedded cervical cancer specimens, the sensitivity and the specificity of HPV16 and 18 detection and viral genome status assessment by ISH-TSA in relation to TaqMan-based 5’exonuclease RT-PCR with type-specific primers.

Material and methods

Patients. The study was performed on cervical cancer biopsies obtained from 85 women treated with concurrent chemoradiotherapy between 2001 and 2005 at the Center of Oncology, Krakow, Poland. Detailed characteristics of patients and tumors are presented in Table 1. Patients were enrolled into this study according to the following criteria: (1) squamous cell carcinoma of the cervix in FIGO (International Federation of Gynecology and Obstetrics) stage I–IV; (2) no treatment before biopsy; (3) concurrent chemoradiotherapy with cisplatin. Patients with coexistent other tumors or distant metastases at the time of diagnosis, treated before biopsy and those with contraindications to chemoradiotherapy were excluded from the study. The study was approved by the Ethical Committee at the Regional Medical Chamber in Krakow (Poland).

Material. Cervical cancer biopsies were taken from 85 patients before treatment. All specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. For RT-PCR, tissue samples were cut into five to eight 5 μm thick sections and spun briefly in a 2.0 mL tube. For ISH-TSA, sections were cut at 4 μm, mounted on Super Frost® Plus slides (Menzel-Gläser, Germany), deparaffinized and hydrated through a series of xylens (100% — 2 × 5 min.), and ethanols (100% — 2 × 1 min. and 95% — 3 × 1 min.). For each patient, hematoxylin and eosin stained sections were reviewed by a pathologist in order to confirm histology and grading.

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DNA extraction. DNA was extracted from paraffin-embeded samples using EX-WAX™ DNA Extraction Kit (Milipore-Chemicon International, USA) according to the manufacturer's instructions. The sections were treated with 1 mL of fresh 100% ethanol at room temperature (RT), gently vortexed for 15 s and spun for 3 min. at 6,450 g. Ethanol was removed and pellets were dried at 50°C for about 20 min. The pellets were incubated overnight at 50°C with 150 µL Protein Digestion Solution and 50 µL Protein Digesting Enzyme Solution. The next day, 100 µL Extraction Solution was added, mixed by inversion for 15 s and spun for 10 min. at 6,450 g. The supernatant was placed in a fresh tube and treated with 150 µL of Precipitation Solution and 900 µL of ice-cold 100% ethanol. The samples were incubated overnight at –20°C. On the third day, the pellets were spun for 10 min. at 6,450 g and the supernatant was discarded. The pellets were dried at 50°C and incubated with 50 µL of Resuspension Solution at 50°C for 60 min. After that, the amount and purity of extracted DNA were evaluated with a spectrophotometer NanoDrop ND-100 (NanoDrop Technologies, Inc. USA). Samples were stored at –20°C until RT-PCR analysis.

Table 1. Clinical and histological features of 85 cervical cancer patients

	Number of patients	Percentage
Age		
≤ 50 years[a]	37	43.5
> 50 years	48	56.5
Menopausal status		
Premenopausal	42	49.4
Postmenopausal	43	50.6
FIGO stage		
IB	14	16.5
IIA	19	22.4
IIB	28	32.9
IIIB	24	28.2
Grade		
G1	13	15.3
G2	53	62.4
G3	19	22.3

[a]The mean age in Poland at which women experience the menopause

Real-time quantitative PCR. Real-time PCR targeting *E6* of HPV16 and 18 was used to determine the infection presence. Based on the assumption that integration disrupts the *E2* gene, RT-PCR targeting *E2* was also performed. To account for the variation in the number of cells in each cervical specimen, the level of housekeeping gene for albumin was also determined for each sample. Quantification of the human albumin gene, *E6* and *E2* genes for HPV16 and 18 was performed using 7500 Fast Real-Time PCR System (Applied Biosystems, USA) and the TaqMan amplification reaction. The primers and probes are presented in Table 2.

