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ORIGINAL ARTICLE

IN SITU HYBRIDIZATION, WITH OR WITHOUT TYRAMIDE SIGNAL AMPLIFICATION, IN EVALUATION OF HUMAN PAPILLOMAVIRUS STATUS INEARLY STAGE CERVICAL CARCINOMA

Kubelka-Sabit KB*, Prodanova ILj, Zografski GD, Basheska NT

***Corresponding Author:** Katerina Kubelka-Sabit, M.D., M.Sci., Department of Histopathology and Clinical Cytology, Institute of Radiotherapy and Oncology, Medical Faculty, Vodnjanska 17, 1000 Skopje, Republic of Macedonia; Tel.: +389-2-3147-902; Fax: +389-2-3111-430 ; E-mail: catkubelka@yahoo.co.uk

ABSTRACT

Conventional *in situ* hybridization (CISH) can be used for detection of human papillomavirus (HPV) DNA, enabling preservation of the tissue morphology and assessment of the physical state of viral DNA, but has low sensitivity. This study compared the sensitivity and efficiency of *in situ* hybridization with tyramide signal amplification (ISH TSA) with those of CISH. The HPV status of 77 cases with early stage cervical carcinoma was evaluated with CISH, using biotinylated probes for HPV types 6/11, 16/18 and 31/33/51, and with ISH TSA using probes for HPV types 6/11, 16/18 and 31/33 or 31/33/51. The HPV DNA was detected in 26 (33.8%) cases using CISH, and in 45 (58.4%) cases using ISH TSA. By adding the TSA step, the sensitivity of CISH was enhanced by 24.7%, thus enabling detection of 20 new HPV-positive cases. Multiple HPV infections were detected in four cases. A dot signal pattern was present in 68.9% (31/45) and more than five positive nuclei per sample were found in 82.2% (37/45) of the cases. We found that the ISH TSA system is a fast and simple method for detection of HPV DNA in cervical carcinoma compared to CISH, and is more sensitive and efficient in the detection and typing of HPV, assessment of HPV DNA physical state and evaluation of the number of positive cells than CISH.

Key words: Cervical carcinoma; Human papillomavirus (HPV); *In situ* hybridization (ISH); Tyramide signal amplification (TSA).

INTRODUCTION

In the 1980s, zur Hausen [1] revealed that human papillomavirus (HPV) could be a possible etiologic agent in cervical carcinogenesis. The association of high-risk HPV types with malignant transformation of epithelial cells of the uterine cervix is now accepted [2-9]. Human papillomavirus type 16 is the most common high-risk HPV type [10-13], being detected in nearly half of all cervical carcinoma and cervical intraepithelial neoplasia (CIN) 2/3 lesions [14-16], and is followed by HPV type 18, which is detected in 14-25% of invasive cervical carcinomas [14,15]. Human papillomavirus types 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 are also included in the high-risk group [17]. Many molecular methods for the detection of HPV DNA have been developed, which are generally based on polymerase chain reaction (PRC) or *in situ* hybridization (ISH) [18]. Introduced by Gall and Pardue in 1969 [19], ISH detects nucleic acids, thus enabling evaluation

* Department of Histopathology and Clinical Cytology, Institute of Radiotherapy and Oncology, Medical Faculty, Skopje, Republic of Macedonia

of results in preserved tissue or cells. By increasing the cellular permeability, contact between probe and target nucleic sequence is enhanced, the morphology of cells or tissue is completely preserved [20-22], and approximative estimation of the number of copies of viral DNA in the host nucleus [21,23] and characterization of the state of the viral DNA, whether in episomal or integrated form [24], are permitted. Conventional *in situ* hybridization (CISH) also allows recognition of multiple HPV infection by detecting the concomitant presence of DNA from more than one type of HPV, but has relatively low sensitivity. With non isotopic indirect fluorescent *in situ* hybridization (FISH) on cytological material, the sensitivity of CISH is limited to the detection of 1 kb of target DNA [23]. In routine work on formalin-fixed tissue, the sensitivity is limited to the detection of 40 kb of target DNA and 10-20 copies of mRNA or viral DNA [6,21].

