Detection of Oncogenic HPV and Identification of 72Arg Polymorphic p53 by In Situ PCR for Clinical Routine Purposes

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Abstract. Background: Ano-genital carcinoma is a poly-factorial and polygenic disease. Certain strains of human papillomavirus (HPV) have been detected in a high percentage of patients. It has been suggested that p53 polymorphisms may be relevant for the interaction with viral proteins that inactivates p53. Materials and Methods: Patients were selected on the basis of HPV infection, clinical history, positive PAP test and type of lesion. In situ PCR was performed on smear samples, in four steps: a) preparation on clean biobond-treated slides, b) permeabilisation and digestion; c) in situ PCR amplification; d) in situ hybridisation with a fluorescent probe. Results: In situ PCR analysis of the smears confirmed the results obtained by classic PCR and by in situ PCR of frozen sections. Conclusion: In situ PCR on smears could be used in targeted-screening for young and post-menopausal women, as well as in the development of large scale studies to establish the connection among the presence of HPV, p53 polymorphisms and the risk of cervical cancer.

Human papillomavirus (HPV) is associated with proliferative disorders, including condylomata and carcinoma of the cervix, bladder, penis and anus and papilloma of the larynx (1-6).

Oncogenic HPV can be detected in 98% of high-grade lesions (7), in most invasive carcinoma of the cervix (8, 9) and in 60-80% of head and neck carcinoma (10, 11). This association is not a simple cause-effect relationship, but is conditioned by a number of factors related to the host and/or virus. A routine and balanced evaluation of such factors is required for more precise prognostic staging and more appropriate therapy, representing a challenge in both technical and economic terms.

In the past few years, the nature of the above-mentioned relationship has been partially clarified by four cornerstone achievements: a) the observation that only a few infected patients develop malignant neoplasia and, especially in younger people, the initial lesions associated with the infection may recover or may not progress (12). b) The fact that only a few of the more than 80 strains of HPV are strictly associated with progression to malignancy; types 16, 18, 31 and 33 HPV have been identified as the strains that are associated with the malignant lesion, including invasive and metastatic carcinoma (13). Molecular biology studies have shown that viral oncogenic proteins E6 and E7 are able to form inactivating complexes, respectively, with oncosuppressor proteins p53 and RB110, which, in turn, are rapidly degraded through the proteosomal pathway (14). c) The hypothesis that polymorphisms of the p53 protein could play a role in determining individual susceptibility to the progression of benign lesions (condylomata, etc.) to malignant tumours (14). The polymorphism at codon 72 seems to be crucial for the interaction between p53 and E6. In particular, the E6 protein from HPV 16 and 18 is more effective at degrading p53Arg than p53Pro (14). d) The observation that variability in the structure of the oncogenic proteins E6 and E7 may also influence the formation of a stable E6+p53 or E7+pRB110 complex with the inactivation of oncospessuring activity (14).

Abbreviations: PCR, polymerase chain reaction; OsO4, osmium tetroxide; HPV, human papilloma virus; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate.

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The last three observations have been the subject of much debate. In fact, while some studies have confirmed a correlation between p53 polymorphism at codon 72 and the occurrence of cervical cancer (15-20), others have failed to corroborate such data (21-29). A recent study showed that, by combining all the published literature on p53 and cervical cancer, it was possible to associate the p53 gene with an increased risk of invasive cervical cancer (30). However, the potential risk differed according to the country, cellular and HPV type and with the viral subtype (variations of E6 and E7 proteins) (30).

Therefore, the prognostic evaluation of a lesion in young individuals should consider, not only the histological grading of the HPV type, but also the susceptibility related to p53 and RB110 polymorphisms and possibly the genetic variation of E6 and E7. This aspect could be easily studied in biopsy samples. However, for routine and screening purposes, biopsies are both impractical and expensive.

The major aim of this study was to develop an effective, reliable, relatively simple and low-cost technique for in situ PCR of cytological smears or cytospin, which could be applied in routine clinical molecular diagnostics. Once this technique had been set up, we showed that the results obtained were totally in agreement with those obtained with classic, but less practical, more expensive and time-consuming techniques.

**Materials and Methods**

*Subjects and samples.* Twelve patients were selected on the basis of their past history of HPV infection, as diagnosed by colposcopy and microcolposcopy. The study was conducted using both biopsies and vaginal smears. Samples were collected after obtaining informed consent from the patient.