Each cervical sample was subjected to RT-PCR for quantification of human albumin gene according to Laurendeau et al. [23]. Serial 10-fold dilutions of the reference human genomic DNA (Roche Diagnostics, GmbH, Germany) in TE buffer, corresponding to 1.1063×10^9, 1.1063×10^8, 1.1063×10^7, 1.1063×10^6, 1.1063×10^5 albumin gene copy numbers were prepared. Amplification was carried out in a reaction volume of 25 µl, containing: 12.5 µl of Fast Universal PCR Master Mix (2 ×) (Applied Biosystems, USA), 100 nM of each primer, 300 nM of TaqMan probe and 2 µl of DNA template. Thermal cycling was initiated with 2 min. incuba-

Table 2. Probes and primers used for type-specific HPV detection by RT-PCR

Probe or primer	5'-3' sequence
Probe Albumin	VIC-ggA-gAg-ATT-TgT-gTg-ggC-ATg-ACA-gg-TAMRA
Primer 1 Albumin	gCT-gTC-ATC-TCT-TgT-ggg-CTg-T
Primer 2 Albumin	ACT-CAT-ggg-AgC-TgC-Tgg-TTC
Probe E2 HPV16	VIC-CAC CCC gCC gCg ACC CAT A – TAMRA
Primer 1 E2 HPV16	AAC gAA gTA TCC TCT CCT gAA ATT ATT Ag
Primer 2 E2 HPV16	CCA Agg CgA Cgg CTT Tg
Probe E6 HPV16	6FAM- CAg gAg CgA CCC AgA AAg TTA CCA CAg TT-TAMRA
Primer 1 E6 HPV16	gAg AAC TgC AAT gTT TCA ggA CC
Primer 2 E6 HPV16	TgT ATA gTT gTT TgC AgC TCT gTg C
Probe E2 HPV18	VIC-TCA ACC CAC TTC TCg gTg CAg C-TAMRA
Primer 1 E2 HPV18	AgA AgC AgC ATT gTg gAC CT
Primer 2 E2 HPV18	ggT CgC TAT gTT TTC gCA AT
Probe E6 HPV18	6FAM-gCC ATT CgT gCT gCA ACC gA-TAMRA
Primer 1 E6 HPV18	TCA CAA CAT AgC Tgg gCA CT
Primer 2 E6 HPV18	CTT gTg TTT CTC TgC gTC gT

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tion at 50°C, followed by 10 min. denaturation at 95°C. Then 40 cycles of 95°C for 20 s and 65°C for 60 s were applied. To achieve a reliable standard curve, three replicates for each dilution point were prepared and standard curves were measured three times. Only samples giving positive albumin amplification were further analyzed for HPV detection.

HPV16 *E2* and *E6* genes were amplified and quantitated according to Si et al. [14] in a 25 μl amplification volume containing: 12.5 μl of Fast Universal PCR Master Mix (2 ×) (Applied Biosystems, USA), 100 nM of each primer, 300 nM of TaqMan probe and 2 μl of DNA template. Thermal cycling consisted of a step of 20 s at 95°C, followed by 45 cycles of 3 s at 95°C and 30 s at 60°C. To generate standard curves for *E2* and *E6* of HPV16, the serially diluted DNA plasmids (ATCC 45113), containing 5×10^4, 5×10^5, 5×10^6, 5×10^7, 5×10^8, 5×10^9 *E2* and *E6* genes copy number were included in each experiment (five times). Each time, three replicates for each dilution point were analyzed.