Many modifications to the protocol have been introduced to increase the sensitivity of the CISH. Some are based on amplification of the target nucleic acid sequence, while others are based on amplification of the hybridization signal [21,23,25,26]. *In situ* hybridization with tyramide signal amplification (ISH TSA) [21] uses biotinylated tyramide and its interaction with horseradish peroxidase (HRP). After an initial binding of streptavidin-peroxidase to the biotinylated probe, the peroxidase oxidizes the biotinyl tyramide, which then binds to nuclear proteins that contain aromatic amino acids, such as tyrosine, tryptophan or phenylalanine, thereby preventing diffusion of the amplification products [26]. We have compared CISH with ISH TSA in the evaluation of the HPV status on formalin-fixed paraffin-embedded tissue sections from early stage invasive cervical carcinoma.

MATERIALS AND METHODS

Materials. Seventy-seven cases of early stage invasive carcinoma of the uterine cervix were studied. The mean age of the patients was 41 (range 24-66) years. All neoplasms were limited to the uterine cervix, without involvement of the parametrial tissue or vaginal wall. In 64 cases (83.1%), the greatest diameter of the neoplasm was 4 cm or less (pT1b1 category), whereas in 13 cases (16.9%), it was greater than 4 cm (pT1b2 category). Regional lymph nodes were involved by the tumor in 22 cases (28.6%). Sixty-seven cases (87%) were squamous cell carcinomas, six cases (7.8%) had adenocarcinomas, and the remaining four cases (5.2%) had mixed carcinomas (adeno-squamous and mucoepidermoid carcinomas). The patients were surgically treated with radical hysterectomy and regional lymphadenectomy (Wertheim-Maigs) at the Clinic for Gynecology and Obstetrics or the Special Gynecology and Obstetrics Hospital, Skopje, Republic of Macedonia. and postoperatively received adjuvant radiation therapy at the Institute of Oncology and Radiotherapy in Skopje, Republic of Macedonia. The operative materials were subjected to uniform and detailed histopathological evaluation at the Department of Histopathology and Clinical Cytology at the Institute of Radiotherapy and Oncology in Skopje, Republic of Macedonia.

Methods. All materials were routinely processed using the protocol for formalin-fixed paraffin embedded tissues and the standard hematoxylin-eosin staining procedure. Other histochemical stains were used in some cases to confirm certain histological types of tumor.

The HPV status was evaluated by CISH and ISH TSA in all cases. Six 4 μ-thick sections were cut from the selected paraffin blocks containing a representative portion of the neoplasm. In some cases, mainly because of poor fixation, paraffin blocks from previous biopsy or diagnostic conization material were used. Commercially available (Enzo Diagnostics, Farmingdale, NY, USA) single and double well slides, especially designed for ISH, were used for the CISH. For the ISH TSA, poly-L-lysine pretreated slides on which the wells were circled using a pen, were used (DAKO, Glostrup, Denmark).

Conventional *In Situ* Hybridization. Detection and typing of HPV in tumor tissues was performed in two steps: pretreatment of tissue and hybridization and detection of HPV DNA. The procedure followed the instructions given in the commercially available *in situ* PathoGene® kit "Typing Assay for Human Papillomavirus" from Enzo Diagnostics, with minor modifications in the pretreatment and in the hybridization step. Mixed DNA probes for HPV types 6/11, 16/18 and 31/33/51 from Enzo Diagnostics were used.

Preparation and Pretreatment of Samples. Three 4 μ-thick sections were cut from each paraffin

block and applied on pretreated single and double well slides. The slides were then baked overnight in an oven at 60-80°C, to enable better adhesion of the tissue sections to the slides. Sections were then deparaffinated in xylol and hydrated at graded alcohol concentrations. In order to increase the permeability of cells to the probes, 0.35-0.5 mL of freshly prepared 1X Proteinase K (Enzo Diagnostics) was applied for 15 min. at 37°C, followed by washing in water and dehydration in graded alcohols.

Hybridization and Detection. One drop (~40 μL) of each biotinylated HPV DNA probe was applied to the matching well in each case. Slides were then transferred to a preheated heating block (slide warmer) at 95 ± 3°C for 8-10 min. For the hybridization step, sections were transferred to a slide warmer preheated at 37°C for 6 hours. After hybridization, slides were washed in SignaSure® buffer (Enzo Diagnostics) at a pH of ~8.0, followed by incubation in a hybridization reagent (buffered formamide) at 37°C. Afterwards, streptavidin-alkaline phosphatase (AP-detection reagent from Enzo Diagnostics) was applied. Finally, application of nitroblue tetrazolium (NBT) as a chromogen and bromochloroindolyl phosphate (BCIP) as a substrate (Enzo Diagnostics), enabled visualization of the whole complex as a dark purple precipitate in the nuclei of the infected cells.

