Human Papillomavirus 16 (HPV16) and it's E2 Polymorphisms in High-Grade Squamous Intraepithelial Lesion (HSIL) and Squamous Cell Carcinomas (SCC)

การวิเคราะห์รูปแบบการเปลี่ยนแปลงของจีน E2 ของ HPV16 ที่อยู่ในรูป Episomal ในตัวอย่างที่เป็น High-Grade Squamous Intraepithelial Lesion (HSIL) และ Squamous Cell Carcinomas (SCC)

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ABSTRACT

The E2 protein of HPV is the viral factor that binds to the specific sites on long control region of the viral genome to regulate genome replication and transcription of the viral oncogenes, E6 and E7. To determine the polymorphisms in the E2 DNA sequence and amino acid change in HPV16 episomal form, this study amplified and sequenced the E2 gene of HPV16 episomal form that selected from 241 paraffin-embedded tissues with HSIL and SCC. Results showed 91.70% of samples were positive for HPV DNA and HPV16 was the most common, 77.68% in HSIL and 85.83% in SCC. 13 of 40 positive HPV16 cases were found containing E2 episomal form that were classified mostly into As variant. Nucleotide and amino acid changes were demonstrated in 5 cases and mostly found in TAD of both HPV16 AA and As variant. These results suggested that polymorphisms of E2 protein may impair the transcriptional regulation of vial oncogenes that induce malignant transformation of HPV infected cell in episomal form.

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บทคัดย่อ

โปรตีน E2 ของเชื้อ HPV เป็นปัจจัยหนึ่งที่สำคัญของไวรัส ที่ใช้จับกับตำแหน่งจำเพาะบริเวณ long control regionในจีโนมของไวรัส เพื่อควบคุมการเพิ่มจำนวนและการถอดรหัสของจีน E6 และ E7 ซึ่งมีความสำคัญในการพัฒนาไปเป็นมะเร็งของเซลล์โฮสต์ การศึกษาครั้งนี้จึงทำการวิเคราะห์รูปแบบการเปลี่ยนแปลงนิวคลีโอไทด์และกรดอะมิโนของจีน E2 ที่อยู่ในรูป episomal form ด้วยวิธี sequencing ในตัวอย่างเนื้อเยื่อปากมดลูกที่ผิดปรกติชนิด HSIL และ SCC จากการทดลองใน 241 ตัวอย่าง ตรวจพบดีเอ็นเอของเชื้อ HPV 91.70% ส่วนใหญ่เป็นการติดเชื้อ HPV16 ซึ่งพบร้อยละ77.68 และ 85.83 ในกลุ่มที่เป็น HSIL และ SCC ตามลำดับและพบว่ามี 13 ตัวอย่างจาก 40 ตัวอย่างของ HPV16 ที่อยู่ในรูป episomal form ซึ่งส่วนใหญ่จัดเป็น As variant นอกจากนี้ 5 ตัวอย่างได้นำมาทำการศึกษาการเปลี่ยนแปลงนิวคลีโอไทด์และกรดอะมิโน ผลการศึกษาพบว่าการเปลี่ยนแปลงเกิดได้มากในบริเวณ transactivation domain ของทั้ง HPV16 AA variant และ As variant จากการศึกษาครั้งนี้ อาจกล่าวได้ว่า การเกิดการเปลี่ยนแปลงรูปแบบของโปรตีน E2 น่าจะมีผลทำให้การควบคุมการถอดรหัสของ oncogenes ของไวรัสบกพร่องไปและทำให้เซลล์ติดเชื้อที่อยู่ในรูป episomal form มีการพัฒนาไปเป็นมะเร็งปากมดลูกได้

คำสำคัญ : เชื้อฮิวแมนแพบพิวโลมาไวรัส 16 E2 episomal form รูปแบบการแปลี่ยนแปลงของจีน

