RESEARCH ARTICLE

Polymorphisms and Functional Analysis of the Intact Human Papillomavirus16 E2 Gene

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Abstract

High risk human papillomavirus (HR-HPV) E2 proteins play roles in transcriptional regulation and are commonly functionally disrupted when the HPV genome integrates into host chromosomes. Some 15-40% of cancer cases, however, contain an intact E2 gene or episomal HPV. In these cases, polymorphism of the E2 gene might be involved. This study aimed to determine polymorphisms of the E2 gene in episomal HPV16 detected in high grade squamous intraepithelial lesions and squamous cell carcinomas and altered functions compared to the E2 prototype. The E2 gene was amplified and sequenced. Two expression vectors containing E2 gene polymorphisms were constructed and transfected in SiHa and C33A cells, then E6 gene as well as Il-10 and TNF-α expression was determined by quantitative RT-PCR. Expression vectors and reporter vectors containing the HPV16 long control region (LCR) were co-transfected and transcriptional activity was determined. The results showed that a total of 32 nucleotides and 23 amino acids were changed in all 20 cases of study, found in the transactivation (TA) domain, hinge (H) region and DNA binding (DB) domain with 14, 5 and 13 nucleotide positions. They mostly caused amino acid change. The expressing vectors containing different E2 gene polymorphisms showed E6 mRNA suppression, TNF-α mRNA suppression and IL-10 induction but no statistically significant differences when compared to the E2 prototype. Moreover, promoter activity in HPV16 LCR was not affected by E2 protein with different gene polymorphisms, in contrast to nucleotide variations in LCR that showed an effect on transcription activity. These results demonstrated that E2 gene polymorphisms of episomal HPV16 did not affect transcriptional regulation and suggested that nucleotide variation as well as epigenetic modification of the LCR might play a role in inducing malignant transformation of cells containing episomal HPV16.

Keywords: Cervical cancer - human papillomavirus - E2 protein - polymorphism - function

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Introduction

Cervical cancer is the third most common cancer among women worldwide. An infection with high risk human papillomavirus (HR-HPV) is necessary for the development of cervical cancer (Walboomers et al., 1999; Natphopsuk et al., 2013). Wallboomers et al. reported that HPV DNA was detected in 99.7% of cervical cancer (Walboomers et al., 1999). HPV16 was the most common type found in cervical carcinoma, accounting alone for about 50% of all cervical cancers (de Sanjose et al., 2010; Shen and Liu, 2013).

In the productive HPV infection phase, E6 and E7 oncogene expressions can be controlled by viral E2 protein that can either activate or repress viral oncogene promoter when binding the distal or proximal E2 binding site (E2BS) to promoter (Doeberitz and Vinokurova, 2009). Dysregulation of HR-HPV E6 and E7 oncogene expressions in basal squamous epithelial cells makes the shift from permissive to transforming of HR-HPV-infected cells (Moody and Laimins, 2010).

HPV DNA integration into the host chromosome and then loss of E2 protein inhibitory function on the viral promoter is believed to be an initial step of cervical cancer development due to the loss of E2 open reading frames, while the long control region as well as the E6 and E7 open reading frames remain intact (Pett and Coleman, 2007; Wang et al., 2013). It is still controversial, however, that integration was an early or late event of cervical cancer progression since an intact E2 gene or episomal form of HR-HPV was detected in 15-40% of cancer cases (Nagao et al., 2002). This observation suggested that other mechanisms besides an integration of the viral genome into host cell chromosomes may contribute to the deregulation of the E6-E7 oncogenes during the transition into the transforming mode of HPV infections. Such mechanisms may include de novo methylation or mutations of E2BSs that might cause up-regulation of

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E6-E7oncogene expression by impairing the ability of E2 to bind to its specific sites (Romanczuk et al., 1990; Nishimura et al., 2000; Bhattacharjee and Sengupta, 2006; Chaiwongkot et al., 2013; Jacquin et al., 2013).

Several studies on DNA methylation of HPV 16 reported that E2BSs methylation blocked the binding of E2 to these sites, consequently E2 could not repress viral oncogene promoters (Bhattacharjee and Sengupta, 2006; Jacquin et al., 2013). Chaiwongkot et al. reported that CpGs within the E2BSs 1, 3, and 4 were more highly methylated in all cervical lesions with only episomal HPV16 genomes rather than lesions displaying single integrated copies and suggested that differential methylation of these E2BSs is related to the activation of viral oncogene expression in cervical lesions as long as the viral genome remains in the episomal state (Chaiwongkot et al., 2013). Genetic variations at E2BSs have been shown by previous mutational and functional experiments that could inhibit the binding of E2 protein on its binding site. E2BSs are attributed to mutations that caused the loss of E2 protein binding capacity (Romanczuk et al., 1990; Nishimura et al., 2000). In addition, Casas L et al. reported that E2 gene variations were detected in carcinoma cases and may be involved in deregulation of E6 and E7 oncogenes (Casas et al., 1999).

