Detection of HPV-DNA within cells of tissue sections from uterine cervix – a comparison of in-situ PCR and conventional PCR

Nachweis von intrazellulärer HPV-DNA in Gewebsschnitten der Cervix uteri – Vergleich von In-situ PCR und konventioneller PCR

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Summary

Polymerase chain reaction allows the detection of DNA of human papillomaviruses, a main risk factor for cervical intraepithelial neoplasia. The combination of PCR-mediated amplification of viral DNA inside cells with subsequent in-situ-detection seems to be an attractive application for localisation of infected cells in a tissue section. This method is described and compared with conventional PCR.

We found corresponding results in 16 of 17 cases (14 positive samples with both methods, 2 negative with both methods, 1 positive in the conventional PCR but the sample detached by the in-situ PCR). Our study allows the following conclusions:

The conventional PCR is of advantage for detection and as known from previous studies for typing of HPV infections. The in-situ PCR is the preferable approach for the localisation of viral DNA infected cells in a tissue section. This technique can support a histological statement referring to the expansion of a viral infection too. The in-situ PCR permits a distinction between viral-DNA-infected and -noninfected cells. So the method could be helpful for histological as well as cytological evaluation as part of cancer prevention programs.

Key words

HPV-DNA – uterine cervix – in-situ PCR

Zusammenfassung

Die Polymerase Chain Reaction ermöglicht den Nachweis von DNA humaner Papillomviren. Diese Gruppe von Viren wird als einer der Hauptrisikofaktoren für die Entstehung der Cervikalen Intraepithelialen Neoplasie betrachtet. Die Verknüpfung von PCR-vermittelter Amplifikation viraler DNA-Sequenzen innerhalb von Zellen mit nachfolgender In-situ-Detektion stellt eine attraktive Anwendung zur Lokalisierung infizierter Zellen in Gewebsschnitten dar. Diese Methode wird beschrieben und mit der konventionellen PCR verglichen.

Wir fanden übereinstimmende Ergebnisse in 16 von 17 Fällen (14 Fälle positiv mit beiden Methoden, 2 Fälle negativ mit beiden Methoden, in einem Fall eine positive konventionelle PCR, aber die In-situ PCR war nicht auswertbar). Unsere Untersuchungen gestatten folgende Schlußfolgerungen: Die konventionelle PCR ist für den Nachweis und für die Typisierung von humanen Papillomviren von Vorteil. Die In-situ PCR ist für die Lokalisation viraler DNA in infizierten Zellen von Gewebsschnitten geeignet. Diese Methode kann die histologische Beurteilung hinsichtlich der Ausdehnung der viralen Infektion unterstützen. Die In-situ PCR ermöglicht eine Differenzierung zwischen infizierten und nichtinfizierten Zellen. Sie kann sowohl für histologische als auch zytologische Untersuchungen im Rahmen von Krebsvorsorgeprogrammen Anwendung finden.

Schlüsselwörter

HPV-DNA – Cervix uteri – In-situ PCR

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Introduction

Several factors that increase the risk of cervical cancer are cigarette smoking, use of oral contraceptives and various aspects of sexual behaviour [1]. Furthermore, sexually transmitted infectious agents seem to be from etiological importance. So human papillomaviruses prove to be one of the main risk factors in the pathogenesis of cervical cancer [2, 3]. Conventional PCR permits the direct detection of viral DNA. This method possesses a very high sensitivity. It allows a high security in diagnosis. In-situ-hybridisation has the advantage of detecting the viral DNA intracellularly. However, it owns a lower sensitivity, especially in cases with low abundant target DNA.

The combination of PCR-mediated amplification of viral DNA inside cells with subsequent In-situ-detection could combine the benefit of both [4]. This so called in-situ PCR permits the type specific localisation of viral infections. The distribution of the infection in the tissue section is of interest in respect with further therapy.

The objective of the study was to compare conventional PCR and in-situ PCR on tissue sections from the uterine cervix.

Materials and Methods

Tissue sections

Seventeen tissue sections from the uterine cervix obtained by conization were investigated. The criterion for conization was a pathological result (two times PAP III, one time PAP IV) in a previous cytological investigation. The tissue was fixed in phosphate-buffered formalin (10%) for 16 hours. Then it was embedded in paraffin and divided into 5 µm sections. The sections were mounted on aminoalkylsilane-treated slides and baked for 12 hours at 65 °C. They were stored at room temperature for about 4 weeks.

Pre-treatment

The sections were dewaxed with xylol for 10 min and rehydrated by a graded ethanol series (99%, 1 min; 96%, 1 min; 80%, 1 min; 70%, 1 min; 60% 1 min; H_2O, 2 min).