Key Words : Human papillomavirus (HPV) 16, E2 episomal form, Polymorphisms

Introduction

High risk human papillomaviruses (HR-HPV) are the single most important risk factor of cervical cancer (zur Hausen 2000). The most common HR-HPV type in squamous cervical ancer is HPV16, found in over 50% of the cases (Bosch et al 1995). Integration of viral DNA into the host cell chromosome is considered to provide a selective growth advantage to the infected cells, because integration often disrupts the E1 and E2 open reading frames (ORF), while the E6 and E7 ORFs and long control region (LCR) remain intact (Kulmala *et al* 2006). The E1 and E2 proteins have the function as an essential for viral DNA replication and control of gene transcription. E2 protein is composed of two functional domains, a N-terminal transactivation domain (TAD) and a C-terminal DNA binding domain separated by hinge region and has been shown to act either as an activator or a repressor, depending on the promoter context. The E2 protein represses transcription of E6 and E7 by binding to E2 binding sites in the long control region (LCR). The E1 facilitates the binding of E2 protein in the promoter region. The disruption of E2 gene results in lack of expression of E2 protein and subsequent upregulation of the oncogenic E6 and E7 proteins. Continuous expression of these proteins contributes to malignant transformation(Cricca *et al* 2007).

Normally, HPV genome exists as an episomal form in pre-neoplastic lesion when infected with low-risk or high-risk oncogenic type but most of high-risk oncogenic HPV DNA detected in malignant and invasive cervical cancer are usually integrated into host chromosomes by interruption of the E2 DNA portion (Hillemanns and Wang 2006). In fact, some investigators detected an early integration in preneoplastic lesions

(Huang *et al* 2008), whereas other observed integration almost exclusively in invasive cervical cancer (Tonon et al 2001).

Although several studies have reported the detection of integrated HPV sequence in the majority of invasive cervical cancer cases, but some studies reported that 16.7% of the CIN 3 group and 10% of the squamous cell carcinomas group harbored pure episomal genomes (Zheng *et al* 2006). It is interesting that why E2 gene from the episomal HPV16 can not control the expression of E6 and E7 oncogene and with this condition the infected cells can progress to cervical neoplasia.

Polymorphisms within the E2 region may have an impact on the oncogenic potential of the virus. In this study, we purposed to investigate for HPV16 from paraffin embedded tissue with HSIL and SCC of Thai women, subsequently determine for E2 gene polymorphisms within HPV16 episomal and amino acid prediction.

Materials and methods

Samples

The clinical material used in the present study consisted of 241 paraffin-embedded cervical tissue biopsies from women in 4 regions of Thailand. These were all histopathologically confirmed as 121 high-grade squamous intraepithelial lesions (HSIL) and 120 squamous cell carcinomas (SCC).

DNA extraction and HPV DNA detection

Two to five pieces of paraffin sections (5-10 μm) were placed in 1.5 ml plastic tube and incubated at 60°C for 30 minutes. The sections were deparaffinized by adding 1200 μl of xylene, and then centrifuged 14,000 rpm for 5 min at room temperature and the supernatant were discarded. The step of deparaffin with xylene was repeated twice. Then 1200 μl of absolute ethanol was added, mixed and centrifuged at 14,000 rpm for 5 min at room temperature and the supernatant was discarded. After removing supernatant, the open tube was incubated at 37°C until the ethanol evaporated. The DNA extraction was performed according to manual instruction of the QIAamp DNA mini kit (Qiagen, germany).

GP5+/6+ consensus primers, which amplify 150 bp DNA sequence within the L1 region of HPV, were used to detect generic HPV DNA. Amplification were carried out in 50 μl of reaction mixture contained 1X PCR buffer, 3 mmole $MgCl_2$, 0.2 mmole of each dNTP, 50 pmole of each primer and 1.25 U Taq DNA polymerase. The amplification were performed for 40 cycles, with initial denaturizing at 94°C for 4 minutes; each cycle was performed as followed at 94°C for 1 minutes, 42°C for 1 minutes and 72°C for 30s; and final extension at 72°C for 4 minutes. The B-globin primers, which amplify a 268 bp region of the human DNA, were used as an internal control. PCR products were analyzed on 1.5% agarose gel with ethidium bromide staining for visualization of DNA under ultraviolet light.