Amplification and quantitative measurements of HPV18 *E2* and *E6* genes were performed according to Damay et al. [9]. The assay was undertaken in a reaction volume of 25 μl containing: 12.5 μl of Fast Universal PCR Master Mix (2 ×) (Applied Biosystems, USA), 0.5 μM of each primer, 0.5 μM of TaqMan probe, 2 μl of DNA template to and 2.5 μl of H_2O. RT-PCR conditions were: 10 min. at 95°C, followed by 45 two-step cycles of 95°C for 15 s and 60°C for 45 s. Standard curve for quantification of HPV18 *E2* and *E6* genes was obtained on the basis of 10-fold serial dilutions of HPV18 plasmid (ATTC 45152), containing $7{,}225 \times 10^2$, $7{,}225 \times 10^3$, $7{,}225 \times 10^4$, $7{,}225 \times 10^5$, $7{,}225 \times 10^6$, $7{,}225 \times 10^7$ genes copy number. Three replicates were prepared for each dilution point and three sets were performed in order to check the reproducibility of standard curve measurement. The validity of RT-PCR applied to assess viral genome state was analyzed by the determination of the physical status of SiHa cell line which harbors pure integrated form of HPV16 genome.

***In situ* hybridization.** *In situ* hybridization for detection of HPV16 and 18 was performed on formalin fixed and paraffin-embedded sections, according to Evans et al. [17] with the modification proposed by Wiedorn et al. [18]. After deparaffinization and rehydration, the slides were placed into the TRS/pepsin (1:100) solution at RT and the solution was heated to 95°C in a water bath. After 20 min. of incubation at 95°C, the staining jar was removed from the water bath, allowed to cool at RT for 20 min. and then slides were rinsed three times in dH_2O. Endogenous peroxidase activity was quenched with 3% H_2O_2 in 70% methanol for 45 min. and the sections were washed in dH_2O for 10 min. One drop (approximately 15 μl) of the HPV types 16/18 biotynylated DNA probe (DAKO Corporation, Carpinteria, CA, USA) was applied to each individual slide and coverslipped with 22 × 22 mm cover glass. Denaturation of target and probe DNAs was conducted by heating on a hot plate (5 min. at 90°C). The slides were hybridized overnight in a humid chamber, at 37°C. The next day, coverslips were removed and the slides were rinsed in Stringent Wash Solution (DAKO Corporation, Carpinteria, CA, USA) for 20 min. at 55°C. GenPoint™ system (DAKO Corporation, Carpinteria, CA, USA) was used to detect hybridized DNAs. The sections were sequentially incubated with primary streptavidin-peroxidase (1:100 dilution) biotynyl-tyramide, and secondary streptavidin-peroxidase, for 30 min. each. The slides were washed in TBS-T buffer (0.05 M Tris-HCl, pH = 7.6, 0.1% Tween 20) for 5 min., three times between each incubation. Diaminobenzidine (DAB, at 1:50 dilution) was applied for 10 min. and then slides were counterstained with hematoxylin. Finally, the sections were dehydrated in ethanol, cleared in xylene and mounted. The sections were analyzed in light microscopy (at 400 × magnification). Tumor specimen with known positive HPV16/18 infections with mixed status of viral genome was added to each series of slides and treated as positive control. For negative control, TBS was substituted for the HPV16/18 probe. Validation of ISH was performed by adding tumor specimen with known positive HPV16/18 infections and mixed form of viral genome to each of ten series of staining.

Tumor cells with brown discrete dot-like product in the nuclei were interpreted as HPVs infected (Figure 1). The percentage of infected tumor cells was estimated and tumors with a value above 5% were assumed to be positive. For each sample, physical status of HPV infection was assessed according to Cooper et al. [19]: diffuse signal throughout the nucleus indicates episomal form, punctuated discrete dots inside nuclei represent viral integration, and the coexistence of diffuse and punctuated signals represents the mixed form (Figure 1).