The E2 protein, which is expressed at the early stage of the HPV life cycle, is mostly nuclear protein containing three functional domains, an amino-terminal transactivation domain (TAD), a proline-rich high region and a carboxy-terminal dimerization DNA binding domain (DBD) (Blakaj et al., 2009). HPV E2 plays essential roles in the viral life cycle by activating viral DNA replication in concert with the E1 viral helicase (Kadaja et al., 2009) and regulating viral genome maintenance by its association with the cellular bromo-domain 4 (Brd4) protein (Oliveira et al., 2006; Helfer et al., 2013). The DBD of E2 protein binds to the promoter proximal E2 binding site on LCR that overlaps binding sites for the cellular SP1 and TFIID transcription factors resulting in repression of E6 and E7 transcription (Demeret et al., 1994). There is the evidence showing that the E2 protein binds to the regulatory region of the human interleukin-10 (IL-10) and telomerase gene and induces promoter activity in epithelial cells (Bermudez-Morales et al., 2008). In addition, the data from the lab of the current authors showed that tumor necrosis factor alpha (TNF-α) mRNA expression was decreased in cervical cancer cells transfected with E2 expression vector. Therefore, an impaired function of the E2 gene may be a factor involved in cervical cancer development.

The genetic variants of HPV16 occur commonly and are different based on geographic distribution (Yamada et al., 1997). HPV16 variants have previously been classified into 4 major lineages; European-Asian (EAS), including the sublineages European (EUR) and Asian (As), African 1 (AFR1), African 2 (AFR2) and North-American/Asian-American (NA/AA) (Cornet et al., 2012). Variations of HPV nucleotides have been found in L1, L2, E2, E5, E6, E7, and the LCR of the HPV16 genome. Previous evidence has indicated that HPV16 As variant was the major causative agent associated with cervical cancer in Northeast Thailand (Chopjitt et al., 2009). Increased risk of cervical neoplasia may be associated with HPV nucleotide variations including variations of the E2 gene.

In order to further clarify the mechanisms that may lead to the deregulation of the HPV oncogenes or the host genes during cervical carcinogenesis, the polymorphisms and amino acid changes of the E2 gene in episomal HPV16 detected in high grade squamous intraepithelial lesions and squamous cell carcinoma were analyzed and the function of the HPV16 E2 gene polymorphisms on HPV E6, IL-10 and TNF-α gene expression in SiHa cells and p97 promoter activity in C33A cells were determined.

Materials and Methods

Clinical specimens

A total of 20 episomal HPV16-positive samples were used. They consisted of 11 fresh cervical tissue biopsies, that were classified as 5 cases of high grade squamous intraepithelial lesions (HSIL), 6 cases of squamous cell carcinoma (SCC) and 9 formalin-fixed, paraffin embedded (FFPE) cervical tissues that were classified as 2 cases of HSIL and 7 cases of SCC. These samples were selected from hospitals from 4 parts of Thailand including central (Bangkok), North, South and Northeast of Thailand.

HPV16 E2 DNA amplification and sequencing

The DNA extraction was performed according to the instruction manual of the QIAamp DNA mini kit (Qiagen, Germany) and then an intact HPV16 E2 gene was amplified using four primers with overlapping fragments of E2A to E2D (Table 2). PCR amplification mixtures contained 1x PCR buffer, 0.2 mmoles of each dNTP, 2 mmoles $MgCl_2$, 10 pmoles of each E2 primer, 5 µl of DNA sample and 1 unit of Taq DNA polymerase (Fermentas Life Science, St. Leon-Rot, Germany). Amplifications were performed for 35 cycles with the following parameters; initial denaturing at 95°C for 5 min; each cycle at 95°C for 1 min, 53°C for 2 min and 72°C for 90 sec; and final extension at 72°C for 10 min. The amplification products of the HPV16 E2 gene were purified and sequenced using the automated system from Molecular Informatic Laboratory, Hong Kong.