Abkürzungen:

CIN = cervical intraepithelial neoplasia
HPV = human papillomavirus
PCR = polymerase chain reaction

One of the immediate adjacent sections was mechanically detached from the slide and placed in a microtube for further handling using conventional PCR. After addition of 500 µl of a non-ionic detergent buffer (Tris-HCl 10 mM, ph 8.3; KCl 50 mM; $MgCl_2$ 2.5 mM; Gelatine 0.001%; NP 40 0.45%; Tween20 0.45%) and 5 µl Proteinase K (10 mg/ml in Tris-HCl 50 mM, ph 8.0; EDTA 10 mM; NaCl 10 mM) the mixture was incubated for 2 hours at 56 °C. Then the enzyme was denatured at 95 °C for 10 min. The section for the in-situ PCR was treated with 100 µg/ml Proteinase K in Tris-HCl (50 mM, ph 8.0; EDTA 10 mM; NaCL 10 mM) for 10 min at room temperature with following degradation of the enzyme at 95 °C for 5 min.

Oligomers

Oligonucleotides were synthesised on a Gene Assembler from Pharmacia LKB. The consensus primers from the L1-region of the virus genome contained wobble-bases [5]. They were complementary to HPV 6-, 11-, 16-, 18- and 33-sequences.

sense-primer : 5' – G C M C A G G G W C A T A A Y A A T G G

antisense-primer: 5' – C G T C C M A R R C G A W A C T G A T C

M ... A and C
R ... A and G
W ... A and T
Y ... C and T

Conventional PCR

The conventional PCR was assayed according to our recently published method [6]. In brief, the PCR-mix consisted of 7.5 µl H_2O, 2.5 µl 10 X PCR-buffer (Tris-HCl 10 mM, pH 8.3; KCl 50 mM; Gelatine 0.001%; $MgCl_2$ 1.5 mM), 2.5 µl dNTP-mix (200 µM each), 1.25 µl of each primer (0.25 µM), 10 µl of the prepared DNA-solution and 1 unit Taq-polymerase. As negative control we used a tube with PCR-mix and 10 µl H_2O instead of the DNA-solution. The Hot-Start-PCR was performed as described by Bassam and Caetano-Anolles [7]. The amplification proceeded in 35 cycles of 30 sec at 94 °C, 45 sec at 53 °C and 1 min at 72 °C on a Thermocycler 9600 from Perkin Elmer, Applied Biosystems GmbH. 7 µl of the amplificated solution was mixed with 3 µl loading buffer (Xylencyanol 5%; Glycerol 5% in H_2O) and run on an agarose-gel (2%). The fragments were differentiated in comparison to a molecular size marker.

In-situ PCR

Amplification

The PCR-mix consisted of 11 µl H_2O, 2.5 µl 10 X PCR-buffer (Tris-HCl 10 mM, pH 8.3; KCl 50 mM; Gelatine 0.001 %), 4.5 µl $MgCl_2$ (4 mM), 4 µl dNTP-mix (200 µM each, dig-dUTP/dTTP = 1/20), 1 µl of each primer (2 µM). 19 µl of this mix were applied to the slide and covered with a coverslip. The remaining 5 µl of the mix were kept on ice. While the slide was heated at the block of the thermocycler 1 unit of Taq-polymerase was added to the tube with the PCR-mix. When the temperature at the block reached 62 °C the Taq-polymerase consisting mix was pipetted under the coverslip. Then the slide was overlaid with preheated mineral oil to prevent evaporation. The amplification proceeded in 35 cycles of 1 min at 94 °C and 2 min at 53 °C on a Thermocycler 9600 from Perkin Elmer, Applied Biosystems GmbH. As negative control we used a PCR-mix without Taq-Polymerase on an adjacent section. After amplification the coverslip was detached and the mineral oil was removed by a 1 min-treatment with xylol. The following rehydratation occured with a graded ethanol series as described above.

Detection

The detection procedure was performed according to the original protocol for the Digoxigenin-system from Boehringer Mannheim. It was started with a bovine serum albumin (2%, in H_2O) treatment at 45 °C for 15 min. Then the slide was covered with blocking-Reagent (1%, in maleic acid 0.1 M, NaCl 0.15 M, pH 7.5). After 30 min the solution was removed and the anti-DIG-Alkaline Phosphatase (AP)-Conjugate (1:500, in Blocking-Reagent 1%, maleic acid 0.1 M, NaCl 0.15 M, pH 7.5) was pipetted on the slide. After 30 min the slide was washed in Maleic-Acid-Buffer (maleic acid 0.1 M, NaCl 0.15 M, pH 7.5) twice and then once in Reagent-Buffer (Tris-HCl 0.1 M, NaCl 0.1 M, $MgCl_2$ 0.05 M, pH 9.5). The colour reaction was performed by distribution of 30 µl Nitroblue-Tetrazolium-Solution (NBT) and 20 µl 5-Bromo-4-Chloro-3-Indolyl-Phosphate (X-Phosphate) in 1 ml Reagent-Buffer for 15 min at 37 °C in the dark. Nuclear fast red was used as counterstaining. The results were evaluated with a light microscope using 200 to 400 times magnification.