Statistical analysis. All statistical procedures were performed two-sided and $p = 0.05$ was considered significant. Descriptive statistics were used to determine mean and median values of continuous variables and standard errors of means (SE). Standard curves were drawn using serial dilutions of known target gene copy numbers (x-axis) vs. corresponding Ct values (y-axis) and fitted by the least-square fit method. The *E2* and *E6* gene copy numbers were estimated by the following calculation: Y (gene copy number) = = ax + b, where: x = *E2* or *E6* Ct, and a and b are parameters of linear equations describing standard curves. Normalization of gene copy number was calculated as *E2* or *E6* gene copy number/(albumin gene copy number/2), because each diploid cell contains two copies of albumin. The distribution of categorized variables (presence of HPV infection, viral genome status) was assessed by the chi-square test. The agreement between HPV detection methods was evaluated by McNemar's chi-square test. Additionally, ISH-TSA sensitivity (i.e. the proportion of ISH-TSA positive samples

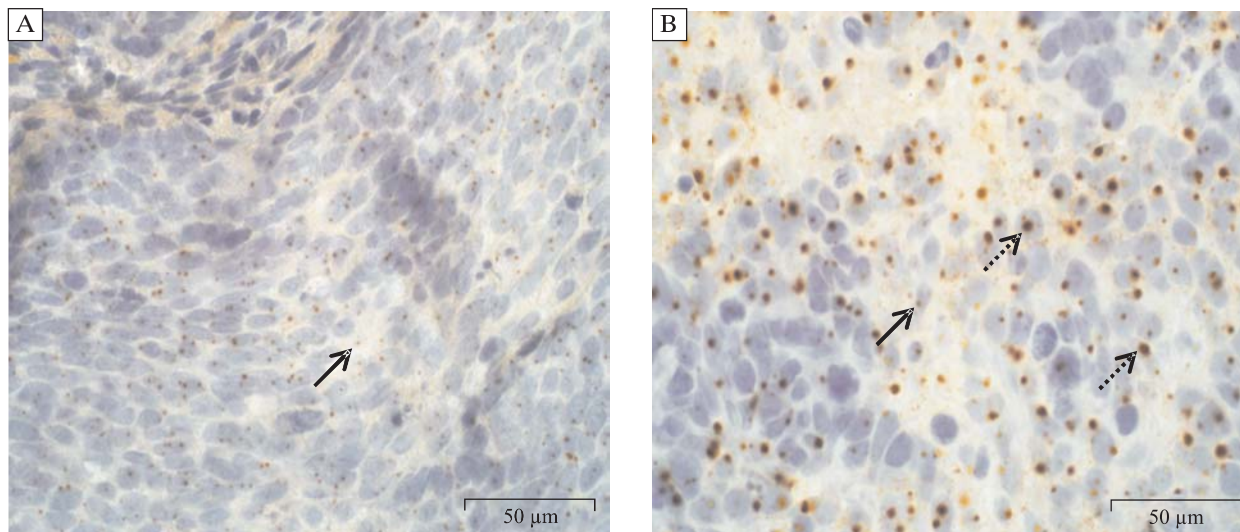

Figure 1. Detection of HPV DNA type 16 and 18 on archival cervical cancer specimens by hybridization *in situ* with tyramide signal amplification. **A.** Cervical cancer cells demonstrating integrated viral genome state (presence of punctuated signals — solid arrows). **B.** Cervical cancer cells demonstrating mixed form of viral genome state (both punctuated and diffuse signals present in the same tissue, presence of diffuse signal — dashed arrows); × 60,000 magnification

among which are RT-PCR positive samples) and specificity (i.e. the proportion of ISH-TSA negative samples among which are RT-PCR negative samples) were calculated [24]. The positive (the proportion of RT-PCR positive results, among which are ISH positive samples) and negative (the proportion of RT-PCR negative samples among which are ISH negative) predictive values were also analyzed [24]. All statistical analyses were carried out using Statistica v.8.0 program.

Results

All the women (n = 85) included in the study had histopathologically proven squamous cell carcinoma of the cervix. The mean patient age was 54.3 years ± ± 1.3 (SE) with median value of 52 years. In this group prevailed cancers with FIGO stages IIB and IIIB (n = 52, 61.2%) in grades G2 and G3 (n = 72, 84.7%). The patient and tumor characteristics are summarized in Table 1.