A single biopsy was divided into four samples. The first sample was embedded in paraffin for histopathological study; the second one was fixed in glutaraldehyde for transmission electron microscopy (TEM); the third was used for molecular biology studies; and the fourth, and largest, was frozen to obtain cryosections for *in situ* PCR (1). Nucleic acids were extracted from the third sample and were utilised for HPV typing and p53 polymorphism detection through PCR amplification, while the same information was obtained from the fourth sample by *in situ* PCR.

Four vaginal cytological smears were obtained. One of these samples was used for a PAP test, while the other three were stored at –30 °C for molecular biology studies. The flow-chart used for the molecular typing of the HPV strain and the p53 polymorphisms are detailed in Figure 1.

As negative controls for HPV typing, smears and tissues from healthy women were used. For p53 polymorphism, a homozygous p53Arg72 was used as a negative control for p53 Pro72, and a homozygous p53Pro72 was used for p53Arg72.

*Standard histology.* Histological samples obtained from the biopsies were fixed in formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin for light microscopy.

*Electron microscopy.* Samples were fixed in glutaraldehyde 2.5% in phosphate buffer 0.1 M, pH 7.2 (for at least 4 h at 4 °C), washed in buffer solution for 2 h and post-fixed in OsO4 (1.33%) for 2 h. Dehydration was performed in a graded ethanol solution series and, finally, in toluene. Infiltration in an Epon 812 mixture (4 h at room temperature) was followed by polymerisation of the resin (24-36 h at 60 °C). Ultrathin sections, after staining with uranyl acetate and lead hydroxide staining, were examined with a CEM-10 Philips transmission electron microscope (TEM).
DNA extraction from tissues. DNA was isolated from the human cervix biopsies by the following procedure: the samples were transferred into 1.5-ml sterile microcentrifuge tubes and gently crushed in digestion buffer (containing SDS, Tris-HCl, EDTA) using sterile pipette tips; after treatment and centrifugation, proteinase K was added and the samples were digested for 3 h at 37°C; following digestion, the nucleic acids were extracted twice with phenol-chloroform (PC-9) and the supernatants, containing DNA, were treated with chloroform and precipitated with cold ethanol; finally, after washing in 70% cold ethanol, the pellets were resuspended in water. Aliquots of DNA (250 ng) were then amplified in PCR buffer containing the reagents in the following proportions: 10X PCR buffer, 0.2 mM dNTPs, 20 pmol each of upstream and downstream primers, MgCl₂ 4 mM and 2.5 units of Taq Polymerase in a final volume of 100 μl.

HPV detection and typing. The PCR assay was used to amplify the L region of the HPV. Two sets of primers were used: the first one to amplify several HPV strains (strains 6, 11, 16, 18, 31, 33, 42, 52 and 58); the second one, named HPV16 or HPV18, designed to specifically amplify either strain 16 or strain 18. The sequences of the primers are listed in Table I. The PCR products were separated on agarose gel using a 100-bp marker as a standard.

p53 polymorphism analysis. The analysis of the p53 genotype at codon 72 was performed by PCR, using the primers listed in Table I. The PCR products were fractionated on agarose gel using a 100-bp marker as a standard.

In situ PCR. In situ PCR, either on tissue sections or smear samples, was performed in five basic steps:

i) Preparation of samples on clean biobond-treated slides: Glass slides (Perkin-Elmer, Wellesley, MA, USA) were washed in distilled water, dried, immersed in the BIOBOND (Electron Microscopy Science, Hatfield, PA, USA) solution and dried in a dust-free environment. These slides were used both for tissue sections and for isolated cells. a) Biopsy tissue specimens were stored in liquid nitrogen at the optimum cutting temperature. Three 5-μm sections were cut with a cryostat at −20°C, collected on glass slides and washed in PBS at 4°C (Electron Microscopy Science). The samples were then fixed in formaldehyde 4% in PBS for 2 h, dehydrated, dried and stored at 4°C. b) Vaginal smears were prepared by using the ThinPrep System (Cytyc Corporation, Marlborough, MA, USA), according to the manufacturer’s instructions. Briefly, exfoliated vaginal cells were collected using a cytobrush device and immediately immersed in a vial containing PreservCyt transport medium (methanol/EDTA). The vial was inserted into the ThinPrep 2000 Processor, where the medium was drawn by negative pressure through a ThinPrep filter that collects the cells, free of mucous and various debris. Subsequently, the cells on the filter were transferred onto a Biobond-treated glass slide and fixed for the subsequent steps.