Results

In 14 cases we detected human papillomavirus DNA with conventional PCR (results not shown, see ref. 6) as well as In-situ PCR. Figure 1 shows an epithelial part of a tissue section with multiple positive signals. The deep blue or black colour of the nucleus indicates the presence of amplified base sequences inside the cells. Diffusion of PCR products and non-specific sticking onto the cellmembrane leads to the staining of the membrane of the infected cells. Areas of noninfected cells can be clearly differentiated in this tissue section.

No specific signals could be found in 2 cases, neither by the in-situ-procedure nor by conventional PCR. Figure 2 displays a part of a HPV-DNA-negative tissue section. The poor infrequent signals in the subepithelial region have to be discussed critically. The signals were found mostly attached at membranes, and in few cases at the nucleus. These signals have to be considered as unspecific amplified DNA also because they were not found to be accumulated in a certain area of the tissue section.

We found corresponding results in 16 of 17 cases. In the remaining one case a main part of the tissue section was detached from the slide. So we can not judge the result without doubt.

Discussion

The incidence of cervical cancer varies in different population groups. They range from 10 to 33 per 100.000 women in Europe [7].

Cervical squamous intraepithelial lesions as preliminary stage of cervical cancer usually contain human papillomavirus DNA [8]. Normal cervical epithelium transfected by human papillomavirus DNA develops the histological changes characteristic of a squamous intraepithelial lesion [9]. So it appears to be proved that an infection by human papillomaviruses is an essential event in the development of squamous intraepithelial lesions. Nuovo et al. detected human papillomavirus DNA in 42% of cases in spite of a negative colposcopic examination for cervical squamous intraepithelial lesions [8]. The detection of human papillomavirus infections is of importance in the cancer prevention strategy. In that context the PCR is very helpful.

Komminoth and Long [11] have critical reviewed the in-situ PCR and have shown the problems, the pitfalls but also the importance of this method. In this study we have performed the in-situ PCR in parallel with the conventional PCR of the identical tissue sections. The conventional PCR was used as the control and standard for the in-situ PCR. Furthermore, the direct detection of in-situ PCR amplified DNA has some disadvantages especially if archival, formaldehyde-fixed, paraffin-embedded materials were analysed. We investigated only fresh prepared tissue sections. But also under these circumstances unspecific signals could not be avoided comple-

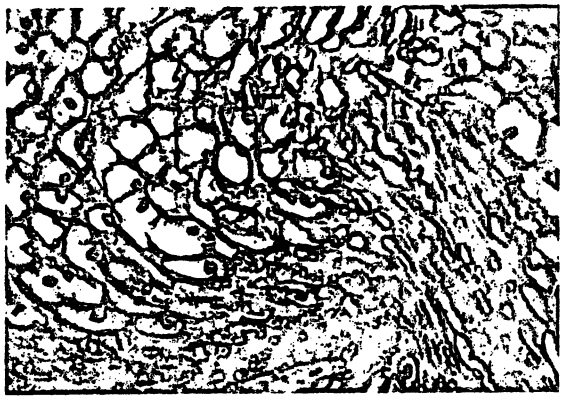

Fig. 1. Epithelial part of a tissue section with multiple positive signals, 400 times magnification, nuclear fast red counterstain. The deep blue or black colour of the nucleus indicates the presence of amplified base sequences inside the cells.

Fig. 2. Part of a HPV-DNA-negative tissue section, 200 times magnification, nuclear fast red counterstain.

tely. However the investigator is able to differentiate between infected and noninfected cells.

The results of this study and the comparison of effort and benefit of conventional PCR and in-situ PCR allow the following conclusions. The conventional PCR is of advantage for detection and as known from previous studies for typing of HPV infections. The in-situ PCR is the preferable approach for the localisation of viral DNA infected cells in a tissue section. The diagnostic application can be attractive if the conventional PCR gives a positive result. They can support a histological statement referring to the expansion of a viral infection too. Different methods for the visualisation of in-situ PCR products are reported (summarised ref. [11]) and especially the indirect detection approaches using labelled specific probes are of potential interest to prevent unspecific staining. In respect to this we have shown, that the in-situ PCR permits an evaluation in respect with viral-DNA-infected and -noninfected cells. So the method could be helpful for cytological diagnosis as part of the cancer prevention program.

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