The quality of DNA extraction was assessed by RT-PCR using primers for albumin housekeeping gene. All cervical specimens were albumin positive and thus adequate for further analysis. Standard curves for albumin, HPV16 and HPV18 plasmids are presented in Figure 2. In all three cases, a linear relationship was found fitted by the least-square method.

Among 85 cervical cancers, RT-PCR analysis revealed single HPV16 infection in 48 cases (63.2%), single HPV18 infection in eight tumors (10.5%), and both HPV types infection in 20 samples (26.3%). For further analysis, tumors with single HPV infection (independent of HPV type) and those with infection of two virus types were considered as HPV16/18 positive (n = 76; 89.4%), whereas nine cancers were recognized as HPV16/18 negative (10.6%). For women after the menopause, a significantly higher percentage of cervical cancer without HPV16/18 infection (15.3%) than for premenopausal patients (4.7%) was found ($p = 0.015$) (Figure 3). A similar difference in HPV16/ /18 distribution among younger (age ≤ 50 years) and older patients (age > 50 years) was observed (data not shown). There were no correlations between HPV16/ /18 infection frequency and FIGO or grade.

From the 85 cervical cancer biopsies, ISH-TSA reaction for HPV16/18 was positive in 81 cancers (95.3%). There was no significant relation between HPV16/18 frequency assessed by ISH-TSA and the patient's menopausal status, age, FIGO or grade.

The results of HPV16/18 detection obtained by ISH-TSA were compared with the results of RT-PCR treated as reference method (Table 3). There were eight false positive and three false negative results in ISH-TSA as compared with RT-PCR results (Table 3). ISH-TSA was characterized by high sensitivity (96.1%), high positive predictive values (90.1%), low specificity (11.1%) and low negative predictive values (25.0%) in relation to RT-PCR. The difference between these two techniques in HPV16/18 presence detection was significant ($p = 0.000$) (Table 3).

RT-PCR was also used to assess physical genome state on the basis of *E2*/*E6* ratio. In all experiments,

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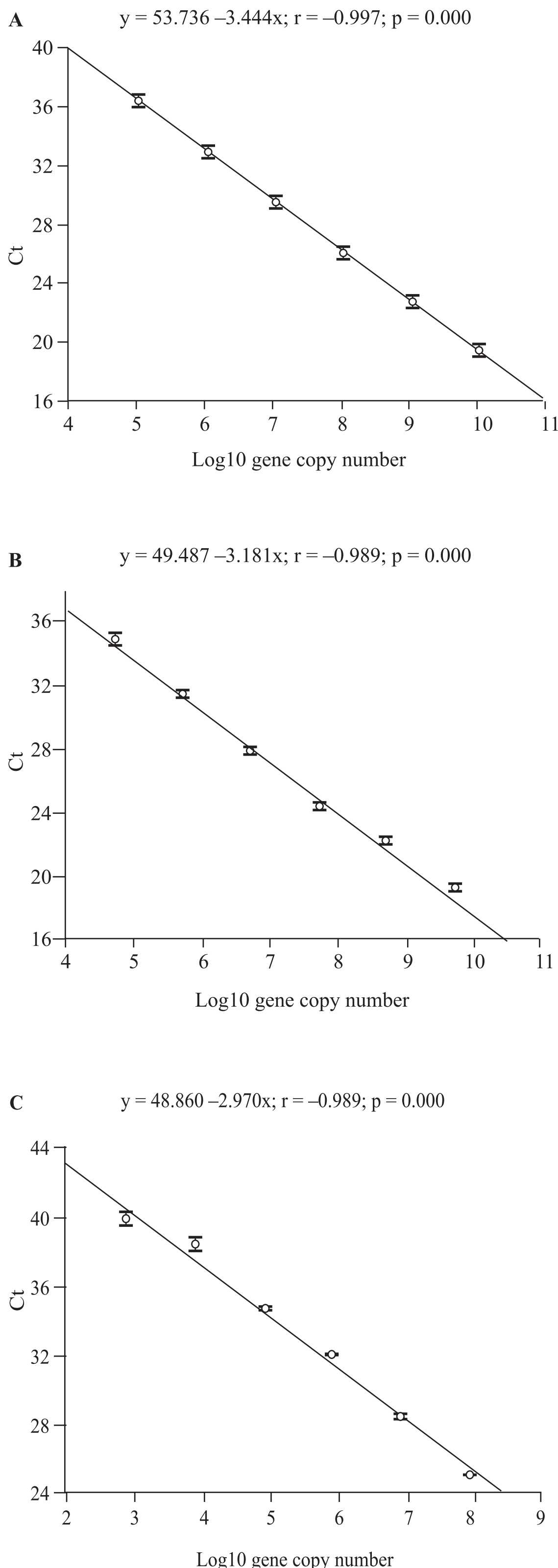

