E4 EXPRESSION OF HUMAN PAPILLOMAVIRUS IN CERVICAL SAMPLES WITH DIFFERENT CYTOLOGY CATEGORIES

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Abstract. In some circumstances, infection with human papillomavirus (HPV), especially HPV type 16 (HPV16), progresses to cervical cancer. Viral E4 expression reflects viral replication and translation and its presence may rule out a latent infectious stage. Twenty cervical cytology samples with known HPV 16 infection of each cytological category namely, negative for intraepithelial lesion (NIL), low grade squamous intraepithelial lesion (LSIL), high grade squamous intraepithelial lesion (HSIL) and squamous carcinoma (SCC) were investigated by single-step quantitative RT PCR for HPV 16 E4 mRNA, which was not found in any NIL sample but in all LSIL and HSIL samples. Thus, E4 expression assay should be useful for determining precancerous states and may be suitable as an adjunct in cervical HPV testing.

Keywords: human papillomavirus, E4, mRNA expression, quantitative RT-PCR, cervical cytology

INTRODUCTION

Although human papillomavirus (HPV) is known to be the major cause of cervical carcinoma, the mechanism of progression from a latent stage infection to precancerous and cancerous stage has been the subject of recent review (Stanley, 2010). HPV 16 is the subtype most commonly found in cervical cancer worldwide. The virus can be detected at any stage of the carcinogenesis process, from normal, low-grade squamous intraepithelial lesion (LSIL) to high-grade intraepithelial lesion (HSIL) and carcinoma (Clifford *et al*, 2005; De Sanjosé *et al*, 2007; Smith *et al*, 2007).

Currently, HPV DNA testing involving detecting L1 or E1 protein or other genes of the virus, has become a useful tool for the triage of women with abnormal cervical Pap smears and as follow-up on women undergoing cancer treatment or antiviral vaccination (Petry *et al*, 2003; Chansaenroj *et al*, 2010). However, the high prevalence of asymptomatic and

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Table 1
Oligonucleotide primers.

Primer	Sequence
HPV_E4_F3566	AAGCCGTSKCCKTGGGCAC
HPV_E4_R3732	TATRGGYGTAGTGTTACYAYTACAG
F85_GAPDH	GTGAAGGTCGGAGTSAACGG
R191_GAPDH	CCATCAATGACCCCTTCATTGA

temporary HPV infections makes the test unsuitable for screening purposes (Cattani *et al*, 2009).

A previous study found HPV DNA-positive women with normal cytology tested positive for HPV E6/E7 mRNA resulting in false-positive results (Molden *et al*, 2005, 2006). Thus, similar to DNA tests, E6/E7 transcript-based RNA tests will lead to overtreatment, additional costs and considerable anxiety for the women concerned.

Contrary to other HPV genes, E4 transcription is only found when the host cell has incorporated abundant viral DNA (Chow *et al*, 2009) and the protein can damage keratinocytes (McIntosh *et al*, 2010). E4 has been postulated as a marker of viral carcinogenesis progression and might be useful for screening purposes. We have examined the presence of E4 mRNA among HPV16 expression in normal, LSIL, HSIL and cervical cancer samples.

MATERIALS AND METHODS

Specimens

Frozen samples tested positive for HPV-16 by nested PCR and direct sequencing in a previous study (Chansaenroj *et al*, 2010) were used. Twenty samples of each cytology category (normal, LSIL, HSIL and cervical cancer) were randomly selected. Samples were labeled with a coding number to ascertain the patients' anonymity.

The study protocol was approved by the institutional review board of the Faculty of Medicine, Chulalongkorn University, and the ethics committee of the National Cancer Institute, Thailand.

Single step quantitative RT-PCR

For primers detecting E4 transcript, one primer pair for single step quantitative RT-PCR was designed based on the E4 gene sequence in Genbank (http://www.ncbi.nlm.nih.gov/). Alignments were performed using BioEdit software (Hall, 1999) to facilitate the identification of HPV E4 genotype 16 specific regions. The primers were designed using FastPCR software (http://www.primerdigital.com/) (Table 1). The designed primers produced an amplicon of 191 bp.

pGEM-T Easy vector was used (Promega Corporation, Madison, WI) to increase HPV E4 gene quantity in a bacterial culture to serve as positive control. The 191 bp cDNA fragment was inserted into pGEM-T vector. The cloned plasmid was sequenced and compared with NCBI database (www.ncbi.nlm.nih.gov).

mRNA was purified using the guanidine thiocyanate method (Cha *et al*, 1991), treated with RQ1 RNase-Free DNase (Promega Corporation) at 37°C for

30 minutes and the reaction terminated with RQ1 DNase stop solution (Promega Corporation) for 10 minutes at 65ºC. A 1.0- l aliquot of RNA sample and a reaction mixture containing 5 l of 2x Reddy Mix PCR Master Mix (Thermo Fisher Scientific, Waltham, MA), 0.25 l of 10 M forward and reverse primers (BioDesign, Bangkok, Thailand**)** and DEPC water to make up 12.5 l were combined. PCR thermo cycling reaction was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) as follows: at 90ºC for 5 minutes; 40 cycles of 95ºC for 30 seconds, 56ºC for 30 seconds, 72ºC for 45 seconds; and a final extension step of 72ºC for 7 minutes. The PCR products were subjected to 2% agarose gel-electrophoresis at 100 volts for 45 minutes and visualized under UV transilluminator after staining with ethidium bromide.