HPV genotyping by Reverse Line Blot Hybridization

HPV DNA was amplified by PCR using biotin-labeled GP5+/6+ primers (Invitrogen Life Technologies, Carlsbad, CA, USA). Fifty micorlitres of amplification mixture contained 1X PCR buffer, 3 mmole MgCl2, 0.2 mmole of each dNTP, 50 pmole of each primer and 1.25 U Taq

DNA polymerase. DNA amplifications were performed for 40 cycles with the following parameters; initial denaturing at 94 °C for 4 min; each cycle at 94°C for 1 min, 42°C for 1 min and 72°C for 30 sec; final extension at 72°C for 4 min. PCR products were analyzed on 1.5% agarose gel with ethidium bromide staining for visualization of DNA under ultraviolet light.

Reverse line blot hybridization was performed using previously described (van den Brule *et al* 2002). The system is based on the use of a miniblotter for spotting in parallel up to 37 different specific oligoprobes containing a 5′-amino group on a carboxyl-coated nylon membrane. Briefly, membrane (Biodyne C) was activated by using 16% (w/v) 1-ethyl-3-(3-dimethy laminopropyl) carbodiimide (EDAC) and placed in miniblotter system. 37 different HPV probes were dropped on the nylon membrane through the slots of miniblotter system in parallel lines. The probes were covalently bound to negative charged membrane. After 1 min of incubation, the channels were aspirated and the membrane was inactivated using 100 mM NaOH. For hybridization, biotinylated GP5+/6+ PCR product was diluted, heat denatured, and rapidly cooled on ice. The PCR products were pipetted into the parallel channels perpendicular to the rows of oligoprobes. Hybridization was performed at 42 °C for 1 h, subsequently incubated with streptavidin-peroxidase conjugate for 1 h at 42 oC (1:6000 dilution in 2X SSPE/0.5% SDS), washed in 2X SSPE/0.5% SDS, and rinsed in 2X SSPE for 5 min. Detection was done by using LumiGLO (KPL Inc., Gaitherburg, MD, USA) for 1 min and exposed to an X-ray film (Eastman Kodak, Co., New York, USA) for 10 min in cassettes. After film exposure, the film was continuously processed in the dark.

E2 amplification by Polymerase Chain Reaction (PCR) and sequencing

HPV-16 positive samples were subjected to PCR amplification of the E2 ORF using a four set of overlapping primers as shown in Table1. The reaction mixture (50 µl) contained 3 µl target DNA, 1X Taq buffer with $(NH_4)_2 SO_4$, 2.5 mM $MgCl_2$, 0.2 mM dNTP and 1 U of Taq DNA polymerase (Fermentus). The primer sequences and amplicon size are summarized in Table 1. The presence of E2 amplicon in agarose gel confirmed an intact E2, while lack of specific band indicated E2 gene disruption. Plasmid HPV16, which contains an intact E2, was used as a positive control.

Table 1 Primer sequences and product size of the E2 gene

E2 primer (5'-3')	Product size (bp)
E2A1 :AGGACGAGGACAAGGAAAA	327
E2A2 : CAGTTAAATACACTTCAAGGCTAAC	
E2B1 :GAAAAGTGGACATTACAAGACGTTA	329
E2B2 :CACAGATGTAGGACATAATATTACT	
E2C1 :TGCAGTTTAAAGATGATGCAG	346
E2C2 :GTTACTATTACAGTTAATCCGTCC	
E2D1 :TCCAATCCTCACTGCATTTAAC	329
E2D2 : GGATGCAGTATCAAGATTTGTC	