Figure 2. Standard curves for albumin (**A**), HPV16 (**B**) and HPV18 (**C**) plasmids obtained by TaqMan-based 5'exonuclease RT-PCR. Each point represents mean value ± SE. The linear equations were obtained on the basis of the least-square fit method

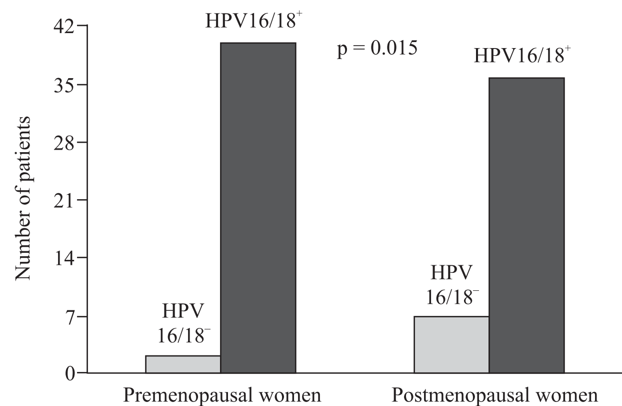

Figure 3. The frequency of HPV16 and 18 infection measured by TaqMan-based 5'exonuclease RT-PCR in relation to menopausal status of cervical cancer patients

SiHa DNA consistently showed *E6* amplification and a lack of *E2* amplification. In 76 cervical cancers, the *E2*/*E6* mean value was 0.916 and ranged from 0.701 to 0.983. Therefore, viral genome was regarded as integrated when the *E2*/*E6* was 0, as episomal when this ratio was 1 or more, and as mixed when *E2*/*E6* was between 0 and 1. Because all HPV18 positive tumors (independent of concurrent HPV16 infection) were characterized by integrated viral genome form for further analysis, we decided to combine the RT-PCR results of HPV16 and 18. Tumors with mixed HPV16 form were considered as those with HPV16/18 mixed infection, and cancers with integrated HPV16 genome as those with HPV16/18 integrated infection. Among 76 HPV16/18 positive cancers in RT-PCR, there were 30 (39.5%) with integrated and 46 (60.5%) with mixed infection. There were no samples with pure episomal form. The frequency of integrated or mixed infection types was similar in respect to patient age, FIGO and grade.

In the group of 76 HPV16/18 positive tumors, considering both RT-PCR and ISH-TSA, as assessed by ISH-TSA there were 39 (51.3%) samples with integrated and 37 (48.7%) with mixed viral genome form. In ISH-TSA, similarly to RT-PCR, no episomal type of HPV16/18 genome was detected. There were also no significant differences in the distribution of cancers with integrated or mixed genome forms between younger and older or pre- and postmenopasal woman and tumors with different FIGO or grade.

