Detection of high-risk HPV (16, 18, 33) in situ cancer of the cervix by PCR technique

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Summary

Objective: The purpose of this study was to collect data about the incidence of high-risk HPV (16, 18, 33) types in in situ cervical cancers, and to evaluate the reliability of the morphological signs of HPV infection by comparing the presence of these signs to the PCR-proven HPV virus infection.

Methods: Fifty patients who underwent conisation at the Department of Obstetrics and Gynecology of Semmelweis University, Budapest, Hungary because of in situ cervical cancer were examined retrospectively for the presence of HPV infection by the PCR technique. The direct and indirect morphological signs of HPV infection identified in the histological and cytological samples were compared to the actual results of virus DNA amplification by PCR in the identical histological sections. The evaluation of the cytological smears and the histological sections was accomplished independently by two different pathologists.

Results: E6 open reading frame of HPV 16, 18 or 33 was detected by PCR in 56% (28 cases) of the histological sections of the 50 examined patients with in situ cancer. In 92% (26 patients) of the 28 HPV positive patients one HPV type was detected, while in one of the remaining two cases two HPV types (16/ 33), or all three types could be detected. The direct morphological signs for HPV infection proved to be 75% sensitive and 50% specific when compared to the results of PCR. Their predictive value for HPV infection was 65%. For the indirect HPV signs the sensitivity was 64% and specificity 31%. The predictive value, prognosticating the presence of HPV 16, 18, 33 infection was 54% in the same sections. Using significance analysis no significant relationship ($p = 0.7728$) could be detected between the positivity of indirect signs and the presence of HPV 16, 18, 33 infection, while in case of direct signs the relationship was almost significant ($p = 0.0675$). The joint testing of the direct and indirect signs did not improve the results ($p = 0.1338$).

During the review of the cytological smears the specificity of the cytology in predicting true HPV infections was found to be 68% and sensitivity was 20%. The predictive value was only 50%. A significance analysis was not accomplished by this diagnostic method because of the missing data (see text).

Conclusion: The method of Nawa et al. seems to be a reliable approach for the detection of HPV DNA in paraffin-embedded material. The three main types of HPV (16, 18, 33) are probably represented in lower percentages in CIN III in Hungary, but a larger survey is needed to obtain reliable data. The direct and indirect morphological signs of HPV infection failed to show a significant relationship with the PCR proven presence of HPV 16, 18, 33.

Key words: Cervix; Colposcopy; Cytology; Histology; Genital infections; Mycoplasma; HPV; HSV; Adenovirus; Chlamydia trachomatis.

Introduction

There are 500,000 new cervix cancer patients every year worldwide, which means, that this cancer is still the second most frequent among gynecological cancers. In the USA despite the well-organized cervix cancer screening programs, there are still 15,000 new cases registered every year, 5000 of whom die of this disease. Scientists are focusing on more and more risk factors [1-5] as a result of decades of pragmatic work. Since the risk factors have become clear and the testing methods more sensitive and accurate [6, 7], it is unanimously accepted that certain subtypes of HPV represent a high risk for the development of cervix cancer [1, 2, 8-11]. However the causal and prognostic significance of HPV infections in preblastomatosis is not so clear-cut [10, 12-16]. Therefore within the scope of a retrospective study, we detected high-risk HPV types by PCR in histologically diagnosed CIN-III cases. Our goal was to gain experience about the detection of HPV DNA using paraffin-embedded sections, to gather data about the incidence of high-risk HPV types in 'in situ' cancers, and to evaluate the reliability of the morphological signs of HPV infection by comparing them to the PCR-proven presence of HPV types 16, 18, 33.

Materials and Methods

A retrospective assessment of the histological and cytological results of 50 patients was carried out at the 1st Obstetrics and Gynecological Department of Semmelweis University, Budapest, Hungary. These patients previously overcame cone biopsies because of colposcopic and/or cytological atypia, and the final histological evaluation of the removed specimens showed in situ cancer of the cervix. Ten μm sections of the paraffin-embedded specimens of these patients were used to detect HPV 16, 18, 33 types by PCR (*Semmelweis University, 1st Dept. of Pathol. and Exp. Cancer Res.*). Simultaneously the direct and indirect histological signs of HPV infection were examined in

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hematoxylin-eosin stained parallel sections. Direct signs of HPV infection are the presence of giant epithelial cells and koilocytes, while indirect signs are the presence of several nuclei, dyskeratosis, amphophil staining of the cytoplasm, nuclear net and hyperchromasia. The cytological smears, in addition to the Papanicolaou classification, have also been evaluated according to the Bethesda System. Direct and indirect signs identified in the histological and cytological samples were compared to the actual presence of the E6 gene by the PCR technique. The evaluation of cytological smears and histological sections was accomplished independently by two pathologists.

