Use of the nested reverse transcription-polymerase chain reaction for the detection of human papillomavirus 16 *E6* transcriptional activity in cervical cancer: *A technical perspective*

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Summary

Aim: The aim of this study was to evaluate HPV 16 *E6* expression using nested RT-PCR in cervical tumour tissue and compare this technique with standard RT-PCR in a group of patients using injectable contraceptive steroids.

Patients and Methods: Tumour DNA was analysed for the presence and type of HPV by polymerase chain reaction (PCR) from 120 cervical cancer samples. Ribonucleic acid (RNA) was extracted from cervical tissue samples and cell-lines. Reverse transcription was carried out on all samples using reverse transcriptase enzyme to form single-stranded cDNA. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) housekeeping gene was used.

Results: The majority of patients had squamous cell carcinoma. Of 120 cervical tissue samples, there were 111 samples with confirmed HPV 16 infection. RNA was extracted in only 86 samples. Of these, 23 samples contained genomic DNA. Of the remaining 63 patients, there were 53 patients who had expression of HPV-type 16, E6 full-length gene expression. In total there were 25 patients (40%) with expression of the HPV 16 E6*I gene and 30 patients with expression of the E6*II gene. The nested PCR method using S1/S2 primers detected 54 patients with the E6*I & E6*II transcripts in comparison to classical PCR which detected only 31 such transcripts.

Conclusion: Nested RT-PCR is the method of choice to determine the role of different E6/E7 splice products in HPV-associated carcinogenesis.

Key words: Nested RT-PCR; HPV-related cervical carcinogenesis.

Introduction

To date, more than 100 genotypes of human papillomaviruses (HPV) have been identified. Several epidemiological studies have established a strong link between infection with high-risk HPV types (16 & 18) and cervical cancer or precursor lesions [1-3]. The *E6* and *E7* early genes of HPV 16 encode the main transforming proteins [4]. These genes are capable of immortalization and oncogenic transformation under appropriate conditions. The protein products of these early genes interfere with the normal function of the products of tumour suppressor genes, *p53* and retinoblastoma genes. The *E6* gene product binds to *p53* tumour suppressor gene product and promotes its degradation [4-6].

Transcription of the HPV E6/E7 open reading frames (ORFs) produces three different splice products, due to alternative splicing, using a common donor site at nucleotide 226 and two different splice acceptor sites at nucleotide 409 and 526. The full length transcript encodes for the functional E6 protein, while the E7 protein is most likely encoded by the *E6*1* and *E6*11* splice products [7-9]. The major splice product is *E6*1* [9]. Using specific outer primer pairs designated S3 and S4 in the reverse transcriptase-polymerase chain reaction (RT-PCR) yields the following fragments: full-length product consisting of 525 base pairs (E6); *E6*1* consisting of 343 base pairs and *E6*11* consisting of 226 base pairs. Further testing using the S1/S2 inner primer pairs in a nested system (nRT-PCR) with the products of the S3/S4 primers yields a *E6* full-length fragment with 395 base pairs; *E6*1* fragment consisting of 213 base pairs and *E6*11* consisting of 95 base pairs [10]. It is thought that the detection of the *E6*1* and *E6*11* splice products are unequivocal proof of HPV-16 *E6* oncogene transcription [8].

The aim of this study was to evaluate HPV-16 *E6* expression using nested RT-PCR in cervical tumour tissue and compare this technique with standard RT-PCR in a group of patients using injectable contraceptive steroids.

Materials and Methods

Institutional ethical approval was obtained prior to commencement of this study.

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Sample collection

A total of 120 cervical tissue samples were obtained from patients with proven cervical cancer. All patients were clinically staged according to the International Federation Obstetrics Gynaecology (FIGO) staging system. Two punch biopsy specimens of the cervical tumour tissue were taken. One specimen was used for typing the HPV 16 virus, while the second specimen was used for the main project if the tumour tissue contained HPV 16. The second specimen was stored at –80°C till the final project was performed.