ISH-TSA results considering viral genome state were compared with reference to RT-PCR method. In respect to viral integration, ISH-TSA showed nine false positive results. The sensitivity and the specificity of this method in detection of integrated viral genome were 70.0% and 60.9%. Positive and negative predictive values were: 53.8% and 75.7%, respective-

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Table 3. Comparison of TaqMan based 5'exonuclease RT-PCR with type-specific primers (treated as reference method) and hybridization *in situ* with tyramide amplification system in HPV16/18 detection and viral genome status assessment

ISH for HPV16/18	RT-PCR for HPV16 and 18		Total	McNemar p value	Sensitivity[a]	Specificity[b]	Positive predictive value[c]	Negative predictive value[d]
	Positive	Negative						
HPV 16/18 detection								
Positive	73	8	81	0.000	96.1%	11.1%	90.1%	25.0%
Negative	3	1	4					
Total	76	9	85					
HPV16/18 genome status assessment								
Integrated	21	18	39	0.391	70.0%	60.9%	53.8%	75.7%
Mixed	9	28	37					
Total	30	46	76					

[a]The proportion of ISH-positive samples, among which are RT-PCR positive samples; [b]the proportion of ISH-negative samples, among which are RT-PCR negative samples; [c]the proportion of RT-PCR-positive samples, among which are ISH positive samples; [d] the proportion of RT-PCR-negative samples, among which are ISH negative samples

ly. The difference between these two tests in detection of HPV physical status was not statistically significant (p = 0.391) (Table 3).

Discussion

In the present study, in 85 archival cervical cancer biopsies we compared detection of HPV16 and 18 infection and viral genome state assessment between RT-PCR (based on TaqMan reaction and viral specific probes system) treated as reference method and ISH-TSA. Our study demonstrates a lack of correlation between RT-PCR and ISH-TSA in HPV16 and 18 detection. We found very low specificity of ISH-TSA (11.1%) in relation to RT-PCR, which means that the negative results obtained by ISH-TSA must be confirmed by other methods, like RT-PCR. However, we obtained a significant correlation between these two techniques in physical state of viral genome detection.

To the best of our knowledge, there is only one paper [21] in which authors have analyzed the sensitivity and specificity of HPV detection by ISH-TSA in relation to TaqMan PCR reaction with HPV16 and 18 specific probes. In contrast to our results, Birner et al. [21] in a group of 86 paraffin-embedded cervical cancer sections, obtained a significant correlation between RT-PCR and ISH-TSA in the assessment of viral presence. In this study, ISH-TSA was characterized by the sensitivity of 86.7% and specificity of 90.0% in relation to RT-PCR and gave no false positive results, whereas in our study ISH-TSA showed eight false positive results. These contrasting results might be related to differences in ISH-TSA methodology. Birner et al. [21] applied three 15 min. incubations in TSA and 20 min. of incubation with 0.3% H_2O_2. However, in the present paper, similarly to Wiedorn et al. [18], we decided to use three 30 min. incubations in TSA system and 45 min. of incubation with H_2O_2. As has been shown by some authors [18], the prolongation of the incubation period up to 30 min. is connected to an increase of TSA sensitivity. However, this procedure must be carefully established due to the possibility of artefacts, because even minimal amounts of the residual endogenous POD may give unspecific staining. Therefore, in this case, there is also the need to lengthen the time of quenching procedure with H_2O_2. TSA system may also give the risk of non-specific staining, because POD catalyzes the deposition of biotynyl tyramides only near, and not precisely at, the target localization [18]. Another reason for discrepancies in HPV detection in cervical biopsy specimens may be related to biopsy heterogeneity. Especially when samples contain low viral loads, sampling variation may lead to false negative results [14].