ii) Triton X-100 permeabilisation and digestion by proteinase: Following fixation, samples on the glass slides were permeabilised. The samples were incubated in HCl 0.02% for 10 min, washed for 2 min in PBS, incubated in 0.01% Triton X100 in PBS for 90 sec and then washed again in PBS for 2 min. The permeabilisation step was achieved by incubating the samples with 50 μl of a solution prewarmed for 5 min at 37°C and containing 1-2 μl of Proteinase K diluted in Tris-EDTA. After a 30-min incubation in a humid chamber, the slides were washed first in glycine/PBS to inactivate the enzyme and then in PBS. The permeabilised samples were dehydrated, dried and stored at 4°C.

iii) In situ amplification: The in situ amplification step was performed in 50-μl reaction volumes containing: 20 pmol of each primer, 200 μM dNTP, 1X PCR buffer, Taq DNA polymerase 20 U/μl and MgCl₂ 4 mM. Forty microlitres of this reaction mixture were applied to the glass slides (used for the in situ reaction) and washed for 5 min in 2x SSC at room temperature. The glass slides were washed again in 2x SSC for 5 min at 37°C and then rinsed in bi-distilled water. Finally, they were incubated for 15 min in 100% EtOH and air dried.

iv) Fluorescence probe labelling: The probe for the detection of intracellular PCR products was labelled with fluorescein-12dUTP using a kit from Boehringer Ingelheim (Ingelheim, Germany), following the manufacturer’s instructions. Briefly, the PCR reaction was performed in 100-μl reaction volumes containing: 1X PCR buffer, MgCl₂ 4 mM, PCR fluorescein labelling mix 200 μM, dNTP 20 pmol of each primer, Taq Polymerase 2.5 U 10 μg of template DNA. PCR was performed for 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. At the end of the PCR reaction, the coverslides were removed from the GeneAmp system and washed for 5 min in 2x SSC at room temperature. The glass slides were washed again in 2x SSC for 5 min at 37°C and then rinsed in bi-distilled water. Finally, they were incubated for 15 min in 100% EtOH and air dried.

v) In situ hybridisation: Two to 5 μl of the labelled PCR reaction product were lyophilised and resuspended in 20 μl of the hybridisation buffer (2 x SSC, 5% dextran sulphate, 0.2% BSA in PBS, 50% formamide). The buffer with the PCR product was applied to the glass slides (used for the in situ amplification). These were then incubated at 95°C for 5 min in the GeneAmp In Situ PCR System 1000. The glass slides were left in a humid chamber overnight. The next day, the samples were washed in SSC 2x at room temperature, in SSC 2x at 60°C for 10 min, in SSC 2x at room temperature for 10 min, in SSC 1x at room temperature for 5 min and finally in SSC 0.2x for 5 min at room temperature. The glass slides were then assembled in glycerol-Tris-HCl buffer.

Results

Diagnostic protocol for molecular typing of HPV and p53 polymorphisms. The general protocol used to validate the