Relative gene expression was detected with the single step quantitative RT-PCR using SYBR Green I dye with melting curve analysis. The reaction mixture consisted of 1.5 l of RNA sample, 6 l of 2.5 RealMasterMix RT-PCR, 0.1 l of RealMasterMix RT Mix, 0.15 l of 10 SYBR Green I dye, 0.375 l of 10 M forward and reverse primers and DEPC water to make up 15 l. The thermo cycling conditions included a reverse transcription step at 42ºC for 30 minutes. After an initial denaturation/RT inactivation step at 90ºC for 5 minutes, amplification was performed for 45 cycles, consisting of 95ºC for 10 seconds, 56ºC for 30 seconds and 72ºC for 20 seconds. The house keeping gene GAPDH was selected to serve as internal control for RNA extraction and to normalize the amount of target gene in the sample. Housekeeping gene detection was accomplished by single step quantitative RT-PCR using GAPDH specific primers (Table1). Single step quantitative RT-PCR was performed in Rotor-Gene RG-3000 (Corbett Research, Sydney, Australia) and analysis of the quantitative PCR assay was carried out with Rotor-Gene software version 6.0 (Corbett Research, Sydney, Australia).

Data and statistical analysis

Relative quantification employed $2^{-\Delta\Delta Ct}$ method. The cutoff value for the Ct value was determined as 0.05 RFU. Statistical analyses were performed with SPSS software, version 16. Comparisons across all cytology grades were performed by one-way ANOVA. The pairwise comparisons were performed by LSD to compare median values in different groups. All tests were performed bilaterally, and p-values <0.01 are considered statistically significant (Cohen, 1988; Keppel and Wickens, 2004).

RESULTS

E4 mRNA transcripts was absent in the 20 samples with the normal cytology and 6 samples of the cancerous category. The mean ages and E4 expression levels normalized with LSIL are shown in Table 2. E4 mRNA was highly expressed in HSIL state, 8.3×10^5 fold in comparison with LSIL ($p < 0.01$) and in cancer cells decreased 1×10^{-1} fold compared to LSIL ($p < 0.01$).

DISCUSSION

The molecular diagnosis of HPV can use different viral genes (E1, E2, E4, E5, E6, E7 and L1) to identify women at risk of developing cervical cancer. It is important that the assay detects only high risk HPV infection and their genes, which are associated with a significantly increased risk for cervical cancer (Molijn *et al*, 2005).

Table 2
Relative HPV-E4 expression in each group.

Cytology	HPV type	Mean (Age)	Sample (*N*)	Sample positive E4 (*N*)	Mean ΔCt (E4 : GAPDH)	Relative E4 expression (Fold)[a]
NIL	16	39	20	0	0	-
LSIL	16	31	20	20	3.63	11
HSIL	16	40	20	20	-3.1	1,152
Cancer	16	48	20	14	7.07	1

[a]Relative E4 expression was calculated by $2^{-\Delta\Delta Ct}$ compared to cancer group ($p< 0.05$)

The results from the quantitative RT-PCR in our study showed expression of HPV-E4 only in infected cervical cells with cytological changes. Using PCR for detecting HPV DNA in cervical cells ranging from normal to cervical cancer showed that HPV 16 was dominant (Chansaenroj *et al*, 2010). This finding is consistent with another study (Schmitt *et al*, 2010). The expression of HPV E4 in this study was found at early phases before development of cancer. We did not find HPV E4 mRNA expression in normal cervical cells although the samples were positive for HPV16. Therefore, absence of HPV E4 transcription in normal cells prevents unnecessary treatment, thereby decreasing cost and reducing anxiety for patients.

From this study, HPV E4 expression was seen with LSIL, which corresponds to previous studies (Doorbar, 2006, 2007). HPV E4 mRNA was most expressed in HSIL which is the prelude to cancer development. This technique may identify patients at risk for cancer. Infection with high risk HPVs in a woman with LSIL lesions can spontaneously regress in more than 60% of the cases without treatment, whereas, a woman with HSIL lesions is at high risk of developing cancer if not treated properly (Insinga *et al*, 2009).

Absence of HPV16 E4 mRNA expression in HPV16-infected cells at the cancer stage collected from Pap smears could be due to inappropriate sampling (Abdali *et al*, 2010; Korfage *et al*, 2011). Hence, the method used for specimen collection is an important factor for detection of HPV16 mRNA E4 expression.

In summary, we identified distinct HPV16 E4 mRNA expression in HPV16-infected cervical samples with cytological abnormalities; indicating that HPV16 E4 mRNA expression is related to the progression to cancer stage. The gene expression was found from the initial LSIL to the cancer stage but not in HPV16-positive cells at the normal stage. Identification of E4 mRNA may constitute a useful screening method for HPV infection leading to cervical cancer.

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