HPV typing

Tumour DNA was analysed for the presence and type of HPV by polymerase chain amplification using the LI consensus primers MY 11 and MY 09 [11]. The PCR product was then cycle sequenced using the MY 09 primer and the Big Dye™ Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems Prism 310 Automated Genetic Analyser. The sequences were analysed using the Sequence Navigator Software (v. 1.0.1 - Applied Biosystems), and aligned manually to the Gene Bank sequence. Three cell lines were grown in culture: CaSki and SiHa cell lines are both human cervical carcinoma cell lines which carry transcriptionally active HPV 16 genomes while the C33A cell line does not contain HPV genome.

RNA extraction and reverse transcription

Extraction of RNA from tissue samples and cell lines was carried out using the TRIZOL RNA extraction reagent (GibcoBRL® Life-Technologies). The quality and quantity of extracted RNA was determined by spectrophotometry at 260 nm. Reverse-transcribed (RT) cDNA was synthesized for all samples including the two positive cell-line controls (Caski, SiHa) and negative control (C33A) cell line. Basically, RT of alliquots of 2 µg of RNA into cDNA was carried out with reverse transcriptase enzyme and random hexamer primers in a final volume of 20 µl. The initial reaction mixture was heated at 70°C for ten minutes and then transferred onto ice for two minutes. Following the addition of the free-strand buffer, oligo-dNTPs and reverse transcriptase enzyme, the mixture was then heated at 37°C for one hour. A second round of mock "cDNA synthesis" was performed without reverse transcriptase to exclude the presence of genomic DNA.

Reverse transcription-polymerase chain reaction (RT-PCR)

Primers for the amplification of a 248 bp product of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) housekeeping gene were used. These were specific to nt 3932 to 3949 and nt 4355 to 4372 of GAPDH cDNA. PCR was performed using primers specific for nucleotide 142 to 161 (primer S3) and nucleotide 666 to 647 (primer S4) of the HPV type 16 (outer primers) (GibcoBRL® Life-Technologies). This was necessary to detect the *E6* unspliced transcript with 525 base pairs. PCR was performed for all samples including the three cell lines. The specific sequences are illustrated below:

S3: 5' - ACA GTT ATG CAC AGA GCT GC – 3'
S4: 5' - CTC CTC CTC TGA GCT GTC ATT – 3'

PCR of reverse transcribed cDNA was performed on a Hybaid Omnigene® (Middlesex, UK) GeneAmp PCR system in a final volume of 50 µl. The PCR amplifications were performed using 2 µl of cDNA and Taq DNA polymerase (GibcoBRL® Life-Technologies). The PCR mixture was subjected to denaturation x 1 cycle at 95°C for one minute; annealing at 56°C for 45 seconds; denaturation at 72°C for five minutes. The first cycle was preceded by five minutes of denaturation at 95°C and the last cycle was followed by a 5-minute incubation at 72°C.

Nested RT-PCR and gel analysis

Nested RT-PCR was performed on all PCR products from reactions with and without reverse transcriptase during the cDNA synthesis. The specific primer pairs used were the S1 primer specific for nucleotides 192 to 211 and S2 primer specific for nucleotides 586 to 567 (inner primers) (GibcoBRL® Life-Technologies). This was necessary to detect spliced variants viz. *E6*I* (213 base pairs) and *E6*II* (95 base pairs) of HPV 16 *E6* oncogene.

The sequences of these primers were:

S1: 5' - GTG TGT ACT GCA AGC AAC AG – 3'
S2: 5' - GCA ATG TAG GTG TAT CTC CA – 3'

A dilution of the RT-PCR product from the S3/S4 reaction was made by adding 1 µl of PCR product to 99 µl of PCR water for each sample including the positive and negative cell-line controls. This product served as the template for the nested RT-PCR. Amplification products were analyzed by gel-electrophoresis on agarose gels and stained with ethidium bromide. The X174 Hae III Digest (Sigma® Aldrich Chemical Co.) molecular weight marker used was able to detect all band sizes of the full-length and spliced products. The gel was examined under UV light to detect the presence or absence of PCR products.

Statistical Methods

Fisher's exact was used to analyse contraceptive use versus HPV 16 *E6* expression, stage versus *E6/E6*1/E6*11* expression; histology versus *E6/E6*1/E6*11* expression. Pearson's chi-square test was used to analyse odds of *E6/E6*1/E6*11* expression versus use of steroids.

Results

HPV 16 DNA was detected in 111/120 tissue samples. Of 111 cervical tissue samples RNA was extracted in 86 samples. Of these, there were 23 samples which contained genomic DNA as determined by mock "cDNA synthesis" without reverse transcriptase enzyme. The majority of patients (92%) had squamous cell carcinoma.