Table 1. Primers utilised for RT-PCR.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sense</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPL</td>
<td>Sense</td>
<td>5'-CGT AAA CGT TTT CCA TAT TTT TT3'</td>
</tr>
<tr>
<td>HPL</td>
<td>Antisense</td>
<td>5'-TAC CCT AAA TAC TCT GTA TTG-3'</td>
</tr>
<tr>
<td>HPV16</td>
<td>Sense</td>
<td>5'-CAG GAC CCA CAG GAG CGA CC3'</td>
</tr>
<tr>
<td>HPV16</td>
<td>Antisense</td>
<td>5'-ATC GAC CGG TCC ACC GAC CC3'</td>
</tr>
<tr>
<td>HPV18</td>
<td>Sense</td>
<td>5'-GCT TTG AGG ATC CAA CAC GG-3'</td>
</tr>
<tr>
<td>HPV18</td>
<td>Antisense</td>
<td>5'-TGC AGC ACG AAT GGC ACT GG-3'</td>
</tr>
<tr>
<td>Arg/p53</td>
<td>Sense</td>
<td>5'-ACT CCC TTG CCG TCC GCT-3'</td>
</tr>
<tr>
<td>Arg/p53</td>
<td>Antisense</td>
<td>5'-ATG GTG CAG GGG CCA ACT-3'</td>
</tr>
<tr>
<td>Pro/p53</td>
<td>Sense</td>
<td>5'-TTA AGA GGC TGC TCC CAA-3'</td>
</tr>
<tr>
<td>Pro/p53</td>
<td>Antisense</td>
<td>5'-ATG GCA ACT AGC ACA CTT-3'</td>
</tr>
</tbody>
</table>
Figure 2. Sample characteristics. a) Haematoxylin and eosin-stained section of condyloma from patient T.D.. Koilocytes are present (x 20). b) ThinPrep from a smear obtained from the same patient and stained with Papanicolaou reagent. Koilocytes are visible (x 40). c) Transmission electron microscopy (TEM) of a biopsy from patient S.G.. Numerous virus particles, apparently devoid of pericapside, are grouped in the nucleus.

Figure 3. HPV typing and p53 polymorphisms. For HPV typing, viral DNA was isolated and amplified from each sample using a common HPV primer or primers specific for HPV strains 16 and 18, as described in "Materials and Methods". For p53 polymorphism analysis, the RT-PCR technique was used to amplify the p53 gene at codon 72, as reported in "Materials and Methods". The PCR products were resolved on agarose gel.
molecular typing of the HPV strain and the p53 polymorphisms by *in situ* PCR on cytological smears is detailed in Figure 1. The validation study was conducted using parallel molecular analysis by standard PCR on DNA extracted from tissue samples, and by *in situ* PCR on tissue cryosections taken from the same patient who had donated the smears.

**Sample characteristics.** A histological preparation of a biopsy from a condyloma in which koilocytes are clearly visible is presented in Figure 2. The same type of cells was found in the preparation from a smear of the same patient obtained with the ThinPrep® technique (Figure 2b). The presence of viral particles was evidenced by TEM in the condyloma cells of the same patient. As shown in Figure 2c, intranuclear viral particles, devoid of the pericapsidic envelope, were present in the nucleus of these cells.

**HPV16 and 18 expressions in sections and smears.** The presence of the virus in the patients selected for this study was confirmed by the PCR technique. The viral DNA extracted from each sample was amplified using common primers for the HPV strains 6, 11, 16, 18, 31, 33, 42, 52 and 58; and specific primers for the HPV strains 16 and 18 were subsequently used in patients positive for the common primer (Table I). Five of the patients were positive for the common HPV primer, represented by a 250-bp DNA species (Figure 3). When the DNA from the biopsies of
these five patients was amplified with the primers specific for HPV16 and HPV18, three showed a positive 400-bp signal for HPV16 and two a positive 420-bp signal for HPV18 (Figure 3).

The presence of HPV16 was studied through indirect in situ PCR. For this purpose, a fluorescent probe was prepared through the amplification of the HPV16 DNA. The same probe was then used to label a histological section. As shown in Figure 4b, the sample from a patient positive for HPV16 according to the PCR showed a green fluorescence indicating the presence of the virus particles. It is important to note that the fluorescence staining had an epithelial, not dermal, distribution (Figure 4b). Sections from a control, uninfected subject did not show any fluorescence staining (Figure 4a). The same fluorescent probe was used to stain cytological smears of the HPV16-positive patient. As shown in Figure 4d, the green fluorescence was clearly visible in the cells, in accordance with the results obtained by PCR and in situ PCR on the section. Again, the control cells from the uninfected individual did not show any fluorescence (Figure 4c).

The presence of HPV18 was investigated, by the same technique as in the case of HPV16, using an HPV18-specific fluorescent probe. In this case, the probe was obtained from the DNA amplification of the HPV18 strain. As shown in Figure 5b, the histological section from an HPV 18-positive patient showed a significant green staining that was concentrated to the epithelial cells according to the PCR. The fluorescence was absent in the control tissue (Figure 5a). Furthermore, cells of the smear from the same patient were labelled with the same probe and showed a fluorescent staining, indicating the presence of HPV18 (Figure 5d). Again, the control cells did not show any fluorescence (Figure 5c).