Fluid phase detection of HPV by nested PCR

The HPV 16, 18, 33 subtypes were detected in formalin-fixed, paraffin-embedded sections. To isolate the DNA, two pieces of 10 μm sections were deparaffinized in microcentrifuge tubes, then digested by 100 μg/ml proteinase-K in 200 μl final volume at 50°C for 24-48 hours. After heat inactivation of the enzyme, amplification was carried out of 1, 0.1 and 0. 01 μl extract by nested PCR as described by Nawa *et al.* [17].

First a pair of primers were used that amplify the E6 ORF sequence of all three subtypes. One μl of the amplified products was used for amplification with the type-specific primers (HPV 16, 18, 33). In order to confirm the specificity of the results gained by PCR, in situ hybridization was accomplished with probes amplified from HPV 16 and 18 cloned virus DNA. The hybridization pattern matched the PCR results in every case, thus the HPV 16 probe was perceptible only in those samples that contained HPV 16 subtype according to the PCR amplification as well (Figures 1a, b).

Southern blot

To confirm the specificity of the PCR reaction, the products were run on 2% agarose gel with ethidium bromide. The samples were blotted onto nylon enforced nitrocellulose membranes by using 0.4 M NaOH for the transfer. Subsequently the filter was neutralized, prehybridized overnight and hybridized with biotin-labeled oligonucleotide probes for 24 hours. Biotin was detected with streptavidine peroxidase, using diaminobenzidine as the chromogen.

In situ hybridization

Paraffin-embedded sections (3-4 mm thick) were placed on silane coated slides and deparaffinized. Samples were digested with 2.5 μg/ml proteinase-K for 30 min at 37°C, then the enzyme was inactivated by heat. To block endogenous alkaline phosphatase slides were treated with 20% acetic acid for one min. After several washings with PBS, slides were prehybridized with 25% formamide, 4x SSC (sodium salt citrate), 1mM EDTA, 50 mM NaH_2PO_4/Na_2HPO_4, 1 mg/ml yeast RNA, 5x Denhardt solution in water. After one hour of prehybridization the sections were denatured at 95°C for 6 min. At the same time the fluorescein labeled probe was denatured in the hybridization solution. Slides were put on ice and the prehybridization solution was quickly replaced by the probe-containing one. Hybridization was carried out overnight at 37°C. After three washings in PBS (phosphate buffer saline) the fluorescein was detected by antifluorescein alkaline phosphatase, using NBT (nitro blue tetrazolium) and BCIP (5-bromo-4chloro-3-indolyl phosphate) as the chromogen.

Statistical analysis

In order to determine the significance levels, the obtained results were submitted to mathematical analysis. Determination of the significance levels and comparison of the individual groups were carried out using the Pearson chi-square statistic ($\chi 2$ -probe), which is used to test for independence between the row and column variables. The efficacy of the method was evaluated by examining sensitivity, specificity and predictive values. For this statistical analysis the BMDP package [18] was used.

Results

HPV 16, 18 and 33 were detected by the PCR technique in 56% (28 cases) of the histological sections of the 50 examined patients with in situ cancer. In 92% (26 patients) of the 28 HPV-positive patients one HPV type was detected, while in one of the remaining two patients two HPV types were found (16/33), and in the others all three types could be detected.

Figure1a. — In situ hybridization control of fluid-phase PCR. Invasive carcinoma specimen harboring a HPV 16 E6 DNA sequence according to the PCR reaction was hybridized with a 150 bp E6 probe amplifided from cloned HPV 16 genome.
In situ hybridization, indicating episomal representation of the virus. Original magnification x 400.

