CHAPER IV
RESULTS

1. HPV DNA detection

A total of 614 FFPE cervical tissues histologically diagnosed by a team of pathologists and comprised of 204 normal, 169 LSIL, 121 HSIL and 120 SCC were included in this study. These cases were selected from the 4 regions of Thailand including 155 cases from the north, 155 cases from the northeast, 182 cases from the south and 122 cases from the central regions. For qualification of the extracted DNA, all of them were tested by PCR for a 268-bp fragment of the β-globin gene (the housekeeping gene). The result showed that all of cases were positive for β-globin gene that showed a good quality of the DNA. Figu re 11 demonstrates the PCR products of β-globin gene (268 bp) from the samples.

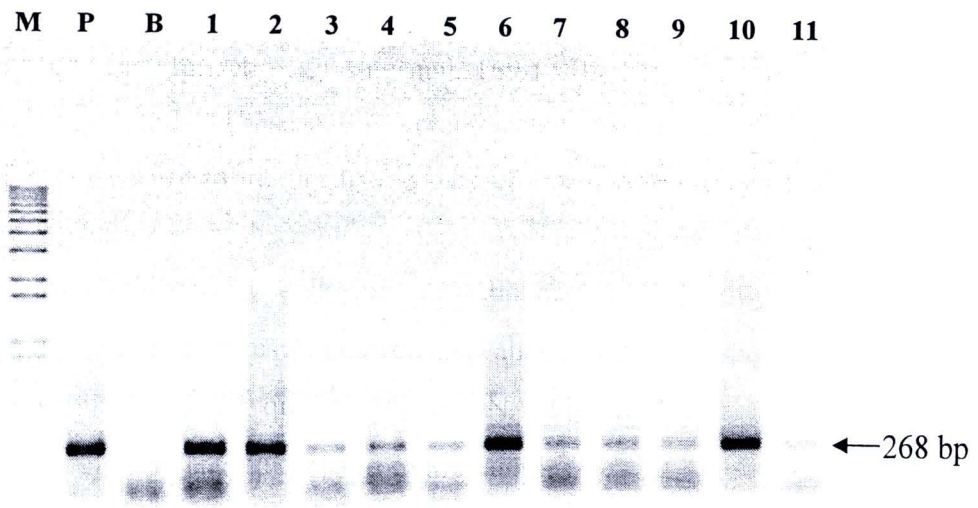

Figure 11 DNA qualification. PCR amplification for a 268-bp fragment of the β-globin gene. PCR products were tested on an ethidium bromide stained 1.5% agarose gel electrophoresis. Lane M; 1 Kb+ marker, Lane P; positive control, Lane B; blank, Lanes no.1-11 represent DNA samples positive for β-globin gene.

All of 614 qualified samples were investigated for HPV DNA by PCR using GP5+/GP6+ consensus primers for a conserved 142-bp segment in the L1 ORF of HPVs. Figure 12 demonstrates the PCR products of HPV DNA positive samples.