In the present study, we also analyzed the correlation in HPV16 and 18 genome state assessment between reference RT-PCR method and ISH-TSA. In RT-PCR, we assessed the detection of the integrated form based on the *E2/E6* ratio, in ISH-TSA by the type of HPV signal. A significant correlation between these two methods in respect of analysis of viral genome status was found. ISH-TSA detected integrated form of HPV16/18 with sensitivity of 70.0% and specificity of 60.9%, as compared with RT-PCR. Fujii et al. [22] for 53 women with cervical neoplasia or cervical cancer, compared frequency of HPV16 integrated form in exfoliated cervical cells between RT-PCR and ISH-TSA. They found a concordance rate of 86.0%. Nevertheless, there are still some controversies of viral genome status assessment consi-

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dering both methods. In RT-PCR, most authors, similarly to us, assessed viral genome status on the basis of *E2*/*E6* ratio, because the most frequently deleted or disrupted part of viral genome following HPV integration in the cellular genome is the *E2* 3'-terminal part [2]. However, it should be noted that viral integration could also lead to disruption of gene regions other than the *E2* [8]. It generates some risk of missing integration that disrupts only viral genome outside the *E2*. Other controversies regarding RT-PCR concern the *E2*/*E6* cut-off value used to determine the viral status. Theoretically, if the integration occurs within *E2,* the ratio ranges from 0 (completely integrated form) to 1 (episomal form only). Values between 0 and 1 reflect a mixed form. Some authors [8, 14, 15], similarly to us, defined an episomal form when *E2*/*E6* = 1 or more, whereas Fujii et al. [22] established the cut-off point on the basis on RT-PCR analysis of two plasmids solutions (prepared in increments of 20%), where one represents a pure episomal, and the other a pure integrated, form. They set the *E2*/*E6* cut-off point at level of 0.79 based on 99% confidence interval calculation. Saunier et al. [12] defined the *E2*/*E6* cut-off point at the level of 0.8, because they found that the variability in experimental *E2*/*E6* was more significant when E2/E6 was higher than 0.8. Cricca et al. [13] found that this ratio in plasmidic DNA solutions ranged from 0.93 to 1.08 and therefore used a cut-off value at the level of 0.93. However, Jiang et al. [10] studied different plasmid constructs, containing a DNA fragment from HPV16 European, Asian-American, African-1 and African-2 variant and found that the *E2*/*E7* ratio for the European variant construct was close to 1. Therefore, we decided to assume an *E2*/*E6* cut-off point at the level of 1.0. Another problem with viral status assessment is related to RT-PCR reaction conditions. Some authors [11, 25], in order to minimize the material and labor costs, assess physical state on the basis of multiplex reaction, where *E2* and *E6* are amplifying in the same tube. However, as indicated from preliminary results presented by Peitsaro et al. [15], the data obtained from multiplex reaction was less reliable than that obtained from separate reactions for *E2* and *E6*. On the basis of these results, we decided to perform RT-PCR separately for *E2* and *E6*.

An alternative technique of HPV genome detection is ISH-TSA. The greatest advantage of *in situ* hybridization is the possibility of microscopic examination of the studied tissues, because viral physical status may differ from cell to cell [14]. However, according to our and other authors' results [22], the sensitivity of ISH-TSA is poorer than that of RT-PCR. This difference may be related to the possibility that ISH-TSA misses some cells with low copies of episomal HPV. As known from previous studies, a high level of episomal forms can mask the presence of low level integrated HPV forms [19, 20]. Episomal DNA can also occur in the form of concatamers comprising multiple HPV copies, and it is possible that these forms could also give punctuated signals [18]. In turn, in RT-PCR, ~8 kilo-base single/low copy episomal sequences may be subject to relatively more degradation and loss from cell during routine processing than integrated DNA [18].

In conclusion, HPV detection with TaqMan RT-PCR system is more sensitive then ISH-TSA based on TSA system. ISH-TSA is characterized by a particularly high risk of false negative results, which means that each negative result obtained on the basis of this technique must be confirmed by other methods. This assay, however, could be applied to the assessment of viral genome status.

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