Sequence analysis of E2

SEQ files thus generated were analyzed using the MegaBACE sequence analyzer software, which assigns quality scores to each base. The MegaBACE outputs for the given amplicons were then aligned using Multalin software (http://bioinfo.genotoul.fr/multalin/multalin.html) and allows identification of sequence differences as well as access to the individual chromatograms to consider each putative variant. Amino acid change was analyzed using ExPASy proteomics tool (http://au.expasy.org/tools/translate)

Results and discussion

Two hundred and fourteen Paraffin-embedded cervical tissue biopsies with HSIL and SCC were tested for HPV DNA by PCR using GP5+/6+ primers. Ninety-one point seven percent (221/241) of them were HPV DNA positive, with a prevalence of 90.08% (109/121) in women with HSIL and 93.33% (112/120) in women with SCC (Table 2). These results corresponded well with the study in Paraffin-embedded cervical tissue biopsies, especially, of SCC cases in Northern and Southern Thailand that HPV DNA was detected in 96.9% (Siriaunkgul *et al* 2008), 95.3% (Chichareon *et al* 1998), respectively. However, it is lower than the prevalence seen in Paraguay (97% in the SCC cases) as well as in the worldwide prevalence (99.7%) (Walboomers *et al* 1999). In addition, the HPV detection in this study is higher than the prevalence found among the same groups of Chinese women (82.7% and 88.5%, respectively) (Bao *et al* 2007) and in Costa Rica showed HPV DNA in 88.4% of high-grade lesions and cancers (Schiffman *et al* 2000). This difference may be due to a variety of sample types and techniques used such as DNA extraction techniques, HPV detection methods, primers used in the PCR and inherent differences in the population.

Table 2 Prevalence of human papillomavirus 16 (HPV16) infection of the cervix among 241 women

Women with abnormal cervical lesions	HPV DNA positive (%)	HPV DNA negative (%)
HSIL (121)	109 (90.08)	12 (9.92)
CA (120)	112 (93.33)	8 (6.67)
Total (241)	221 (91.70)	20 (8.3)

Among women with HPV infection, 13 HPV genotypes were detected (data not shown) and HPV16 was the most common in 77.68% and 85.83% of HSIL and SCC, respectively. HPV infections with multiple types were found in 54.54% and 56.67% of HPV positive women (91.70%). In this result, HPV16 as well as multiple infection was higher than reports by others parts of Thailand that was reported about 57-59% of HPV16 and 4-21% of multiple infection (Chichareon et al 1998; Siriaunkgul et al 2008). In Taiwan women, Huang LW,et al (Huang et al 2004) reported the prevalence of HPV16 and multiple HPV infection in paraffin embed tissues of SCC cases about 51.5% and 26.5% respectively. These differences are most likely related to techniques used. We used nested PCR that can amplify HPV even in the cases of low viral load or fragmented DNA sequences, leading to a higher rate of detection and also used RLBH providing for high rate of multiple type detection.

HPV16 E2 episomal

Forty cases of HPV16 positive samples were random selected for E2 full length amplification using overlap primers, only 13 samples were positive for the presence of E2 episomal form, whereas 27 samples were negative suggesting an integrated forms of HPV genome or may be disrupted from chemicals in paraffin embedded process. All of 13 samples with E2 episomal were compared to previous study (Unpublished data) and proved to be HPV16 variants as showed in Table 3. and HPV16 As variant was the most common.

E2 polymorphisms

Five cases containing E2 episomal form were performed for nucleic acid polymorphism and amino acid change as shown in Table 4. In all 5 cases of E2 episomal, nucleotide changes were identified in a total 24 positions, 11 positions in transactivation domain and DNA binding domain and 2 positions in hinge region. 17 of them had amino acid changes.