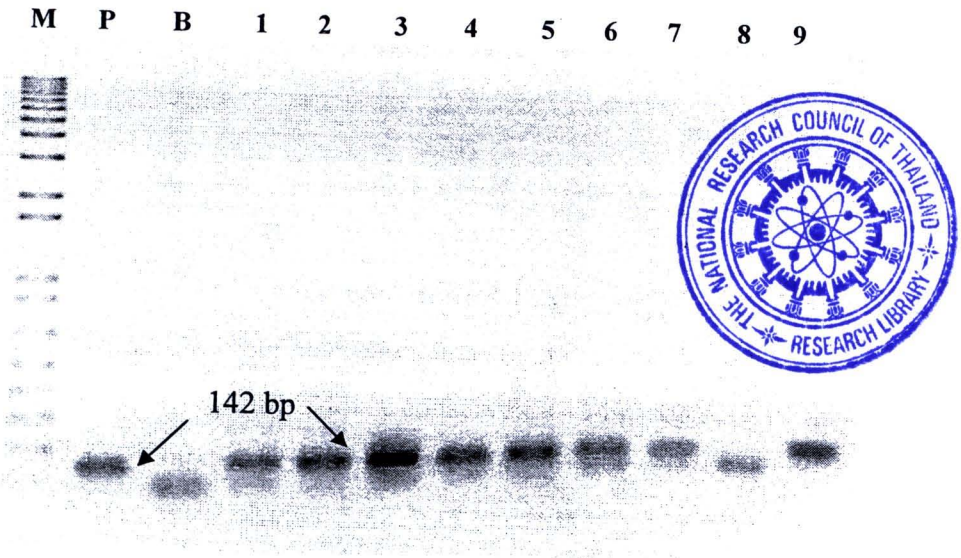

Figure 12 HPV DNA detection. PCR products were tested on an ethidium bromide stained 1.5% agarose gel electrophoresis. Lane M; 1 Kb+ marker, Lane P; positive control, Lane B; blank, Lanes 1-7 and 9 represent HPV DNA positive samples, Lane 8 represents HPV DNA negative sample.

The prevalence of HPV DNA was 91.19% (186/204) of normal, 84.62% (143/169) of LSIL, 90.08% (109/121) of HSIL and 93.33% (112/120) of SCC as shown in Table 8

Table 8 The prevalence of HPV DNA in 614 FFPE cervical tissues histological diagnosed as normal, LSIL, HSIL and SCC using GP5+/GP6+ primers PCR amplification.

Histological diagnosis*	Tatal no.	HPV DNA Detection	
		Positive n, (%)	Negative n, (%)
Normal	204	186 (91.18%)	18 (8.82%)
LSIL	169	143 (84.62%)	26 (15.38%)
HSIL	121	109 (90.08%)	12 (9.92%)
SCC	120	112 (93.33%)	8 (6.67%)
Total	614	550 (89.58%)	64 (10.42%)

* LSIL, low grade squamous intraepithelial lesion; HSIL, high grade squamous intraepithelial lesion; SCC, squamous cervical cell carcinoma

The highest prevalence of HPV DNA detection was found in SCC cases and the prevalence decrease with the degree of disease severity, exception to the normal case.

2. Prevalence of HPV genotypes in Thai women

For determination of the distribution of HPV genotype infection, all of HPV positive samples were subjected for HPV genotyping by RLBH assay using 37 HPV types–specific 5'-amino-linked oligonucleotide probe. Figure 13 shows the positive hybridization signal of HPV genotypes that were detectable as black spots. The difference of spot intensity might reflect the amount of PCR products from different viral load samples. Thirteen of 550 HPV DNA positive samples (8 of normal, 2 of LSIL, 2 of HSIL and 1 of SCC) could not be genotyped. These samples might contain other HPV DNA genotypes out of 37 HPV oligonucleotide probes used in RLBH assay.