The obtained nucleotide sequences of each gene region were aligned and compared with those of known HPV16R NC_001526 types available through the GeneBank database (NCBI, National Institute of Health, Bethesda, MD, USA) by using BLAST 2.0 software server (http://www.ncbi.nih.gov/BLAST/) BioEdit and Multalin programs (http://bioinfo.gonopole-toulouse.prd.fr/multalin/). The nucleotide sequences were translated to proteins for at least 6 frames of DNA databases. The identity or similarity of proteins were aligned and were compared with known HPV16 prototype genes through the BioEdit program and GeneBank database as described above.

Plasmids and constructs

The HPV16 E2 expression vector pCMV4-16E2 was provided by Dr. Alison McBride (National Institute of Allergy and Infectious Diseases, NIH, USA). The reporter

vectors containing LCR of HPV16 prototype (pGL3-16LCR) and the reporter vectors containing LCR with nucleotide variations of HPV16 including pGL3-LCR As (no.36) and pGL3-LCR As-sv14 (no.42) were previously constructed from the current group (Pientong et al., 2013).

For construction of expression vectors containing HPV 16 E2 gene polymorphisms, the DNA from samples no.38 (As) and no.32 (As sub-variant) with different E2 gene polymorphisms (Table 1) were PCR-amplified using P*fu* polymerase and specific primers containing restriction enzyme recognition sequence F:5'-ACT AGA TCT AAC GAT GGA GAC TCT TTG CC'-3 with the artificial Bgl II site underlined and R:5'-TGT CTA GAG CCG TCA TAT AGA CAT AAA TCC AG -3', with the artificial Xba I site underlined. Restriction-digested PCR products were cloned into the pCMV4 expression vector giving two expression vectors pCMV4-16E2no.38 and pCMV4-16E2no.32. The constructs were verified by restriction digestion with Bgl II and Xba I and gel electrophoresis for the E2 gene.

Cell culture and DNA transfection conditions

HPV negative human cervical cancer cells (C33A cells) and HPV16 positive human cervical cancer cells (SiHa cells) were grown in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 1% nonessential amino acids, 1% Glutamax-I and 10% fetal calf serum. The cells were maintained in the presence of 5% CO_2 at 37°C.

The day before transfection, $3x10^5$ SiHa cells per well were seeded in a 12-well plate. The cells were transiently transfected with 0.8 μg of expression vector pCMV4-16E2 or vectors pCMV4 containing E2 gene polymorphisms using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The expression of E2 was firstly determined at 24 and 48 hr after transfection. In these transfected cells, the function of E2 was evaluated for the expression of IL10 and TNF-α. The expressing level was evaluated by quantitative RT-PCR.

To investigate for the function on transcriptional regulation of E2, 1 ug of vector pCMV4-16E2 or vectors pCMV4 containing E2 gene polymorphisms were co-transfected in C33A cells with 0.2 ug of pGL3-16LCR or pGL3 containing LCR variation reporter vectors for transcriptional analysis. The pSV-β galactosidase control vector was co-transfected as the positive control. The transcriptional activity was analyzed using a Lucifererase assay.

Quantitative RT-PCR

Total RNA was isolated from the transfected C33A and SiHa cells by Trizol reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA was then DNase-treatd and reverse-transcribed to the cDNA using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Quntitative RT-PCR was performed with gene-specific primers for HPV16 E6, IL-10, TNF-α and the house-keeping gene β-actin (Sigma-Aldrich, MO, USA) as shown in Table 2 using the SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA). Threshold cycle numbers (CT) were determined with LightCycler® 480 SYBR Green I Master Real-time PCR (Roche Diagnostics, Penzberg, Germany) with the following thermal cycling conditions: an initial denaturation step at 95°C for 10 min, followed by 45 cycles at 95°C for 10 sec, at 56°C

Table 1. HPV16 E2 Nucleotide Sequence Variation Using for Vector Construction

HPV/sample no.	Nucleotide of HPV16 E2 gene									Variant classification
	TAD					Hinge region		DBD		
Nucleotide position	2828	3159	3249	3384	3410	3446	3524	3684	3787	
HPV16/Reference	G	C	G	T	C	G	T	A	C	Prototype
HPV16/no.38		A	A	C	T	A	C	A		As variant
HPV16/no.32		A	A	C	T	A	C	A	A	As sub variant

*TAD=transactivation domain, DBD=DNA binding domain, As variant=HPV16 Asian variant, As sub variant=HPV16 Asian sub variant