The result showed that variation in TAD was prominent such as amino acid position 25 change from Asp to Asn and position 35 change from His to Glu suggesting that the regulation of viral gene transcription may be impaired since this region is respond to bind to E1helicase and to cellular transcriptional factors. The previous study supported that variation at the TAD as the H35Q mutation (position 2860 C to A) within the E1 interaction site might affect the formation of E1-E2 heterodimer, which are known to be important in viral DNA replication (Graham and Herrington 2000). In particular, substitutions of arginine 37 for alanine and of isoleucine 73 for leucine or alanine have been shown to impair the ability of E2 to activate and repress transcription (Abroi *et al* 1996; Nishimura *et al* 2000) since these amino acids are located near each other in the 3-dimensional structure of the TAD and they define a conserved surface involved in binding Brd4 to regulate viral gene transcription (Senechal *et al* 2007).

Table 3 HPV16 variant among women with HSIL and SCC

Samples no.	Histological diagnostic	Variant
130	CA	Java 135 C
133	CA	AA
137	CA	AS
138	CA	E
144	CA	AA
147	CA	AA
155	CA	AS
297	HSIL	AS
303	HSIL	AS
587	CA	AS
615	CA	AS

However, amino acid changes in DNA binding domain (DBD) were frequently detected in only AA variants. The 3684 C-A variation (Thr to Lys) in the DNA binding domain was identified in all samples. Sathish and colleauge suggested that this position variation is near the DNA binding helix of the E2 protein, so it could alter the conformational structure of this helix and the conformation of the E2 protein (Sathish *et al* 2004). Moreover, in this study, variation pattern of E2 As variants are similar to AA variant

that was suggested to have higher oncogenic potential than prototype (Veress *et al* 1999). This result suggested that E2 episomal gene polymorphism, may modulate E2 function and support hypothesis that although the presence of an intact E2 gene, the numerical chromosome abnormalities in HPV infected cell may be induced.

Conclusions

In this study demonstrated that HPV16 was the most common type in HSIL and SCC. Some HPV16 positive samples contained HPV episomals that were classified mostly into HPV16 As variant. Polymorphism of E2 in episomal form occurred in both two functional domain. Interestingly, variation in TAD was dominant in both HPV16 AA and As variants supporting functional modulation of E2 protein in the intact genome of HPV16 found in episomal form of HPV16 associated cervical carcinomas development.

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Table 4 Polymorphisms of E2 DNA sequence and amino acid change of HPV16 E2 episomal form

Nucleotide	Amino acid	130 (Java 135 C)	138 (E)	147 (AA)	587 (AS)	615 (AS)
TAD						
2828 G	25 Asp				A(Asn)	A(Asn)
2860 C	35 His			A(Gln)		
3118 T	121Phe	C (Phe)				
3159 C	135Thr			A(Lys)	A(Lys)	A(Lys)
3181 A	142Glu			C(Asp)		
3182 G	143Ala			A(Thr)		
3224 T	157Leu			A(Ile)		
3249 G	165Arg			A(Gln)	A(Gln)	A(Gln)
3313 T	186 Val			C(Val)		
3384 T	210 Ile			C(Thr)	C(Thr)	C(Thr)
3410 C	219 Pro	T(Ser)	T(Ser)	T(Ser)	T(Ser)	T(Ser)
Hinge						
3449 G	232Glu				A(Lys)	A(Lys)
3524 T	257Leu				C(Leu)	C(Leu)
DBD						
3650 A	299 lys	G(Arg)				
3664 T	303 Try			C(Try)		
3670 T	305Phe		A(Leu)			

Table 4 Polymorphisms of E2 DNA sequence and amino acid change of HPV16 E2 episomal form (cont.)

Nucleotide	Amino acid	130 (Java 135 C)	138 (E)	147 (AA)	587 (AS)	615 (AS)
3673 A	306Lys			C(Asn)		
3684 C	310 Thr	A(Lys)	A(Lys)	A(Lys)	G(Lys)	A(Lys)
3694 T	313 Thr			A(Thr)		
3706 T	317 Ser			T(Ser)		
3778 G	341Trp			T(Cys)		
3787 C	344Asp			A(Glu)		A(Glu)
3792 T	346Phe	G(Cys)				
3805 T	350 Val			G(Val)		

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