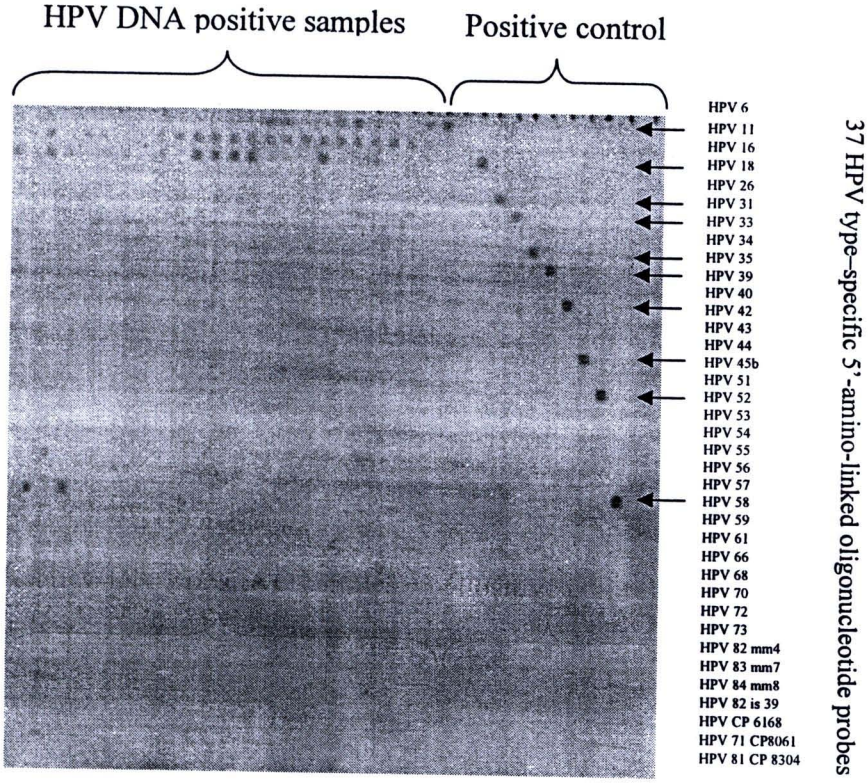

Figure 13 HPV genotype detection by RLBH assay using 37 HPV type–specific 5' amino-linked oligonucleotide probes hybridized with GP5+/biotin labeled GP6+ PCR products derived from HPV DNA positive samples and 10 types HPV plasmid were used as positive control including HPV 11, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 42, HPV 45, HPV 52 and HPV 58.

A total of 20 HPV genotypes were identified in Thai women. The most common HPV genotype was HPV 16 (86.00%). Figure 14 shows HPV genotype distribution and frequency of infections among HPV positive samples including HPV 18 (67.27%), HPV 11 (34%), HPV 58 (6.36%), HPV 45 (4.36%), HPV 52 (2.91%), HPV 56 (2.18%), HPV 31, 39 and 70 (2%), HPV 33 (1.82%), HPV 40 and 66 (1.09%), HPV 43 (0.91%), HPV 6 (0.73%), HPV 51 (0.55%), and HPV 35 and 42 (0.36%), HPV 82-IS39 and 81-CP8304 (0.18%) The top five of HR-HPV infection in

Thai women are HPV 16 (85.82%), HPV 18 (67.27%), HPV 58 (6.36%), HPV 45 (4.36%) and HPV 52 (2.91%).