Of the remaining 63 patients, there were 53 patients who had expression of HPV type 16, *E6* full-length gene expression. Of all patients with *E6*1/E6*11* gene expression, the mean age was 52 years (SD 12 years) compared to patients without *E6*1/E6*11* gene expression (mean age 47; SD 12 years) ($p = 0.14$). In total there were 25 patients (40%) with *E6*1* gene expression and 30 patients with *E6*11* gene expression. Of all patients who had expression of the *E6*1/E6*11* gene, 57% (n = 17) used injectable medroxy-progesterone acetate steroid contraception ($p = 0.800$) (Table 1). Of all patients with early stage disease (FIGO < IIa), 78% (n = 23) had expression of the HPV 16 *E6* gene, in contrast to late stage disease (88%) ($p = 0.48$) (Table 2).

Overall, the relative risk of HPV 16 *E6*1/E6*11* gene expression in contraceptive users versus non-users for early and late stage disease was 1.10 (CI 0.69 - 1.74). There were 57% (n

Table 1. — *Contraceptive use versus HPV-16 E6*1/E6*11 oncogene expression*

Contraceptive use	E6*1/11 absent	E6*1/11 present	p value
	N (%)	N (%)	
Yes	13 (43)	17 (57)	
No	16 (48)	17 (52)	0.800

Table 2. — *Contraceptive use versus HPV-16 E6*1/E6*11 oncogene expression*

E6*1/E6*11	Early Stage	Late Stage
	N (%)	N (%)
Present	13 (57)	21 (53)
Absent	10 (43.4)	19 (47.5)

= 13) of patients with early stage disease with expression of the *E6*1/E6*11* gene, compared to 53% (n = 21) with late stage disease (p = 0.80) (Table 2). The full-length *E6* oncogene transcript was detected in 29 patients using both the S3/4 as well as the S1/2 primers (Figure 1). However, the nested RT-PCR method using S1/S2 primers detected 54 patients with the *E6*1 & E6*11* transcripts in comparison to classical RT-PCR which detected only 31 such transcripts (Figure 2).

Discussion

Invasive cervical squamous cancer is the commonest cancer in developing countries such as South Africa [12]. The *E6* and *E7* early genes encode the main transforming proteins [4]. These genes are thought to play a role in the initiation and oncogenic progression of tumours. Key host regulatory factors are thought to play a role in cell cycle control. These include cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDIs) [13, 14]. Cell cycle arrest at the G1/S point occurs as a result of cyclin-kinase inhibition by p21 CDI. Loss of functional *p53* due to *E6* binding results in failure of G1/S arrest [15-17].

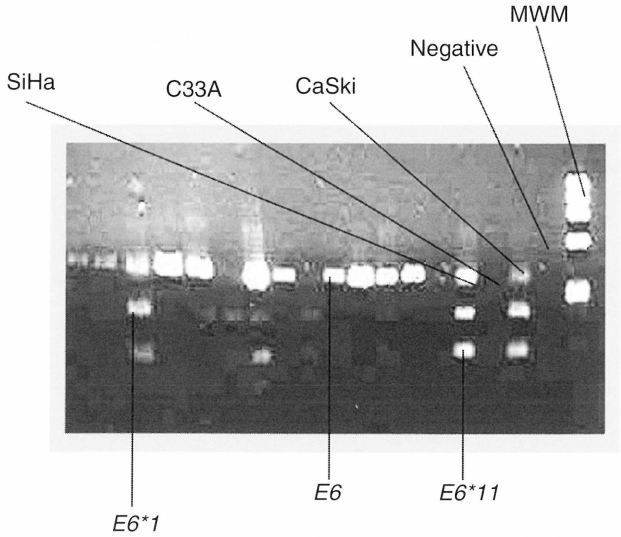

Figure 1. — RT-PCR products demonstrating *E6/E6*1/E6*11* genes with S3/S4 primers in samples & cell lines (MWM = Molecular Weight Marker).

