The use of general primers in the polymerase chain reaction permits the
detection of a broad spectrum of human papillomavirus genotypes

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A novel polymerase chain reaction (PCR) method was
developed that permits the detection of 11 different
human papillomavirus (HPV) genotypes using two
general primer sets. By computer-assisted sequence
analysis, two pairs of general primers were selected
from the conserved L1 open reading frame and tested
in the PCR on a set of cloned HPV genotypes.
Experimental analysis showed that up to three mis-
matches between primers and target DNA did not
influence the efficiency of the assay. The use of these
primers in the PCR enabled the detection of HPV
genotypes HPV-1a, -6, -8, -11, -13, -16, -18, -30, -31,
-32 and -33, and was also successfully applied to well
characterized cervical carcinoma cell lines and clinical
samples. For the HPV types tested sub-picogram
amounts of cloned DNA could be detected after general
primer-mediated PCR and subsequent hybridization.
The specificity of the amplification products was
confirmed by blot hybridization procedures and Rsal
restriction enzyme digestion. The results indicate that
this PCR method can be a powerful tool for identifying
novel HPV genotypes in dysplasias and squamous cell
carcinomas suspected of having an HPV aetiology.

Introduction

During the last few years increasing efforts have been
made to determine the role of human papillomavirus
(HPV) in malignant transformation of squamous cell
epithelium. In particular the finding that almost all
carcinomas of the uterine cervix harbour specific HPV
genotypes (Dürst et al., 1983; van den Brule et al., 1989),
supports a causal relationship between certain HPV
types and squamous cell carcinoma of the genital tract
and has contributed to a growing field of interest in HPV
research. Moreover, a role for HPV in the development
of carcinomas of the respiratory tract has been supported
as well (Löning et al., 1985; de Villiers et al., 1985; Kahn
et al., 1986; Byrne et al., 1987). Because several of these
carcinomas were found to contain HPV-related DNA
(Abramson et al., 1985; Brandsma et al., 1986, 1989;
Stremlau et al., 1985), it has been speculated that
presently unidentified HPV types could be specifically
associated with carcinomas of the respiratory tract.

In this study the capability of the polymerase chain
reaction (PCR) (Saiki et al., 1988) for the detection of a
broad spectrum of HPV genotypes was investigated, in
order to obtain a powerful tool for the demonstration of
unknown HPV types. Based on short regions of
homology conserved amongst HPV genotypes whose
complete sequences have been determined, two pairs of
general primers were designed that match the same
sequences in the L1 open reading frame (ORF). A model
system of several cloned HPV types (pHPVs) was
employed to test these primer pairs in the PCR. With this
system experimental conditions could be determined to
amplify target DNA of the examined HPV types 1a, 6, 8,
11, 13, 16, 18, 30, 31, 32 and 33. Furthermore the use of
one of the primer pairs in the PCR allowed the detection
of different HPV genotypes in cervical carcinoma cell
lines and clinical specimens that are known to harbour
HPV DNA. The results indicate that the primer
matched sequences are highly conserved among a broad
spectrum of HPV types, suggesting that analogous PCR
products can be generated from other, unknown HPV
genotypes. This general primer-mediated PCR method
can therefore be a powerful tool for the detection of
presently unidentified HPV types in lesions of the
respiratory tract.

Methods

Cell cultures, tissue specimens and HPV clones. The human cervical
cancer cell lines CaSki, C4-1 and Siha were obtained from the
American Type Culture Collection. HeLa cell line 229 was obtained
from Dr K. H. Thian (Rotterdam, The Netherlands). CaSki/Siha and
C4-1/HeLa are known to contain HPV types 16 and 18, respectively (Boshart et al., 1984; Schwarz et al., 1985; Yee et al., 1985). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum. After growth to near confluence cells were harvested by trypsinization, washed with phosphate-buffered saline, spun down and subsequently suspended in 10 mM-Tris·HCl, 1 mM-EDTA pH 7.5.