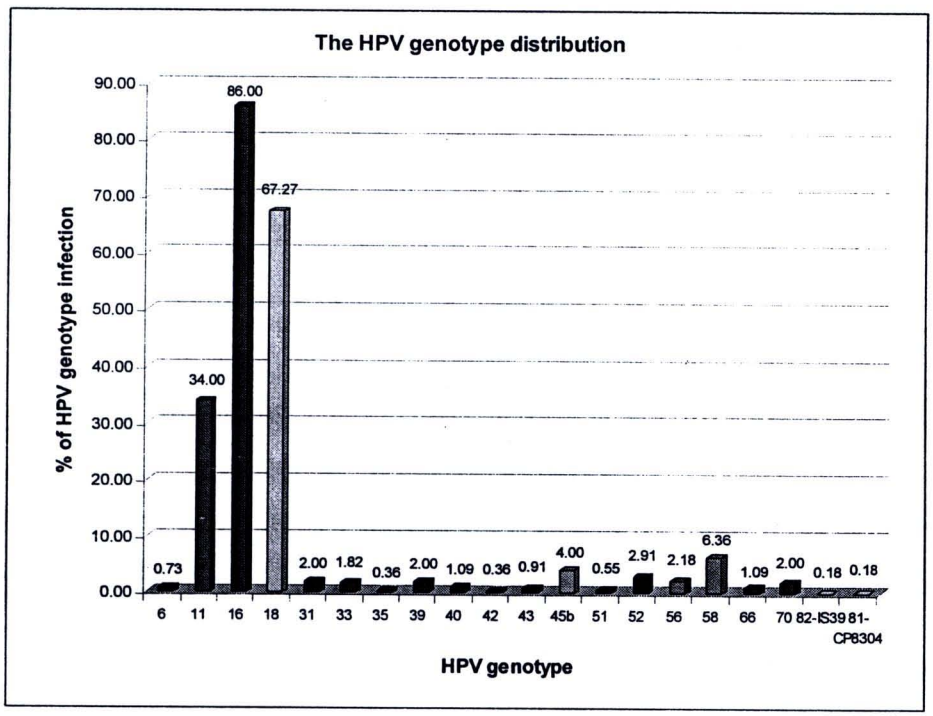

Figure 14 HPV genotype distribution and frequency of infection among HPV DNA positive samples from Thai women histologically diagnosed with normal, LSIL, HSIL and SCC.

This study detected three patterns of HPV infection that were single (one HPV type) infection, double (two HPV types) infections and triple+ (three or more than three HPV types) infections. The prevalence of HPV triple+ infections that was mostly detected in normal cases, decreased according to disease severity, whereas, the HPV single infection increased. The most frequently found in SCC cases was the single HPV type infection (37.5%), whereas only 12.19% in normal cases as shown in the Figure 15.

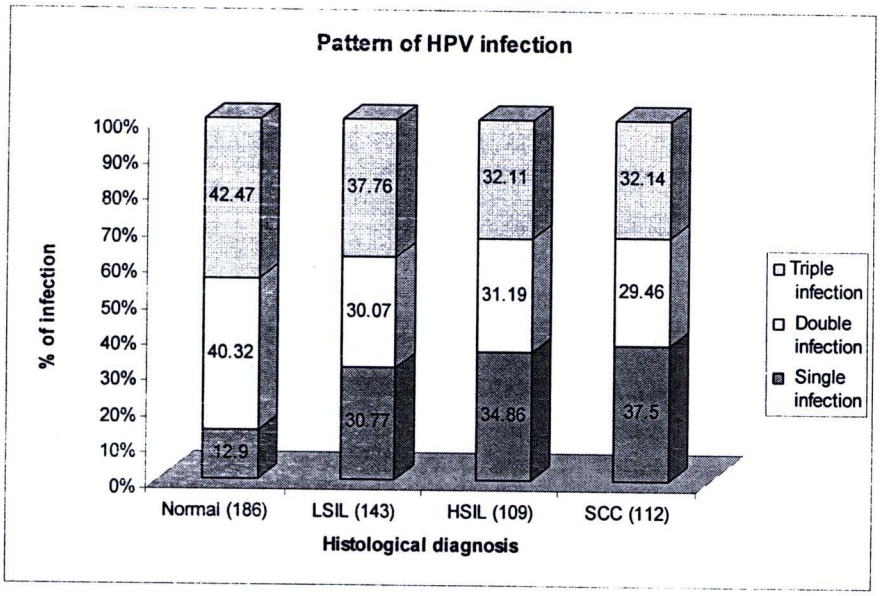

Figure 15 Pattern of HPV infection in FFPE sample from Thai women with different grade lesion.

The top five HR-HPV genotypes in each group were demonstrated in Table 9. HPV 16 showed the highest frequency of infection followed by HPV 18, HPV 58, HPV 52 and HPV 45, respectively.

Table 9 Number of the top five HR-HPV genotypes found in each histological diagnosis.