Figure 5. In situ PCR analysis of HPV18 in tissue sections and smears. The presence of HPV18 was evidenced by in situ PCR on both tissue sections and smear cells, as described in "Materials and Methods". a) Negative control of a tissue section from an uninfected subject. b) The HPV18 stain was present in the epithelial layers, but not in the sub-epithelial cells of a histological section. c) Negative control of a cytological smear from the same uninfected subject. d) HPV18 fluorescence appeared to be present in the cytological smear from the same patient positive for HPV16.
p53 polymorphisms in sections and smears. To study the polymorphisms at codon 72 of the p53 gene, the RT-PCR technique was employed. RNA was extracted from the tissue obtained from the five patients positive for HPV16 and 18. cDNA was produced from the RNA and amplified through PCR using specific primers, as reported in Table I. The results of the PCR amplification are provided in Figure 3. Among the five patients studied, all showed polymorphism with arginine at codon 72, represented by a 141-bp DNA species, while only four showed polymorphism with proline at the same codon visualised as a 177-bp DNA species. Therefore, among the five patients considered, one was homozygous for arginine-72, while the other four were heterozygous, Pro72/Arg72 (Figure 3). The p53 polymorphism was also studied through in situ PCR on the vaginal smears. As shown in Figure 6b, cells from a smear of a patient with a p53Pro72 polymorphism revealed green fluorescent staining when hybridised with the probe obtained with the primers for p53Pro72. As expected, the fluorescent was apparent both in the nuclei and in the cytosol, although more evident in the nuclei (Figure 6b). The cells obtained from the patient homozygous for Arg72 and treated with the Pro72-specific primer, appeared clearly negative (Figure 6a).

Similarly, a green fluorescent probe was obtained with the primers for p53Arg72. This probe was then used to stain cells of a vaginal smear from a patient with a homozygotic p53Arg72 polymorphism, as previously shown through PCR amplification (Figure 3). As shown in Figure 6d, when these
cells were incubated with the p53$^\text{Arg72}$ probe, green fluorescent staining was observed in both the nuclei and the cytosol of the cells (Figure 6d). As a negative control, cells from a previously selected patient homozygous for p53$^\text{Pro72}$ were used. The staining with the same p53$^\text{Arg72}$-specific probe appeared negative (Figure 6c).

Correlation of clinical and histological diagnosis with analysis of HPV infection and p53 polymorphisms. The results from five patients selected in this study are summarised in Table II. The following analysis was performed on each patient: a) cytological and histological diagnosis; b) HPV typing by standard PCR; c) HPV typing by in situ PCR on sections and on smears; and d) p53 polymorphism detection by standard PCR, as compared with analysis done on ThinPrep® smears by in situ PCR.

It is important to note that there was a perfect correlation between the HPV typing done by standard PCR and that done with in situ PCR on both sections and smears. Furthermore, the results obtained for the p53 polymorphism by in situ PCR matched those obtained by the in situ PCR done on the smears.

Discussion

The major goal of this investigation was to set up a reliable in situ PCR technique to be used on cytological specimens for either HPV typing and/or protein polymorphism determination.

By performing in situ PCR on four smears for each patient, we were able to determine the precise HPV type and the Pro/Arg p53 polymorphism. In particular, all the results obtained with in situ PCR on smears from the studied patients were completely concordant with those obtained by the classic PCR performed on DNA after its extraction and with those obtained by in situ PCR on the frozen tissue sections of the individual lesion. These observations suggest that the methodological procedure, set up and used in our laboratory, is a reliable and practical substitute for classic PCR and for in situ hybridisation.

The advantages of this technique are summarised in Table III and include: the use of a smear, instead of an invasive and less practical cervical biopsy or a large number of samples is needed. In particular, this technique could be used to resolve the debate involving the association of p53 or Rb polymorphism with increased risk of cervical cancer.

In conclusion, the in situ molecular analysis of cytological smears by PCR may provide a practical, useful and specific laboratory routine for assessing the different components of cervical carcinoma dependent on HPV/host interaction, including HPV typing, host-protein polymorphisms and viral protein variability. The relatively low cost of the overall procedure also suggests that in situ PCR on smears could be used in targeted-screening for young and for post-menopausal women and in the development of large-scale studies focused on the presence of HPV and p53 polymorphisms and the risk of cervical cancer.

References


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