Tissue specimens of a laryngeal squamous cell hyperplasia and a juvenile papilloma were snap-frozen and stored in liquid nitrogen. Scrapes were processed as described by van den Brule et al. (1988). The presence of HPV DNA in these samples was determined by Southern blot hybridization and PCR with HPV types 6, 11, 16, 18 and 33-specific primers, as previously described (Melchers et al., 1989). The cloned HPV types 6b, 11, 16, 18 and 30 (pHPVs) were used as targets in a model system for general primer directed amplification. The cloned HPV types 6b, 11, 16, 18 and 30 were kindly provided by Drs H. zur Hausen and L. Gissmann (Heidelberg, F.R.G.), HPV type 31 by Dr A. Lorincz (Gaithersburg, Md., U.S.A.), HPV type 33 by Dr G. Orth (Paris, France) and HPV types 1a, 2a, 8, 13 and 32 by Dr E.-M. de Villiers (Heidelberg, F.R.G.).

Polymerase chain reaction. A slight modification of the PCR method described by Saiki et al. (1988) was used. The PCR was performed on 1 ng DNA of cloned pHVs or 100 to 500 ng of cellular DNA. In addition dilutions of several pHPV DNAs mixed with 100 ng human placental DNA or diluted SiHa DNA were also subjected to PCR, in order to assess the sensitivity of the assay. The reaction mixture of 50 μl also contained 50 mM-KCl, 10 mM-Tris·HCl pH 8.3, 0.01% (w/v) gelatin, 200 μM of each dNTP, MgCl2 at between 1.5 and 10 mM, 1 unit of a thermostable DNA polymerase (Thermus aquaticus; Cetus) and 50 pmol of each primer of either the GP5/6 or GP11/12 primer combination (Fig. 1). The mixture was overlaid with several drops of paraffin oil and incubated for 5 min at 94°C for DNA denaturation, followed by 40 cycles of amplification using a PCR processor (Biomet). Each cycle included a denaturation step to 94°C for 1 min, an annealing step to 40°C for 2 min and a chain elongation step to 72°C for 1.5 min. To ensure complete extension of the amplified DNA, each individual elongation step was increased by 1 s and the final elongation step was prolonged for another 4 min. To avoid contamination by preparation and the amplification reaction were performed in strictly separated rooms. Samples containing distilled water were included as negative controls, none of which showed a successful amplification. A total of 10 μl of each of the PCR mixtures was finally analysed by agarose gel electrophoresis.

Southern blot analysis of PCR products. Electrophoretically separated DNA fragments were transferred onto nylon membranes (GeneScreen Plus; DuPont) by diffusion blotting in 0.5 M-NaOH, 0.6 M-NaCl, GP5/6- or GP11/12-directed, HPV-specific PCR products were used as probes after 32P-labelling by the random priming method. Hybridization was performed at 65°C (Tm − 23°C, for high stringency analysis) or 55°C (Tm − 33°C, for low stringency analysis) in 0.5 M-sodium phosphate pH 7.4, 7% SDS, 1 mM-EDTA for 16 h. Subsequent washings were carried out at high (Tm) or low (Tm − 33°C) stringency in 0.1 x SSC (1 x SSC is 0.15 M-sodium chloride and 0.015 M-sodium citrate), 0.5% SDS at 65°C or 3 x SSC, 0.5% SDS at 56°C, respectively. Autoradiography was performed for 1 day at −70°C with Kodak Royal X-Omat film and intensifying screens.

Restriction enzyme analysis. Analysis of PCR products by restriction endonuclease digestion was performed directly on a 10 μl sample of the reaction mixture, without prior purification and resuspension of the DNA in the recommended restriction buffer (Carman & Kidd, 1989).

Two units of RsaI (Boehringer) were added and the digestion was allowed to proceed for 2 h at 37°C. Digestion products were analysed on composite gels consisting of 3% NuSieve agarose (FMC Bioproducts) and 1% type 1 agarose (Sigma) to obtain adequate resolution of low M, DNA fragments.

Dot blot analysis. For dot blotting 1 μg pHPV DNA and pBR322 vector DNA was dotted onto nylon membranes (GeneScreen Plus; DuPont). HPV-specific amplification products of 140 to 150 bp were used as probes. The fragments were electrophoretically separated in low melting point agarose (Bio-Rad), excised from the gel and directly labelled by random primed labelling. Hybridization was performed under high stringency, as described above. Subsequent washings were carried out at high stringency (Tm) down to 0.1 x SSC, 0.5% SDS at 65°C. Autoradiography was performed for 1 day at −70°C with intensifying screens.