For visualization of the cellular morphology, a counter stain with eosin was used. At the end of the procedure, the sections were dehydrated at graded alcohol concentrations and xylol, and were mounted with a permanent mounting medium (Enthelan; Merck, Darmstadt, Germany).

Controls. Two types of control slides were used: i) for the pretreatment, hybridization and detection steps, slides with formalin-fixed, paraffin-embedded tissue sections infected with HPV 6/11 were used; ii) slides containing commercially available cervical carcinoma cells (CaSki cells, Figure 1a) which have 400-600 copies of HPV type 16 integrated in at least 11 different places in the chromosomes [16].

***In Situ* Hybridization Tyramide Signal Amplification.** The necessary reagents were provided in the GenPoint kit from DAKO. Biotinylated HPV DNA probes for HPV types 6/11, 16/18 and 31/33 were used. For four cases, HPV DNA probes from Enzo Diagnostics were used. In two others, which were positive for HPV types 31/33/51 when using

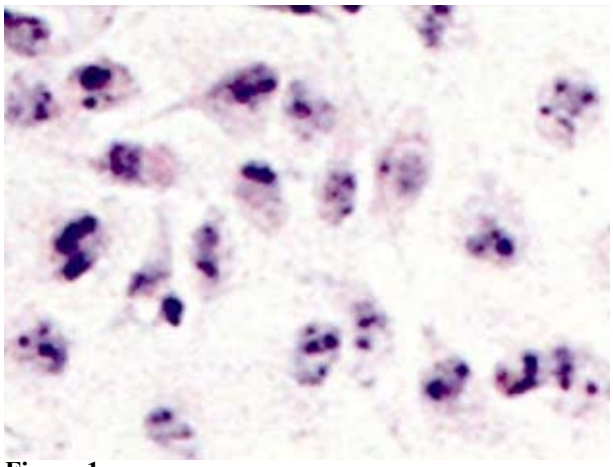


Figure 1a.

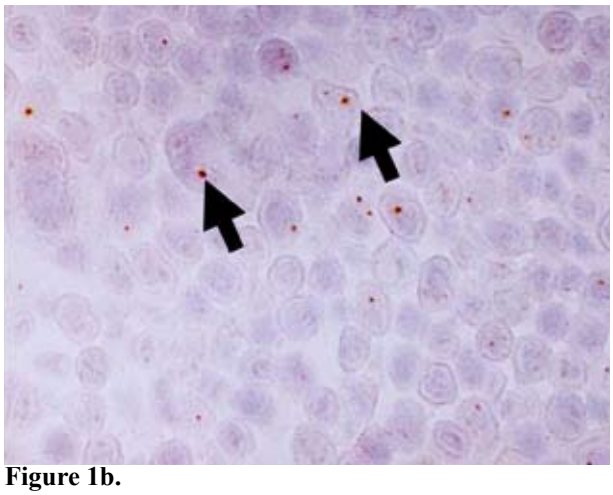


Figure 1b.

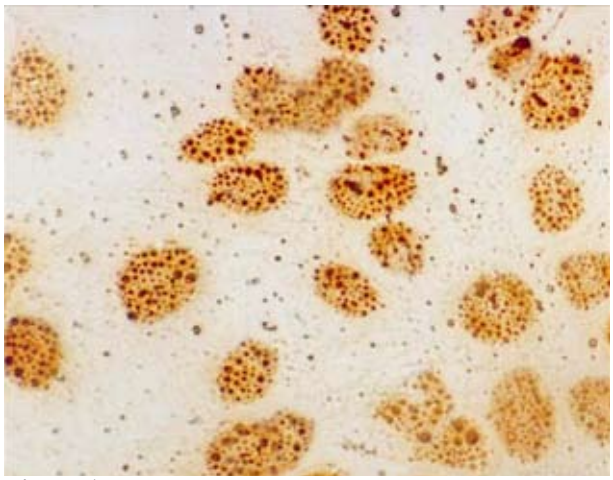


Figure 1c.

Figure 1. Positive control slides: CaSki cells containing 400-600 copies of HPV DNA type 16 (a, CISH, counter stained with eosin, 400X magnification); SiHa cells containing 1-2 copies of HPV DNA type 16 (b, ISH TSA, counter stained with hematoxylin, magnification, arrow); CaSki cells (c, ISH TSA, counter stained with hematoxylin, 400X magnification).