Table 2. Primers used for HPV16 E2/E6 and Host Genes Amplification

Genes	Primer sequence (5' to 3')	Positions	Product size (bp)
E2A	F: AGGACGAGGACAAGGAAAA	nt 2735-2753	334
	R: CAGTTAAATACACTTCAAGGCTAAC	nt 3044-3068	
E2B	F: GAAAAGTGGACATTACAAGACGTTA	nt 3023-3047	330
	R: CACAGATGTAGGACATAATATTACTG	nt 3327-3352	
E2C	F: TGCAGTTTAAAGATGATGCAG	nt 3261-3281	347
	R: GTTACTATTACAGTTAATCCGTCC	nt 3584-3607	
E2D	F: TCCAATCCTCACTGCATTTAAC	nt 3550-3571	324
	R: GGATGCAGTATCAAGATTTGTC	nt 3852-3873	
E6	F: GCAACAGTTACTGCGACGTG	nt 205-224	234
	R: GGACACAGTGGCTTTTGACA	nt 419-438	
TNF-α	F: TCTTCTCGAACCCCGAGTGA		151
	R: CCTCTGATGGCACCACCAG		
IL-10	F: GTGATGCCCCAAGCTGAGA		138
	R: CACGGCCTTGCTCTTGTTTT		
β-actin	F: TCACCCACACTGTGCCCATCTACGA		295
	R: CAGCGGAACCGCTCATTGCCAATGG		

(exception: IL-10 and TNF-α at 60°C) for 10 sec, and at 72°C for 1 min. The relative quantities of mRNA per sample were calculated using the ΔΔCT method and β-actin as the calibrator gene.

Luciferase assay

The co-transfected cells were analyzed at 24 hr by adding the Bright-Glo™ luciferase assay reagent (Promega, Madison, WI, USA) to the each well. Luciferase activity was measured with a Modulus Single tube Multimode reader (Turner BioSystems, Sunnyvale, CA, USA) following the manufacturer's instructions.

Statistical analyses

All real-time PCR data were expressed as mean±SD. Statistical analysis was done using an unpaired, Student's t-test by SPSS software. If $p \leq 0.05$, results were considered statistically significant.

Results

E2 gene sequence analysis

The results showed that in nucleotide sequences of E2 gene in all samples studied the HPV16 E2As variant was found the most as shown in Table 3. Four isolates (no. 143, 147, N47 and AL71) were the Asian-American (AA) variant and had E2 gene variations in the TAD region (A3181C, G3182A, G3249A and T3224A/C), and in the hinge region (C3516A and T3517A/C) and DBD (A3538C, T3566G, C3684A and T3694A). These sequence variations corresponded with the results of Veress's study (Veress et al., 1999). A few new positions of sequence variation such as 3313, 3368, 3507, 3650, 3670 and 3792 were identified in this study.

Twenty samples of episomal HPV16 genomes were investigated for E2 polymorphism. The E2 nucleotide sequence of DNA samples from FFPE cervical tissue samples (7 SCC and 2 HSIL) corresponded well to 11 cases from fresh cervical biopsy samples (6 SCC and 5 HSIL). Of the 32 consistent nucleotide changes, there were changes of 24 amino acids and 2 contiguous mutated (C3516A and T3517C) amino acids in the same position of 254. 14 nucleotide variations were found in the TAD. In this region, nucleotide changes at position 3118 and 3313 failed to show amino acid changes, while the remaining 12 nucleotide variations resulted in amino acid changes as indicated in Table 3. There were 5 and 13 nucleotide variations in the hinge region and the DBD. Most of nucleotide variations showed similar patterns to several previous studies.

The results of E2 gene sequence variations in the As variant were also found but fewer than the variations in AA variants such as C3159A, G3249A and T3384C in TA region, G3446A and T3524C in the hinge region and C3684A in the DB region (Table 3).

Expression of HPV16 E2 mRNA from the constructed vectors in SiHa cells

To confirm the constructed vectors containing E2 gene polymorphisms, each of the constructed pCMV4 expressing vectors containing E2 gene polymorphisms was transiently transfected into SiHa cell lines. The pCMV4-16E2 prototype was used as control. The results indicated that E2 mRNA was detected at 24 hr higher than 48 hr and showed similar patterns in all constructed vectors, but E2 mRNA was not detected in non-transfected SiHa cells (Figure 1).

The effect of HPV16 E2 gene polymorphism on HPV E6 mRNA expression in SiHa cells

To determine the effect of HPV16 E2 gene polymorphisms on regulation of HPV E6 transcription, the pCMV4 expressing vectors containing HPV16 E2 gene polymorphisms and HPV16 E2 prototype were transiently transfected into SiHa cells. Figure 2 shows the E6 mRNA levels that confirmed the function of E2 protein to regulate E6 mRNA expression that was continuously decreased at 24 and 48 hr. The expression level of E6 mRNA was lower in transfection with both vectors of E2 gene polymorphism than in the E2 prototype, but not with a statistically significant difference ($p=0.056$) (Figure 2). The E2 gene polymorphisms from two vectors showed an effect with similar patterns. These data demonstrated that E2 gene polymorphisms of episomal HPV16 did not affect regulation of HPV E6 transcription.