Computer analysis and primer synthesis. All matrix, homology and restriction site analyses were executed with the Microgenie sequence analysis program [GenBank (Release No. 54), Beckman] developed by Queen & Korn (1984). From the sequence data two sets of general primer sequences were selected that match the same regions within the L1 ORF of all sequenced HPV types (Fig. 1). The primers were synthesized on a DNA synthesizer (Applied Biosystems 380A) by the methoxy-phosphoramidite method.

Results

Selection of general HPV primer sequences within the conserved L1 open reading frame

Matrix comparison of the sequenced HPV types 1a, 6b, 16 and 18 revealed that the most conserved regions are localized within the E1 and L1 ORFs (Giri & Danos, 1986). As a consequence general oligonucleotide sequences that could be used for the detection of multiple HPV genotypes (general primers), were sought in those ORFs. Although the overall homology between different HPV isolates allows cross-hybridization between the individual HPV genotypes under conditions of low stringency (Tm − 40°C), striking heterogeneity is observed at the nucleotide level using the sequence analysis program. This excluded the possibility of selecting linked 20 bp regions that are completely similar in all sequenced HPV types. However, two 20 bp sequences within the L1 ORF were found that are highly conserved but divergent amongst the examined types. Based on these homologies, two pairs of oligomers with a length of 20 nucleotides were designed (names GP5/6 and GP11/12) that are mainly homologous to the corresponding region of the HPV types 6b, 11, 16, 18, 31 and 33 (GP5/6; Fig. 1a) and HPV-1a, -5 and -8 (GP11/12; Fig. 1b), respectively. The oligomer-resembling sequences span a region of approximately 140 to 150 bp in all examined HPV genomes (Table 1).
Table 1. Sizes of DNA fragments of the sequenced HPV types that are spanned by the GP primer sets

<table>
<thead>
<tr>
<th>HPV type</th>
<th>Total length (bp)</th>
<th>Length of RsaI restriction fragments (bp)</th>
</tr>
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<tbody>
<tr>
<td>HPV-1a</td>
<td>139</td>
<td>66</td>
</tr>
<tr>
<td>HPV-5</td>
<td>154</td>
<td>29</td>
</tr>
<tr>
<td>HPV-6b</td>
<td>139</td>
<td>30</td>
</tr>
<tr>
<td>HPV-8</td>
<td>154</td>
<td>154</td>
</tr>
<tr>
<td>HPV-11</td>
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<td>30</td>
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</tr>
<tr>
<td>HPV-33</td>
<td>139</td>
<td>112</td>
</tr>
</tbody>
</table>

Application of general primers in the PCR on pHPV DNAs

With both primer pairs the PCR was performed under moderate stringency conditions (3.5 mM-Mg²⁺) on 1 ng DNA from several different pHVPs (Fig. 2). In addition to the sequenced HPV types 6b, 11, 16, 18, 31 and 33, the GP5/6 primer set yielded 140 to 150 bp PCR products with the presently unsequenced types 13, 30 and 32. The GP5 and GP6 oligomers did not direct amplification of HPV-1a, -2a and -8 DNA. In contrast, with primer pair GP11/12 successful amplification resulting in a PCR fragment that was clearly detectable on the gel under u.v. with ethidium bromide staining, was limited to HPV-8 DNA. Although amplified DNA specific for HPV-1a appeared after hybridization (not shown), lower stringency conditions of annealing were required to yield gel-detectable quantities of a GP11/12-directed PCR product of HPV 1a (Fig. 3, lane HPV1a/10 mM-Mg²⁺). However, even under lower stringency conditions (10 mM-Mg²⁺) none of the primer pairs was found to direct amplification of a 140 to 150 bp DNA fragment specific for HPV-2a (Fig. 2 and results not shown). Neither GP5/6 nor GP11/12 gave rise to amplification of pBR322 DNA (Fig. 2), indicating that the PCR products were HPV-specific and were not the result of cross-reaction with vector sequences. In some instances, co-amplified DNA of lower (40 to 45 bp) and higher M, was observed (Fig. 2, lanes HPV-18, HPV-30 and HPV-32). The low M, DNA fragments could reflect the ligation and amplification of the primers, whereas the fragments of higher M, were presumably the result of primer...
annealing to additional target sequences within the HPV genome.