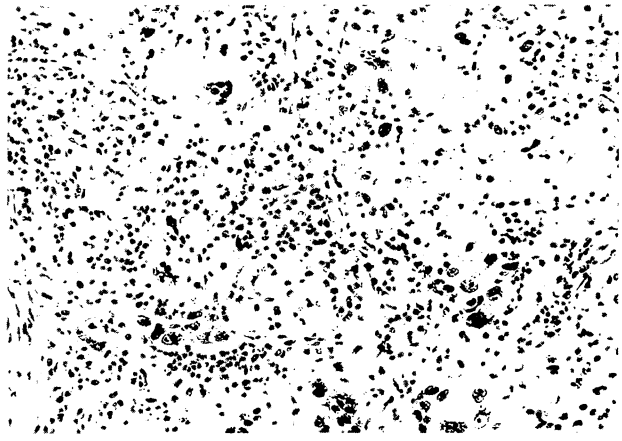

Figure 1b. — Histological features of the same tumor with conventional hematoxylin-eosin staining. Histological features of invasive cervical cancer with conventional H-E staining. Small nests of anaplastic tumor cells surrounded by abundant connective tissue with lymphocytic infiltration. Original magnification x 400.

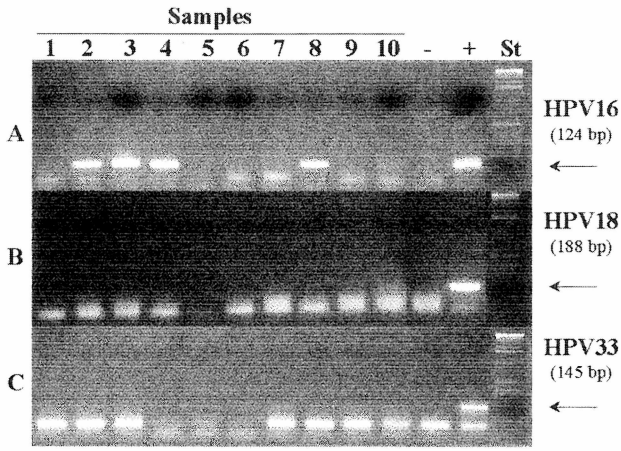

Figure 2. — Detection of HPV 16 by nested polymerase chain reaction from CIN III lesions. Ten representative samples from the investigated specimens showing HPV 16 positivity in four cases. All samples were negative for HPV 18 and 33. DNA from paraffin-embedded specimens was amplified (described in Material and Methods) and run on 2% agarose gel.

Review of the histological sections of the 50 examined patients revealed direct HPV signs in 32 cases (64%) and indirect HPV signs in 33 patients (66%). The revision of the cytological smears could be carried out only in 41 cases because the cytological examination in the missing nine cases was performed in other institutes; therefore the original smears were not at our disposal. Thirty (73%) of the 41 attainable smears showed signs of HPV infection.

Among those patients where the direct HPV signs proved positive during the review of their histological section (32/50), some subtype of HPV could be detected in 21 cases (65.6%) (true HPV positivity, TP). However in 11 cases (34.4%) the presence of HPV could not be proved (false HPV positivity, FP). During the review of the histological sections no direct signs were recognized in 18 patients (18/50). The HPV detection carried out among these patients showed some type of HPV in seven cases (39%) (false HPV negative, FN), while HPV could not be detected in the histological sections of the remaining 11 (61%) patients (true HPV negative, TN). Based on the previous data the mathematical analysis gave the following results regarding direct HPV signs of the histological sections: sensitivity 75%, specificity 50%, and predictive value for HPV 16, 18, 33 infection was 65% (Table 1). The sensitivity was 64% and specificity was 31% for the indirect HPV signs, while the predictive value, prognosticating the presence of HPV 16, 18, 33 infection was 54% in the same sections. Using significance analysis no significant relationship ($p = 0.7728$) could be detected between the positivity of indirect signs and the presence of HPV 16, 18, 33 infection, while in cases of direct signs the relationship was significant ($p = 0.0675$). The joint testing of the direct and indirect signs did not improve the results ($p = 0.1338$).

During a similar review of cytological smears TP was 36% (15 cases), TN was 9% (4 cases), FN was 17% (7 cases), and FP was 36% (15 cases). The specificity of the cytology in predicting true HPV infections was found to be 68% while sensitivity was 20%. The predictive value was only 50%. A significance analysis was not carried out by this diagnostic method because of the missing data (9 cases).