CISH but negative for HPV types 31/33 using ISH TSA, the latter procedure was repeated using the mixed probe for HPV types 31/33/51 from Enzo Diagnostics.

Preparation and Pretreatment of the Samples. Preparation of the slides was the same as for CISH, but with modifications in the pretreatment step. To increase the permeability of the cells for the probes, the slides were incubated in a demasking solution, commercially available from DAKO as a "target retrieval solution" (sodium citrate buffer, pH 6.0) in a water bath at 95°C for 50 min. They were then immersed in 0.01% pepsin solution for 10 min. to enable proteolytic digestion of the tissue. After washing in distilled water, the slides were incubated in 0.3% hydrogen peroxide in methanol for 30 min. to block the endogenous avidin-binding ability of the tissue.

Hybridization and Detection. Detection and typing of HPV was the same as for CISH, but with modifications. We introduced the signal amplification step in the post hybridization and detection step, with biotinylated tyramide as a reporter molecule. The detection step begins with application of conjugated primary streptavidin and HRP (DAKO). After the initial binding of streptavidin-peroxidase to the biotinylated probe, the peroxidase oxidizes the biotinyl tyramide, which immediately forms covalent bonds with aromatic amino acids of proteins in the specimen. The additional biotin is then used to capture more streptavidin-peroxidase. The amplified hybridization signal uses diaminobenzidine (DAB) as a chromogen, which when oxidized by HRP, turns into a black-brown precipitate in cell nuclei. A counter stain with hematoxylin was used for the visualization of the cellular morphology. At the end of the procedure, the slides were dehydrated and mounted with a permanent mounting medium (Enthelan; Merck).

Controls. Control slides were included in the kit, and contained SiHa cells infected with one or two copies of HPV type 16 (Figure 1b), integrated at chromosome 13q21-q31. A negative control probe (biotinylated plasmid DNA probe) was also used on the SiHa cells, to determine the specificity of the hybridization reaction. To visualize different types of hybridization signal, control slides containing CaSki cells (Figure 1c) from Enzo Diagnostics were also used.

Evaluation of the Human Papillomavirus Status. This included detection of HPV DNA, identification of the type of HPV, determination of the number of infected cells and the type of hybridization signal. The results were evaluated using light microscopy with 400X magnification. A result was considered to be positive if at least one cell contained a purple or brown precipitate in the nucleus. All positive cases were photodocumented. According to the number of positive cells in the tissue sample, cases were divided into groups that contained 1-5, 6-10, or more than 10 positive cells per sample. The cases were categorized into dot, mixed and diffuse types for evaluation of the type of hybridization signal as was initially recommended [27] and is widely accepted [22,24,28].

Statistical Analysis. We used McNemar's test for the analysis of correlated proportions [29].

RESULTS

Using CISH, HPV DNA was detected in 26 cases (33.8%). However, using ISH TSA, 20 more HPV-positive cases were detected (Table 1, Figure 2). In one case, the HPV was detected with CISH, but the result could not be confirmed with ISH TSA.

Human papillomavirus types 6/11 was detected in one case (3.9%) of verrucous invasive squamous cell carcinoma by both methods. Human papillomavirus types 16/18 was detected in 20 cases (76.9%) using CISH, whereas 13 new positive cases were revealed using ISH TSA. One case was positive for HPV 16/18 using CISH, but the presence of HPV was not confirmed using ISH TSA. Human papillomavirus types 31/33/51 was detected in five cases (19.2%) using CISH. The ISH TSA system detected five new cases of HPV types 31/33 and also confirmed the presence of HPV types 31/33 in three of the five initially positive cases. The remaining two cases were confirmed as positive only when they were retested with the probe for HPV types 31/33/51 (Table 1).

Introduction of the ISH TSA system enabled detection of multiple HPV infections in four cases (Figure 3), of whom two were positive for HPV types 16/18 and 31/33/ 51 and negative for HPV types 31/33, suggesting that probably one of the HPV types present was 51. In the other two cases,

Table 1. Comparison of two *in situ* hybridization methods in detection and typing of HPV, evaluation of the physical state of HPV DNA, and estimation of the number of positive cells.