To investigate the number of mismatches acceptable between primer sequences and target DNA the PCR was performed under different stringency conditions by varying the Mg$^{2+}$ concentration. For that purpose HPV genotypes that show two (HPV-16 and -33), three (HPV-8), four (HPV-1a), or an unknown number (HPV-30) of mismatches with one or both primers were subjected to PCR in the presence of 1.5, 2.5, 3.5 and 10 mM-MgCl$_2$ (Fig. 3). The figure shows that two and three mismatches between primer and target DNA, localized more than four bases from the 3' end, are accepted. Under conditions of both high and low stringency GP11/12 and GP5/6 reacted with HPV-8 and HPV-16/-33, respectively. With HPV-16 additional faint signals representing a slightly lower Mr, can be observed that decrease at higher stringency conditions. It still has to be elucidated whether these fragments result from incompletely extended or ssDNA from the specific region, or from the co-amplification of additional target sequences within the HPV-16 genome. Furthermore, it was revealed that lower stringency conditions are required to accept four mismatches in the case of HPV-1a and GP11/12. A 139 bp specific DNA fragment became visible on the gel only when the Mg$^{2+}$ concentration was raised to 10 mM. Weak signals, slightly increasing in intensity at lower stringency conditions, were also obtained with HPV-30 and the GP5/6 primer pair. Additionally, it appeared that low stringency conditions (10 mM-Mg$^{2+}$) of the annealing step resulted in an increase of co-amplified DNA fragments.

The sensitivity of GP-directed PCR was determined by the examination of different concentrations of pHV and Siha (HPV-16) DNA diluted in human placental DNA. For most pHVs that show up to three mis-
matches with one or both primers a detection level, as determined after hybridization of the PCR products with a GP-amplified homologous DNA probe, of 0-1 to 1 fg DNA was found (Fig. 4, HPV-8 and -33). This corresponds to approximately seven to 70 viral genomes. In addition, as little as 10 pg of Siha DNA could be detected after GP5/6-directed amplification (Fig. 4, Siha). Assuming that Siha cells contain one to 10 copies of HPV-16 DNA per genome and that a human diploid cell contains about 5 pg DNA, this implies that two to 20 copies of HPV-16 could be detected. A detection level of between 10 fg and 1 pg pHV DNA, corresponding to 700 to 70000 viral copies was found with HPV-1a and HPV-30 (Fig. 4, HPV-30).

Analysis of PCR products

The type specificity of the PCR was determined by dot blot analysis. For this purpose 140 to 150 bp PCR products, generated with the GP5/6 or GP11/12 primer set were isolated, labelled and hybridized to a panel of dotted pHV DNAs (Fig. 5). As can be seen clearly, the PCR products were type-specific and did not originate from contamination with other HPV types. Further analysis by Rsal restriction enzyme digestion confirmed the specificity of the PCR. In Table 1 the sizes of Rsal restriction fragments are given for the predicted GP-directed 140 to 150 bp PCR products of the sequenced HPV types. Fig. 6 shows the result of Rsal digestion of the PCR products. In some instances it was obvious that the non-specific 40 to 45 bp PCR products, which can be seen in Fig. 2, interfere with the restriction fragments derived from the 140 to 150 bp products (Fig. 6, lanes HPV-18, HPV-30 and HPV-32). However, despite this interference the calculated sizes of specific restriction fragments from the sequenced HPV types are in agreement with the expected sizes, as estimated from the sequence data. From the PCR products of the unsequenced HPVs, restriction fragments were obtained of approximately 73, 40 and 30 bp (HPV-13), 140 bp (HPV-30), and 110 and 30 bp (HPV-32). This indicated that the HPV-13 product contains two Rsal sites, the product of HPV-32 contains one Rsal site, whereas an Rsal site is absent in the HPV-30 fragment.

General primers in the PCR on cellular DNA

Having established the optimal conditions of PCR with general primers, the method was performed with the GP5/6 primer pair on DNAs of cervical cancer cell lines.
Fig. 6. Restriction enzyme analysis of PCR products obtained with the GP11/12 (HPV-1a and -8) or GP5/6 (HPV-6b, -11, -13, -16, -18, -30, -31, -32 and -33) primer pair. PCR products were digested with RsaI and electrophoresed on a composite agarose gel. All DNAs except HPV-1a were amplified under moderate stringency conditions (3.5 mM Mg$^{2+}$). HPV-1a DNA was amplified in the presence of 10 mM-Mg$^{2+}$. Filled markers indicate the specific restriction fragments derived from the 140 to 150 bp products, determined after exclusion of non-specific PCR products that can be seen in Fig. 2. In all cases the sums of lengths of the indicated restriction fragments are consistent with the total length of the undigested PCR product. M, pBR322 fragments (bp) digested with HaellII.