The effect of HPV16 E2 gene polymorphism on IL-10 and TNF-α mRNA expression in SiHa cells

To determine the effect of HPV16 E2 gene polymorphism on regulation of host genes (IL-10 and TNF-α) transcription, the pCMV4 expressing vectors containing HPV16 E2 gene polymorphisms and HPV16 E2 prototype were transiently transfected into SiHa cell. Figure 3 shows the expression of the IL-10 gene that was induced and increased with duration time of incubation. These results corresponded to the study of Bermudez-Morales VH et al. that showed binding activity of HPV16 E2 protein to the regulatory region of the IL-10 gene and induced levels of IL-10 mRNA in HPV infected cells (Bermudez-Morales et al., 2008). In addition, they also found that HPV E2 protein binds to the regulatory region of the human IL-10 gene and induces high promoter activity in epithelial cells (Bermudez-Morales et al., 2011). This study showed that HPV16 E2 prototype had higher activity of induction than HPV16 E2 gene polymorphisms, but not with a statistically significant difference ($p=0.061$). Interestingly, TNF-α was suppressed by all of E2 expressed vectors that had similar activity as shown in Figure 4. These results suggested that natural nucleotide variations of HPV16 E2 at the C-terminal or N-terminal may not affect E2 function.

The effect of HPV16 E2 gene polymorphism on p97 promoter activity in C33A cells

To determine the effect of HPV16 E2 gene polymorphisms on p97 promoter transcriptional activity, the reporter vectors containing LCR of HPV16 prototype (pGL3-16LCR) were co-transfected with pCMV4 containing E2 gene polymorphisms or the E2 prototype into C33A cells. The results showed that the p97 promoter activity was increased by HPV16 E2 when compared with LCR without HPV16 E2. The transcriptional

Table 3. Nucleotide Sequence Variation and Amino acid Prediction in the HPV16 E2 Gene Compared with the Reference Sequence

Samples no.	Nucleotide of HPV16 E2 gene																																			Variant classification
	TAD														Hinge region					DBD																	
Nucleotide position	2828	2860	3118	3159	3181	3182	3224	3249	3313	3362	3377	3384	3386	3410	3446	3507	3516	3517	3524	3538	3566	3650	3664	3670	3673	3684	3694	3706	3778	3787	3792	3805				
HPV16 prototype	G	C	T	C	A	G	T	G	T	A	C	T	A	C	G	G	C	T	T	A	T	A	T	T	A	C	T	T	G	C	T	T				
130	-	-	C	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	G	-	-	-	A	-	-	-	-	G	-	JAVA135C			
N6	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E350G			
N43	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E350G			
138	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	A	-	A	-	-	-	-	-	-	E			
143	-	A	-	A	C	A	A	A	-	G	G	-	C	T	A	T	A	C	-	C	G	-	C	-	-	A	A	C	-	-	-	-	AA			
147	-	A	-	A	C	A	A	A	C	C	-	-	-	T	-	-	-	-	-	-	-	-	C	-	G	A	A	-	T	A	-	G	AA			
N47	-	A	-	A	C	A	A	A	-	G	G	-	C	T	A	T	A	C	-	C	G	-	C	-	-	A	A	C	-	-	-	-	AA			
AL71	-	A	-	A	C	A	A	A	-	G	G	-	C	T	A	T	A	C	-	C	G	-	C	-	-	A	A	C	-	-	-	-	AA			
155	-	-	-	A	-	-	-	A	-	-	-	C	-	T	A	-	-	-	C	-	-	-	-	-	-	A	-	-	-	-	-	-	As			
297	-	-	-	A	-	-	-	A	-	-	-	C	-	T	A	-	-	-	C	-	-	-	-	-	-	A	-	-	-	-	-	-	As			
303	-	-	-	A	-	-	-	A	-	-	-	C	-	T	A	-	-	-	C	-	-	-	-	-	-	A	-	-	-	-	-	-	As			
587	A	-	-	A	-	-	-	A	-	-	-	C	-	T	A	-	-	-	C	-	-	-	-	-	-	G	-	-	-	-	-	-	As			
615	A	-	-	A	-	-	-	A	-	-	-	C	-	T	A	-	-	-	C	-	-	-	-	-	-	A	-	-	-	A	-	-	As			
N19	A	-	-	A	-	-	-	A	-	-	-	-	-	T	-	-	-	-	C	-	-	-	-	-	-	A	-	-	-	-	-	-	As			
N21	A	-	-	A	-	-	-	A	-	-	-	C	-	T	A	-	-	-	C	-	-	-	-	-	-	A	-	-	-	-	-	-	As			
N32	-	-	-	A	-	-	-	A	-	-	-	C	-	T	A	-	-	-	C	-	-	-	-	-	-	A	-	-	-	A	-	-	As			
N38	-	-	-	A	-	-	-	A	-	-	-	C	-	T	A	-	-	-	C	-	-	-	-	-	-	A	-	-	-	-	-	-	As			
AL74	A	-	-	A	-	-	-	A	-	-	-	C	-	T	A	-	-	-	C	-	-	-	-	-	-	A	-	-	-	-	-	-	As			
AL255	A	-	-	A	-	-	-	A	-	-	-	C	-	T	A	-	-	-	C	-	-	-	-	-	-	A	-	-	-	A	-	-	As			
	Predicted amino acid substitution																																			
Amino acid Position	25	35	121	135	142	143	157	165	186	203	208	210	211	219	232	251	254	254	257	261	271	299	303	305	306	310	313	317	341	344	346	350				
HPV16 prototype	D	H	F	T	E	A	L	R	V	N	P	I	I	P	E	C	T	T	L	S	F	K	Y	F	K	T	T	S	W	D	F	V				
Mutation	N	Q	F	K	D	T	I	Q	V	D	A	T	L	S	K	F	N	T	L	S	V	E	Y	L	N	K	T	S	C	E	C	V				