	CISH	%	ISH TSA	%	*p* Value[a]
HPV presence					**<0.0001**
HPV negative	**51**	**66.2**	**32**	**41.6**	
HPV positive	**26**	**33.8**	**45**	**58.4**	
HPV type					**<0.00001**
6/11	**1**	**3.8**	**1**	**2.2**	
16/18	**20**[b]	**76.9**	**32**	**71.1**	
31/33 or 31/33/51	**5**	**19.2**	**8**	**17.8**	
Multiple infection	**0**	**0.0**	**4**[c]	**8.9**	
Type of signal					**<0.00001**
Dot	**12**	**46.2**	**31**	**68.9**	
Mixed	**2**	**7.7**	**12**	**26.7**	
Diffuse	**12**	**46.2**	**2**	**4.4**	
Number of positive cells					**<0.0001**
1-5	**7**	**26.9**	**8**	**17.8**	
6-10	**0**	**0.0**	**3**	**6.7**	
>10	19	73.1	34	75.6	

[a] McNemar's test.
[b] In one of the cases in which HPV type 16/18 was detected using a conventional hybridization method, the presence of the HPV DNA was not confirmed using ISH TSA.
[c] Concomitant infection with HPV types 16/18 and 31/33/51 was detected in two cases, whereas concomitant infection with HPV types 16/18 and 31/33 was detected in another two cases.

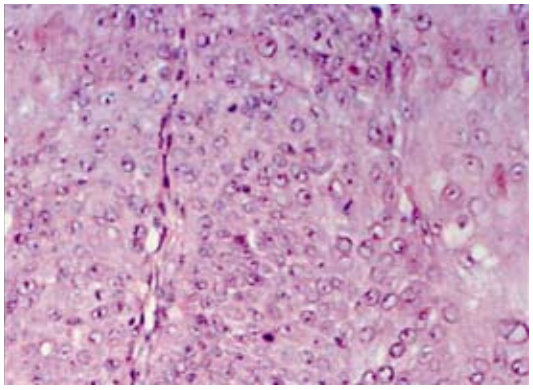


Figure 2a.

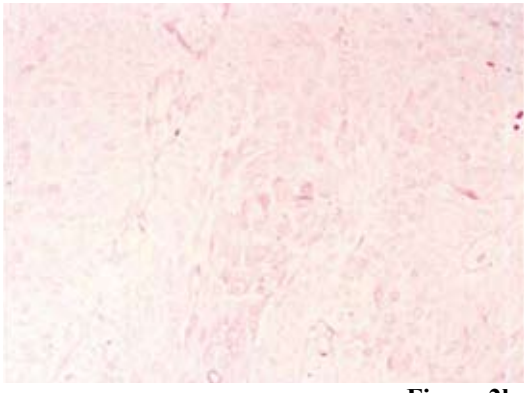


Figure 2b.

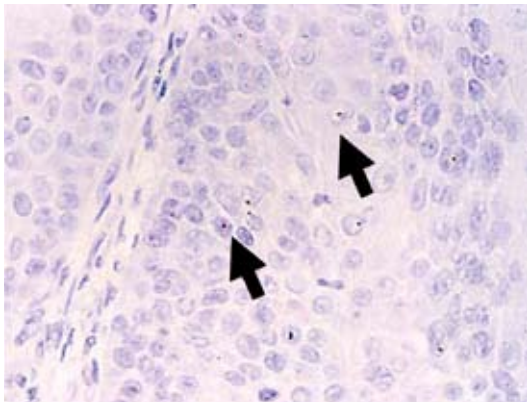


Figure 2c.

Figure 2. Squamous cell carcinoma (a, hematoxylin and eosin stain, 400X magnification) negative for HPV DNA using CISH (b, CISH, counter stained with eosin, 400X magnification), but positive for HPV DNA type 16/18 using ISH TSA (c, ISH TSA, counter stained with hematoxylin, 400X magnification, dot hybridization signal, arrow).

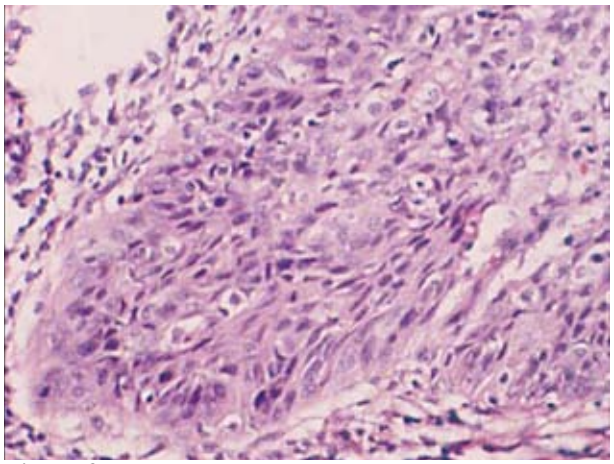


Figure 3a.