Fig. 7. PCR with the GP5/6 pair on cellular DNA of cervical cancer cell lines, cervical scrapes (sl to s6), a laryngeal squamous cell papilloma (pl) and hyperplasia (h1) under moderate stringency conditions (3.5 mM-Mg$^{2+}$). Products are shown after electrophoresis, ethidium bromide staining and u.v. irradiation (a), or after blotting and hybridization under low stringency conditions with an amplified product specific for HPV-16 (b). The positions of the 140 to 150 bp fragments are marked on the right. The presence of HPV DNA was confirmed by PCR with type-specific primers. sl, no HPV; s2, HPV-6; s3, HPV-33; s4, HPV-11; s5, HPV-16; s6, HPV-18; pl, HPV-6; h1, HPV-6; M, pBR322 fragments (bp) digested with HaellII (see Fig. 2).

cervical scrapes and laryngeal lesions that had been well characterized by Southern blot analysis and PCR with HPV type-specific primers (van den Brule et al., 1989). In Fig. 7(a) the PCR products are shown after agarose gel electrophoresis. The presence of additional bands was obvious in most cases. Furthermore, a clear distinction in the detection level between high and low copy numbers of a certain HPV type was demonstrated. From DNA of the CaSki cell line, which contains more than 500 copies of HPV-16 per cell, an HPV-specific PCR product was generated that appeared as a strong band, clearly visible after electrophoresis. In contrast, the HPV product of the low HPV copy number cell line Siha (one to 10 copies of HPV-16 per cell) appeared as a weak signal. In addition, more additional bands were obtained using Siha DNA as target, with a gel band pattern that was hardly distinguishable from that of human placental DNA (not shown) or the cervical scrape (s1) that was negative for a broad spectrum of HPVs. Identical results were obtained with the HPV-18-containing HeLa cell line (10 to 50 copies of HPV-18 per cell) and C4-1 (one to five copies of HPV-18 per cell), and also with cervical scrape s2, laryngeal papilloma pl and hyperplasia h1, of which the latter two contain low quantities of HPV-6 DNA. HPV-specific PCR products were also generated from DNA of cervical scrapes containing HPV-33 (s3), HPV-11 (s4), HPV-16 (s5) and HPV-18 (s6).

After blotting and low stringency hybridization with a GP5/6-amplified DNA probe specific for HPV-16, all HPV-specific 140 to 150 bp fragments could be detected without interference of the additional bands (Fig. 7b).
Several samples yielded additional hybridizing DNA fragments of higher Mr. As the HPV-negative sample (s1) did not yield any hybridizing DNA, it is unlikely that these signals appeared from a cross-hybridization to co-amplified cellular DNA.

Discussion

This study was initiated to develop a convenient and sensitive method for the detection of a broad spectrum of HPV genotypes. Because of its sensitivity, the recently developed PCR (Saiki et al., 1988) is rapidly becoming the preferred means of detecting HPV DNA in cervical scrapes and biopsy specimens (Melchers et al., 1989; van den Brule et al., 1989). Consequently efforts were made to use the PCR for the detection of various unrelated HPV genotypes at one time. This required the selection of primer sequences that are conserved among a broad spectrum of HPV types. As only nine HPV types have been completely sequenced so far, the extent of conservation of a selected primer sequence could only be determined experimentally by subjecting a large group of HPV genotypes to PCR. Two general primer sequences were found within the L1 ORF that match perfectly to HPV-11 and did not differ for more than two bases from the corresponding sequences of the HPV types 6b, 16, 18, 31 and 33. Therefore these oligonucleotides (GP5 and GP6) were considered as general primers for genital HPV's. However, for the skin-associated HPV types 1a, 5 and 8, both primers appeared to be more heterogeneous (Fig. 1a), making it unlikely that these HPV genotypes would serve as targets for GP5/6-directed amplification. By changing some bases within the GP5 and GP6 sequences, an additional primer pair was designed (GP11/12) that match, with a maximum of four mispaired bases, to a higher degree to the skin-associated types (Fig. 1b).