*TAD=transactivation domain, DBD=DNA binding domain, E=HPV16 European variant, AA=HPV16 Asian-American variant, As=HPV16 Asian variant

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E2 Gene Polymorphisms and Functions of the Episomal HPV16 E2 Gene

activity of p97 promoter was higher with HPV16 E2 gene polymorphisms (RLU=1,580) than with HPV16 E2 prototype (RLU=1,358). These promoter transcriptional activities were not significantly different (Figure 5). These results suggested that there were natural nucleotide variations of E2 that did not influence binding to the E2 binding site on LCR or alter the p97 promoter activity.

This study further hypothesized that the LCR containing nucleotide sequence variations (LCR variants), especially on the E2 binding site, may have an effect on promoter activity rather than on E2 gene sequence variations. Therefore, this experiment investigated the effect of the HPV16 E2 prototype on p97 promoter transcriptional activity of HPV16 LCR with nucleotide variations that consisted of LCR As (no.36) and LCR As-sv14 (no.42) reporter vectors. The pGL3-16LCR prototype was used as the control. These pGL3-16LCR reporter vectors were co-transfected with or without pCMV4-16E2 prototype into the C33A cell line. The transcriptional activities of p97 promoter on reporter vectors were analyzed by luciferase assay. The results showed that the p97 promoter activity was increased by HPV16 E2 when

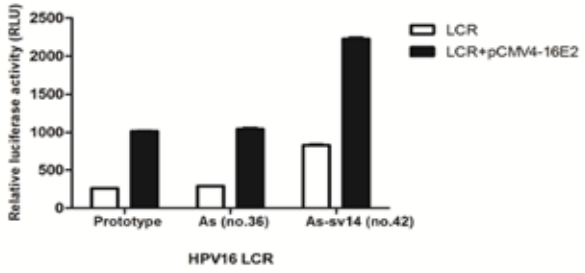

Figure 1. Expression Levels of HPV16 E2 mRNA from the Constructed Vectors in SiHa Cells. The pCMV4 containing E2 gene polymorphisms (Samples no.38; As variant and no.32; As sub-variant) and pCMV4-16E2 prototype were transiently transfected into the SiHa cell line. After 0, 24 and 48 hr of transfection, the expression levels of E2 mRNA were determined by quantitative RT-PCR

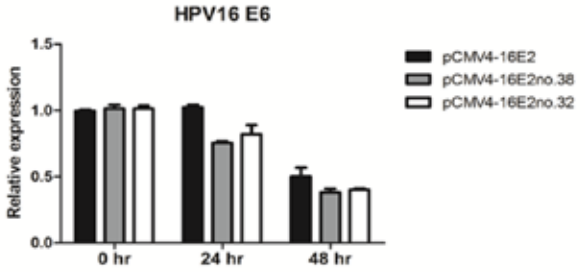

Figure 2. HPV16 E2 Gene Polymorphisms Effect on HPV16 E6 Expression in SiHa Cells. The pCMV4 containing E2 gene polymorphisms (Samples no.38; As variant and no.32; As sub-variant) and pCMV4-16E2 prototype were transiently transfected into SiHa cell line. After 0, 24 and 48 hr of transfection, the expression levels of E6 mRNA were determined by quantitative RT-PCR