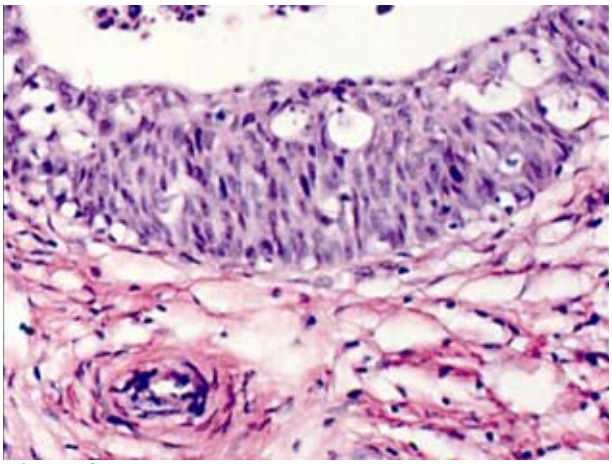
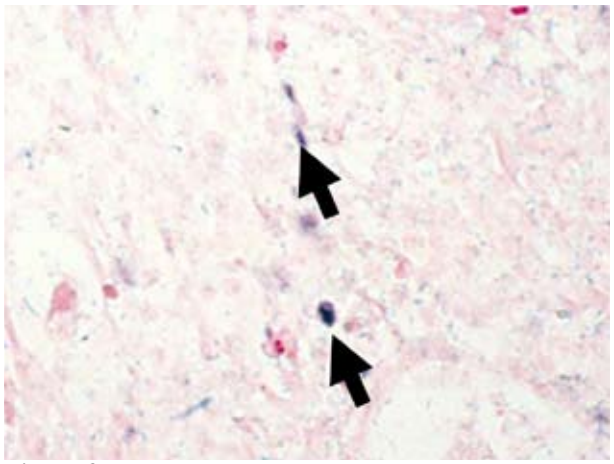


Figure 3d.



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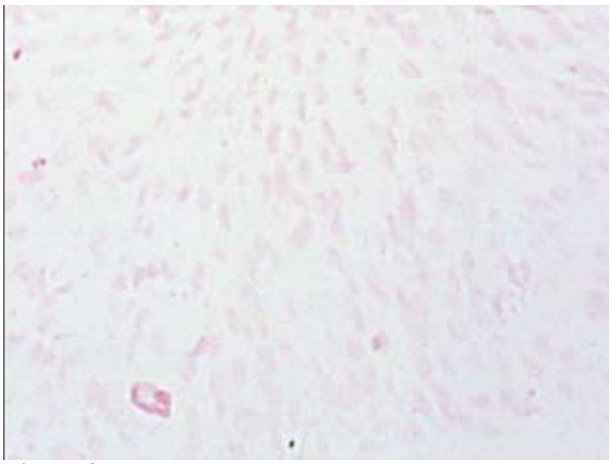

Figure 3e.

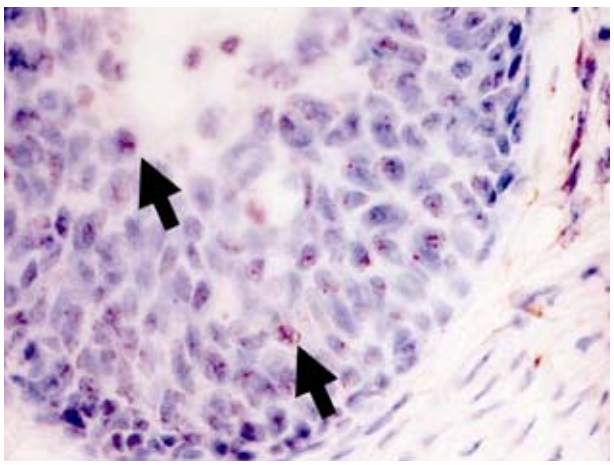

Figure 3c.