Table 1. — *Relation between direct and indirect histological signs and HPV 16, 18, 33 positivity proven by PCR.*

No. of cases	Total	Direct histological signs of HPV		Indirect histological signs of HPV	
	50	+	–	+	–
		32	18	33	17
HPV 16, 18, 33 positive by PCR	28/50 56%	21/32 65.6% (TP)	7/18 39% (FN)	18/33 54.5% (TP)	10/17 58.8% (FN)
HPV 16, 18, 33 negative by PCR	22/50 44%	11/32 34.4% (FP)	11/18 61% (TN)	15/33 45.5% (FP)	7/17 41.2% (TN)

Discussion

HPV DNA can be detected in almost every invasive cervix cancer- verifiability close to 100% depending on the sensitivity of the applied method [8]. The mechanism of the development of cervical cancers has not yet been completely elucidated, and the factors that lead from latent infections of high prevalence which show regression in the majority of the cases, through preblastomatosis to infiltrative cancer are not known in detail [8, 19, 20, 21]. Relatively little data is at our disposal on the connection and prognostic significance of CIN III – the stage that directly precedes invasive processes – and certain types of HPV [1-5]. Trying to find answers to these questions we detected the presence of the most common HPV types (16, 18, 33) of cervix cancer in 50 cases where the histological diagnosis was CIN III. The traceability of our material was 56%. The incidence, which is less than expected, could reflect the real situation thus indicating that the examined types occurred relatively more seldom in our material, and the negative cases regarding these types would have shown regression later. The low incidence could be explained by the fact that detection was carried out in formalin-fixed, paraffin-embedded samples. Nevertheless regarding the given samples our method can be evaluated as correct, since Nawa *et al.* – who described the method we applied [17] – proved the presence of HPV 16, 18, 33 in only 66.7% of the paraffin-embedded sections of cervix cancer, which proves a good correlation and the reliability of our method. The couple of percent difference can be explained by the fact that in preblastomatosis many more types of HPV [15, 21, 22, 23] – not rarely low risk types – can be present than in infiltrative cancers. Sawaya and coworkers [25] detected the three types of HPV examined by us as well albeit more frequently (75%) – but they applied a different method. Lombard *et al.* [3] detected a rate of 83% in cases of cervix cancer by examining frozen tumor samples. However it should be mentioned

that Tsang and co-workers [16], applying their own method, detected HPV types 16 and 18 in almost 79% of the formalin-fixed, paraffin-embedded samples in cases of extended cervix cancer. In the Czech female population – which is similar to ours – Tacheczy *et al.* [26] verified the presence of HPV in 58% of the cytological samples in high-grade squamous intraepithelial lesions.

The low incidence of HPV type 18 in our samples is remarkable. This type could be detected in only one mixed infection. This result is surprising even despite the small number of our examined samples, since a study by others, supposedly with a similar population, proved a much higher prevalence of HPV 18 [26]. Verification of the presence of the virus in the case of one mixed infection contradicts the possibility of a methodical error. The reliability of the detection of the subtypes is indicated by the fact that using in situ hybridization only the subtype specified by PCR gave hybridization signs and no cross-hybridization was experienced.

Our results lead to the conclusion that the method described by Nawa *et al.* for the detection of HPV types 16,18, 33 is reliable in cases of preblastomatosis as well as to a similar extent in cases of invasive cancer. The examined HPV types occurred with a lower incidence in the CIN III cases than in invasive processes. No trustworthy conclusions can be drawn from the observed low incidence of HPV 18 in our small number of cases.

Verification of the presence of HPV and comparison of the morphological signs indicating viral infection is always an exciting question, despite the fact that the morphological signs only propose but do not prove the presence of viral infection. In our study the connection between direct and indirect signs indicating the presence of the virus and the fact or lack of proven HPV 16, 18, 33 infection gave small sensitivity, specificity and low positive predictive value, which can query the value of the morphological marks. The statistical evaluation that more than 20 members of the anogenital-HPV group could induce the morphological marks mentioned above has to be taken into consideration. Direct and indirect signs of HPV infection (eg. the association of low and high risk types), and the method applied in this study were restricted to the detection of only three types – as emphasized before.

The well-known fact mentioned before can serve as an explanation for the statistical results – that a wider scale of HPV types can be detected in preblastomatosis than in invasive processes – thus in the false positive and false negative cases the types not examined could be responsible for the presence or lack of morphological signs. The lack of morphological marks naturally does not exclude the presence of viral DNA (false negative cases). Since in the histological sections of high degree squamous intraepithelial lesions and in CIN III cases the proliferating basal type cells were the results of malign transformation and not of productive HPV infection, it can not be expected that the well-known direct and indirect morphological marks should develop in the transformed cells.

The registration of morphological marks that render HPV infection probable is indispensable since they can indicate the presence of productive viral infection or the possibility of infection. Thus typing of the virus can be helpful in the further control of the patient and in the decision making about the therapy.

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