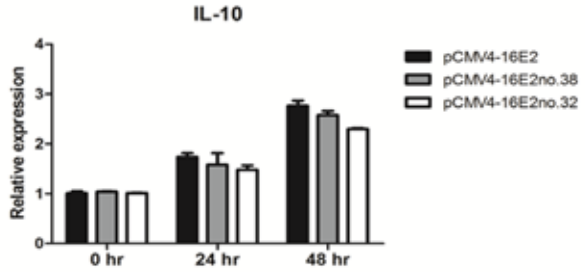

Figure 3. HPV16 E2 Gene Polymorphisms Effect on IL-10 Expression in SiHa Cells. The pCMV4 containing E2 gene polymorphisms (Samples no.38; As variant and no.32; As sub-variant) and pCMV4-16E2 prototypes were transiently transfected into SiHa cell line. After 0, 24 and 48 hr of transfection, the expression levels of IL-10 mRNA were determined by quantitative RT-PCR

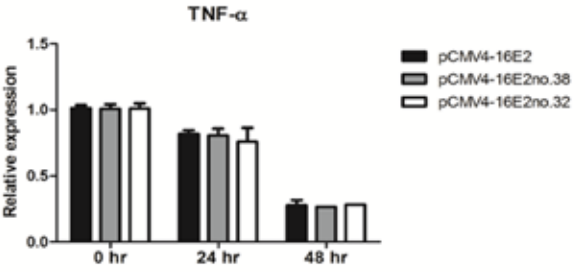

Figure 4. HPV16 E2 Gene Polymorphisms effect on TNF-α Expression in SiHa Cells. The pCMV4 containing E2 gene polymorphisms (Samples no.38; As variant and no.32; As sub-variant) and pCMV4-16E2 prototypes were transiently transfected into SiHa cell line. After 0, 24 and 48 hr of transfection, the expression levels of TNF-α mRNA were determined by quantitative RT-PCR

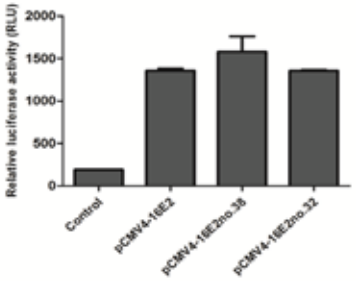

Figure 5. HPV16 E2 Gene Polymorphisms effect on p97 Promoter Activity in C33A Cells. The pCMV4 containing E2 gene polymorphisms (Samples no.38; As variant and no.32; As sub-variant) and pCMV4-16E2 prototype were co-transfected with pGL3-16LCR into the C33A cell line. After 24 hr of co-transfection, luciferase activity levels were measured

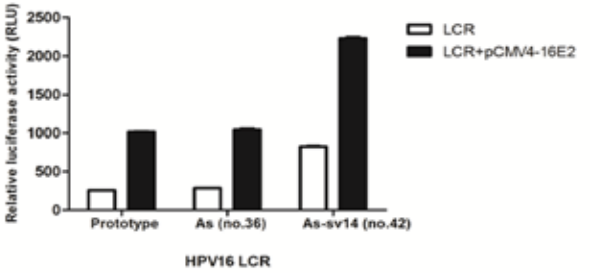

Figure 6. Transcriptional Activity of the LCRs from the HPV16 Prototype, As and As-sv14. The pGL3 containing LCR with nucleotide variation (As; no.36 and As-sv14; no.42) and pGL3-16LCR prototype were co-transfected with or without pCMV4-16E2 prototype into the C33A cell line. After a 24 hr of co-transfection, luciferase activity levels were measured

compared with transcriptional activity without the E2 gene. The pGL3-LCR As-sv14 reporter vector (no.42) showed the highest transcription activity (RLU=2,224), whereas transcriptional activity between pGL3-LCR As (no.36) (RLU=1,043) and the pGL3-16LCR prototype (RLU=1,014) were not significantly different (Figure 6). These results suggested that transcriptional activity of p97 promoter may be affected by nucleotide sequence variation on the LCRs.

Discussion

In the present study, the polymorphisms and amino acid changes of the E2 gene in episomal HPV16 detected in HSIL and SCC that may be the cause of dysregulation of the HPV oncogenes or the host genes during cervical carcinogenesis were analyzed.