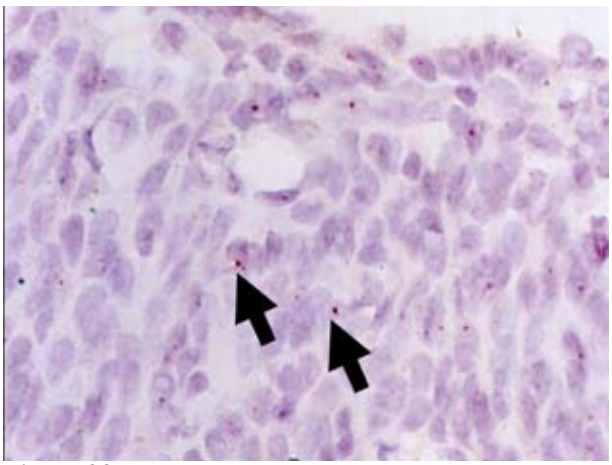

Figure 3f.

Figure 3. Squamous cell carcinoma (a and b, hematoxylin and eosin stain, 200X magnification) with double HPV infection, positive for HPV type 31/33/51 with CISH (c, CISH, counter stained with eosin, 400X magnification, arrow), and with ISH TSA (d, ISH TSA, counter stained with hematoxylin, 400X magnification), mixed hybridization signal, arrow). The tumor was negative for HPV type 16/18 using CISH (e, CISH, counter stained with eosin, 400X magnification), but positive for HPV DNA type 16/18 using ISH TSA (f, ISH TSA, counter stained with hematoxylin, 400X magnification, dot hybridization signal, arrow).

multiple HPV infections were detected using probes for HPV types 16/18 and 31/33 which had not been detected by CISH (Table 1).

The number of cases with a dot hybridization signal (one or a few copies of possibly integrated HPV DNA) was higher with ISH TSA than with CISH (31/45 or 68.9% *vs.* 12/26 or 46.2%). In five cases in which a diffuse hybridization signal was visualized with CISH (Figure 3c), more cells with a dot hybridization signal were visualized (mixed hybridization signal, Figure 3d) with the ISH TSA system (Table 1).

More than five positive cells per tissue sample were detected in 37/45 cases (82.2%, Figure 2c, Figure 3d and 3f) by ISH TSA, in comparison to 19/26 (73.1%) detected by CISH (Table 1). Furthermore, in five of the seven cases in which 1-5 positive cells per tissue sample were detected by CISH, more than five positive cells were detected by ISH TSA. In four of these cases, the number of positive cells increased to more than 10. However, in three other cases, the number of infected cells was higher with CISH than with ISH TSA. When the results on HPV status obtained by the two methods were compared using McNemar's test for correlated proportions, the differences for each of the parameters were statistically significant, in favor of the ISH TSA system (Table 1).

DISCUSSION

In situ hybridization is a valuable molecular method that permits evaluation of the results in the context of preserved cellular morphology. It was introduced and standardized in our department almost two decades ago. The working protocol is simple to perform and results are available in less than 24 hours. In comparison to other molecular methods for detection of HPV DNA, ISH has many advantages. In contrast to polymerase chain reaction (PCR), expensive equipment is not needed, and the risk of contamination of samples is avoided. The major disadvantage of CISH is its limited sensitivity. To detect the presence of one or a few HPV DNA copies in invasive carcinoma cells, we chose the method of catalyzed signal amplification ISH as the most appropriate.

Our study was performed on archival material (5-13 year-old formalin-fixed paraffin embedded tissue samples). Even though archival material could present problems with non specific staining, the age of the paraffin blocks did not significantly influence the results we obtained, as reported by others [25,30] before our study was made. In order to adapt the method of ISH TSA to the conditions in our department, some modifications in the working protocol were necessary. We achieved an optimal balance between intensity of signal and preservation of tissue morphology by prolonging the pretreatment time for 10 min. and increasing the temperature by 5°C, thus increasing the permeability of the cellular membrane with no effect on the tissue morphology. When using biotinylated probes, the non specific background staining, caused by the presence of endogenous streptavidin binding sites in the tissues, can be a serious problem [31]. In our study, the increased non specific background staining occurred only in small biopsies and mucin-producing neoplasms, but except in one case, did not significantly interfere with the specific staining.