In addition to all known HPV DNA sequences, some unsequenced types were found to serve as templates to yield HPV-specific PCR products (Fig. 2), thereby confirming the conserved nature of the selected primer sequences among a broad group of HPV types. This finding suggests that additional HPV types could serve as template to yield analogous PCR products from the same region within the L1 ORF.

Furthermore it was empirically demonstrated that up to three mismatches between primers and target DNA had no effect on the efficiency and sensitivity of the assay. HPV genotypes that possess two (HPV-16 and -33) or three (HPV-8) bases mispairing with one of the primers all showed a sensitive amplification directed by either GP5/6 (HPV-16 and -33) or GP11/12 (HPV-8) pair (Fig. 4). Therefore, both the lower sensitivity of amplification and the faint amplification signals of HPV-30 were supposed to reflect the occurrence of at least three mismatches with one or both primers, possibly in combination with unfavourable positions of the incorrectly matched bases (Newton et al., 1989). Lower stringency conditions were required to ensure the acceptance of four mismatches, as is the case with HPV-1a and the GP11/12 primer pair (Fig. 1). Since it resulted in a decreased efficiency and rather low sensitivity, it seems likely that the presence of more than four mismatches between primer and target DNA would not give rise to a successful amplification by PCR. Although the sensitivity of amplification of HPV-1a and -30 DNA appeared to be 700 to 70000 viral genomes, the GP-directed PCR method still enabled the detection of amounts of these HPV's that range between 0-035 and 3-5 copies per cell in 100 ng input DNA (20000 cells). Hence, this method showed a better sensitivity than genomic Southern blot analysis and is also less laborious.

It was also demonstrated in the present study that the GP5/6 primer pair allowed a successful amplification of different HPV targets in DNA from cervical scrapes, laryngeal biopsy specimens and cervical cancer cell lines. However, many co-amplified cellular DNA fragments were predominantly observed in PCR products of HPV-negative or low copy number DNAs. This could be the result of the lack of competition for the primers, since limited or no specific targets were available to withdraw primers from non-specific targets. These co-amplified products made it necessary to confirm the presence of HPV by hybridization and would probably cause problems in the isolation and characterization of an HPV-specific amplification product from a low copy number sample. Therefore, for general primer-mediated PCR, low stringency annealing conditions (3-5 to 10 mM-Mg 2+ at 40°C) are recommended for initial PCRs followed by a stepwise increasing of the stringency (by raising the annealing temperature or lowering the Mg 2+ concentration) to reduce background until an optimal signal to background ratio is achieved.

Homology analysis of the PCR products of the sequenced HPV types revealed a striking sequence homology ranging from 55-40% (between HPV-1a and HPV-11 products) to 87-8% homology (between HPV-6b and HPV-11 products). This indicates that low stringency conditions of Southern blot analysis should enable the detection of a broad spectrum of HPV-specific amplification products, as could be shown by using the HPV-16-specific PCR product as a probe to detect different amplified HPV sequences from cellular DNA (Fig. 7b). Additional hybridizing fragments of higher Mr, appeared in all samples containing HPV-18, the CaSki cell line and a cervical scrape containing HPV-11. In part these signals could reflect a cross-hybridization to...
co-amplified HPV DNA, generated from other regions within the HPV genome. Such co-amplified DNA has already been demonstrated in PCR products from certain pHPV DNAs (in particular pHPV-18; see Fig. 2). Alternatively as CaSki DNA was shown to be the only HPV-16-containing target with additional hybridizing DNA, the aberrant fragments could also be generated as a consequence of integration events or rearrangements of the HPV genome.

From the results it can be concluded that the application of the selected primer sequences in the PCR opens new ways of detecting novel HPV genotypes in dysplasias and squamous cell carcinomas suspected to be due to HPV.

Since this manuscript was submitted for publication two additional HPV types, namely HPV-45 (kindly provided by Dr E.-M. de Villiers, with permission from Dr K. V. Shah) and HPV-51 (kindly provided by Dr G. Nuovo) have been subjected to GP-mediated PCR. Both types have been found to react successfully with the GP5/6 primer pair under moderate stringency conditions of primer annealing.

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