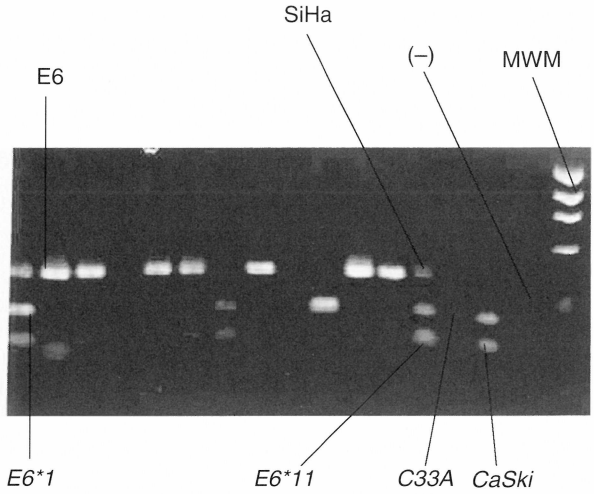

Figure 2. — Nested RT-PCR products demonstrating *E6/E6*1/E6*11* genes with S1/S2 primers in samples & cell-lines (MWM = Molecular Weight Marker); (–) = negative cell line.

Transcriptional activity of *E6* and *E7* is under control of p97, which is mainly suppressed by the viral E2 product [18]. The major splice product, *E6*1* [9], is thought to be unequivocal proof of HPV-16 *E6/E7* oncogene transcription [8]. The presence of the *E6*1* spliced product is also thought to correlate with lesion severity from cervical scrapes [10].

In our study the RNA extraction method using the TRIZOL® reagent, proved unsuccessful in 25 tissue samples. At the outset there were 111 cervical tissue samples with HPV 16 proven by typing and following the RNA extraction step, there were 86 samples, of which 23 contained genomic DNA. The HPV 16 detection rate was much higher compared to previous reports. The absence of RNA in 25 samples may either reflect absence of RNA in the tissue samples (degradation) or technical problems in the extraction process. The nested RT-PCR method using S1/S2 primers detected 54 patients with the *E6*1 & E6*11* transcripts in comparison to classical RT-PCR where only 31 such transcripts were detected. This underscores the value of the nested RT-PCR technique as being superior to conventional RT-PCR. Further, using this technique the HPV 16 *E6*11* spliced product was detected in ten samples using S3/4 (outer) primers and in a further 29 samples using S1/2 (inner) primers. This is in contrast to the report by Sotlar *et al.* [10], in which the *E6*11* product was not detected by use of the S3/4 primers. These authors also reported the presence of the *E6*1* transcript by nested RT-PCR from all biopsies from cervical cancer tissues. In addition, they also found that a template of only ten picograms of CaSki cDNA was sufficient for the detection of the 395 base-pair *E6*1* fragment using the combined primer pairs S3/4 (outer primers) and S1/2 (inner primers) in the nested RT-PCR technique. This technique can be applied even to paraffin-embedded biopsies to differentiate between the presence of HPV genomic DNA and transcriptionally active

viruses, albeit with a reduced sensitivity compared to unfixed fresh tissue. However, this report documented the use of the RT-PCR and nested RT-PCR method in only two cervical carcinomas.

In a study among children it was found that the nested RT-PCR method was able to detect 52% of HPV 16 transcripts compared to generic RT-PCR which detected only 17% of such transcripts [19]. The use of the nested RT-PCR method to detect the presence of HPV 16 transcripts has been described only twice before [10, 19]. Our study, presented purely from a technical perspective, is therefore the first to evaluate the role of nested RT-PCR in a large number of samples from women with invasive cervical cancer and validates the technique as being superior to standard RT-PCR. Although there were no differences in *E6* expression between steroid users and non-users, the main thrust of this study was to evaluate the role of the nested RT-PCR technique.

The nested RT-PCR method is invaluable in studying the role of HPV full-length and spliced transcripts. It has been shown that the detection of these transcripts increased with the progression of disease from low-grade CIN to invasive carcinomas. Since not all HPV infections lead to cervical dysplasia or invasive carcinoma, nested RT-PCR can be employed to study the effects of co-carcinogens which influence transcriptional activity of HPV genes. It has been postulated that HPV-16 *E6* transcripts can provide targets for gene therapy of cervical cancers. The method may also be utilized to monitor the level of HPV *E6/E7* transcripts before and after application of ribozyme-mediated antiviral gene therapy.

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