With CISH, we detected HPV DNA in 33.7% cases of early stage invasive cervical carcinoma. However, using ISH TSA, the sensitivity of CISH was increased by 24.7%, and even multiple HPV infections were detected. Several studies have compared hybridization techniques for detection of HPV DNA [22,32-34]. For example, Lizard *et al.* [32] evaluated the catalyzed signal amplification system for the ISH using biotinylated DNA probes on CaSki, SiHa, and HeLa cells and found the GenPoint (DAKO) method to be superior to immuno-enzymatic procedures, easily allowing detection of 1-2 copies of HPV DNA in the isolated cells. Wiedorn *et al.* [33] compared the sensitivity of the CISH, ISH TSA and *in situ* PCR (direct and indirect) methods on 100 cervical biopsies with precursor lesions and concluded that ISH TSA has sensitivity similar to that of the *in situ* PCR method. In a similar study [34] that compared the sensitivity of CISH, ISH TSA and *in situ* PCR, on HeLa, CaSki cells and 30 biopsies from precursor cervical lesions, the sensitivity of ISH was increased by 6.6-13.3% in low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL), when the ISH TSA step was added to the protocol. The authors of that study hypothesized that, when detecting HPV DNA on archival material, ISH and conventional PCR methods are compatible and are

not mutually exclusive. Poddighe *et al.* [6] tested SiHa, HeLa, CaSki cells and 20 cervical biopsies containing normal epithelium, precursor lesions or invasive carcinomas for the presence of HPV DNA using the CISH, ISH with TSA and PCR. As in our study, these authors detected 20% improvement in sensitivity of the ISH with the TSA step.

Sano *et al.* [22] tested 28 cases of cervical carcinomas and 53 CIN lesions for the presence of HPV type 16, using the ISH TSA method. Eighteen of 28 carcinomas (64%) contained HPV DNA type 16. However, in our study, the percentage of HPV type 16/18 positive carcinomas was lower (46.8%), probably due to the differences in the case selection.

The presence of multiple types of HPV is not rare in cervical carcinomas. According to the literature, multiple HPV infections occur in 5-10% of invasive cervical carcinomas [35,36]. The most common types in multiple infections are HPV types 16/18 [35,36]. According to Bosch *et al.* [37], multiple HPV infection can be encountered in 20% of invasive cervical carcinomas, but only when PCR and ISH were used concomitantly. In our study, multiple HPV infections were detected in 8.9% of the cases. All the above mentioned studies suggest that ISH TSA is superior to CISH when it comes to detection of HPV in cervical lesions that contain only few copies of the viral DNA, such as invasive cervical carcinomas. As suggested by others [6,32-34], the added step of TSA should increase the sensitivity of conventional ISH by up to 25%.

Although we used a highly sensitive ISH method for detection of HPV DNA, we found no HPV in 40.3% of our cases. This may be explained by infection with types of HPV for which probes were not included, by sampling error, by loss of the HPV during the tumor progression, or because the tumor was not HPV-associated. Sampling error could explain the occurrence of the one case in which presence of HPV types 16/18 was detected with CISH but could not be confirmed with ISH TSA.

A major advantage of ISH is the possibility to estimate the physical state of HPV DNA. With ISH TSA, we detected 20 new HPV-positive cervical carcinomas that had dot hybridization signal (one or a few copies of possibly integrated HPV DNA). Similar results were reported in a comparison of the sensitivity of CISH and ISH TSA on SiHa, HeLa and CaSki cells, and 17 samples from genito-urinary tract, using GenPoint kit from DAKO [38]. Unger *et al.* [28] tested 47 cervical carcinomas for the presence of HPV DNA using CISH and PCR and found integrated HPV DNA in 77% of their cases.

In situ hybridization with TSA was also superior to CISH in detection of a higher number of positive cells per sample. Accordingly, detection of more than five positive cells per tissue sample was increased by 9% when the TSA step was added. Our results compare with those reported by others [20,25]. In three of the cases in our study, the number of infected cells was higher when CISH was used. This conflicting result was probably caused by a sampling error, namely, in the preparation step, tissue sections were cut in different levels, which presumably contained cells with a different number of viral DNA copies.

In conclusion, we found ISH TSA to be a fast and simple method for detection of HPV DNA in tissues containing only one or a few copies of HPV DNA, such as in early stage invasive cervical carcinoma. The addition of the TSA step increased significantly the sensitivity and the efficiency of the CISH in detection of the presence of HPV. The efficiency of ISH TSA was also higher in detection of the HPV type, number of positive cells and assessment of the physical state of HPV DNA.

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