The data indicated that the HPV16 intact E2 gene contains extensive base changes distributed throughout the entire gene, including the TAD, hinge region and DBD. According to E2 gene variation, most of HPV16 intact E2 samples were classified to Asian variants (12 samples) and Asian-American variants (4 samples). The results also found that 4 isolates (no. 143, 147, N47 and AJ71), which belong to the AA variant had nucleotide variations in the TAD (A3181C, G3182A, G3249A and T3224A/C), hinge region (C3516A and T3517A/C) and DBD (A3538C, T3566G, C3684A and T364A) corresponding with the reports by Veress's study (Veress et al., 1999).

The HPV16 E2 protein at TAD has been shown to interact with several cellular proteins such as Spl, TBP, TFIIB and TFIID (Demeret et al., 1994; Tan et al., 1994; Steger et al., 1995; Yao et al., 1998). DBD has been shown to bind to the DNA binding site on LCR and regulates several gene expressions such as HPVE6/E7 oncogenes and the host gene (IL-10 and telomerase) (Bermudez-Morales et al., 2008). In addition, DBD can also bind to viral and cellular proteins such as E6, Mdm-2 and PARP (Lee et al., 2002; Grm et al., 2005; Gammoh et al., 2009). The hinge region forms a flexible link between the TAD and DBD and has shown to interact with Sp1 (Zou et al., 2000). These interactions of HPV16 E2 contributed to the regulation of viral and host gene expression. Therefore, sequence variations of HPV16 E2 may lead to the deregulation of the HPV oncogenes or the host genes in transforming HPV infections.

The function of HPV16 E2 gene polymorphisms on the HPV E6 oncogene and host genes (IL-10 and TNF-α) expression in SiHa cells were determined. The results showed that the functions of HPV16 E2 gene polymorphisms had no affect on HPV E6, IL-10 and TNF-α gene expression compared with the HPV16 E2 prototype. This result corresponded with the study of Veress, et al. about the function of HPV16 E2 gene sequence variation on the LCR of the HPV16. They found that the HPV16 E2 gene sequence variation was not the major mechanism in the regulation of transcription on the p97 early promoter of HPV 16 (Veress et al., 1999). This experiment suggested that HPV16 E2 with nucleotide variations at the C-terminal or N-terminal did not affect viral and host gene expression compared to the HPV16 E2 prototype, suggesting that the nucleotide sequence variation of other regions of the HPV genome may have an effect on gene expression that is associated with carcinogenesis.

To confirm the function of HPV16 E2 gene polymorphisms on p97 promoter activity, pCMV4 expression vectors containing HPV16 E2 gene polymorphisms were co-transfected with reporter vectors containing the LCR of HPV16 prototype (pGL3-16LCR). The result showed that both HPV16 E2 gene polymorphisms did not show different effects from HPV16 E2 prototype on p97 promoter activity. When reporter vectors containing LCR of HPV16 with nucleotide variations such as pGL3-LCR As (no.36) and pGL3-LCR As-sv14 (no.42) that were co-transfected with pCMV4-16E2, the promoter transcriptional activity of pGL3-LCR As (no.36) was higher than the LCR prototype and pGL3-LCR As-sv14 (no.42) and showed the highest transcriptional activity (Figure 6). These results suggested that the oncogenic potential of HPV16 could be influenced by sequence variation in the HPV16 LCR regulatory region. The reporter vector pGL3-LCR As-sv14 (no.42) contains sequence variations at the nucleotide position in binding sites of Sp1 (28G > A) and the E2 binding sites proximal to the promoter (46T>G and 61T>G). The HPV16 E2 proteins bind to binding sites that overlap binding sites of the cellular SP1 and TFIID transcription factors suggesting that the variation of this site may affect affinity of HPV16 E2 protein resulting in uncontrolled repression of the p97 promoter and may lead to over-expression of HPV E6 and E7 oncogenes. These results were similar to a previous report. Veress et al. reported that the transcriptional activity of the HPV16 LCR variants (HPV16 AA variant) in C33A cells had higher transcriptional activity than the HPV16 LCR prototype (Veress et al., 1999). In addition, the HPV16 LCR with mutation showed increased promoter activity higher than the wild type (Chen et al., 1997; Pientong et al., 2013).

In summary, this result revealed that E2 gene polymorphisms of episomal HPV16 did not affect HPV oncogenes or host gene transcriptional regulation and suggested that nucleotide sequence variation as well as epigenetic modification on the LCR might play a role to induce malignant transformation of cells containing episomal HPV16.

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