Histology results	Number of cases with HR-HPV genotypes (%)				
	HPV 16	**HPV18**	**HPV58**	**HPV45**	**HPV52**
Normal (186)	169 (89.42%)	154 (82.60%)	1 (0.54%)	8 (24.19%)	1 (0.54%)
LSIL (143)	106 (74.13%)	83 (58.04%)	9 (6.29%)	4 (2.80%)	7 (4.9%)
HSIL (109)	95 (87.16%)	68 (62.39%)	19 (17.43%)	5 (4.59%)	6 (5.51%)
CA (112)	103 (91.96%)	65 (58.04%)	6 (5.36%)	5 (4.59%)	2 (1.79%)
Total (550)	473 (86.00%)	370 (67.27%)	35 (6.36%)	22 (4.00%)	16 (2.91%)

These results demonstrated that HPV 16 was the most common HPV genotype and its higher prevalence was found in the more severe disease, i.e. 74.13% of LSIL, 87.16% of HSIL and 91.96% of SCC, except for the normal cases. The prevalence of HPV 16 in Thai women from each region of Thailand was investigated as shown in Table 10. In all of abnormal lesions (LSIL, HSIL and SCC), the lowest prevalence of HPV 16 was found in the northeast region, whereas the highest found in the north region exception to the SCC cases. This study shows that HPV 16 is the most common g enotype found in women from all 4 regions of Thailand so the most common HPV genotype in Thai women is HPV 16.

Table 10 The prevalence of HPV 16 in 550 FFPE HPV positive cases from women in 4 regions of Thailand.

Histology results	HPV 16 DNA detection				
	North (n=149)	Northeast (n=144)	South (n=162)	Central (n=95)	Total (n=550)
	Pos/Neg (%)	Pos/Neg (%)	Pos/Neg (%)	Pos/Neg (%)	Pos/Neg (%)
Normal (186)	44/4 (91.67/8.33)	43/6 (91.84 /8.16)	49/4 (98.11/1.89)	33/3 (91.67/8.33)	169/20 (89.42/10.58)
LSIL (143)	38/3 (86.36/13.67)	20/18 (52.63/47.37)	29/10 (74.36/26.64)	19/6 (76.00/24.00)	106/37 (74.13/25.87)
HSIL (109)	30/0 (100//0)	21/8 (72.41/27.59)	32/2 (94.12/5.88)	12/4 (75.00/25.00)	95/14 (87.16/12.84)
SCC (112)	28/2 (93.33/6.67)	25/3 (83.33/16.67)	33/3 (91.67/8.33)	17/1 (94.44/5.56)	103/9 (91.96/8.04)
Total (550)	140/9 (93.93/6.07)	111/33 (77.08/22.92)	146/16 (90.12/9.88)	81/14 (85.26/14.74)	473/77 (86.00/14.00)

3. HPV 16 As variant

For identification of HPV 16 As variant, HPV 16 positive samples were determined by PCR using three primer pairs spanning at nt; 83-559 of HPV 16 E6 gene. The HPV 16 E6 amplified products were used to classify HPV 16 variant by comparing with the modified data from previous studies as shown in Table 6. Ten of

normal and 4 of LSIL from HPV 16 positive cases were recruited as normal and low grade cervical lesion in this study whereas 30 HSIL and 30 CA cases were selected to represent of true-precancer lesion and SCC for identification of HPV 16 variant. Six difference HPV 16 variants were found: European prototype (E), European variant (E350G), Asian (As), Asian-American (AA), African-2 (Af2), and J135C. A new variant found such as J135C was identified in Indonesian samples [63]. HPV 16 As variant (58.11%) was found with the highest prevalence that corresponded well to the previous study (Chopjitt *et al*). This study demonstrated that HPV 16 As variant was detected in 4 of 14 normal-LSIL cases (28.57%), 19 of 30 HSIL (63.33%) and 20 of 30 SCC (66.67%) as shown in Table 11.

Table 11 Distribution of HPV 16 variants in normal-LSIL cases, HSIL cases and SCC cases.

HPV 16 Variant	Histology grade lesion				*p*-Value	OR	95%CI
	normal-LSIL n=14	HSIL n=30	SCC n =30	Total n = 74 (%)			
E prototype	4	5	3	12 (16.22%)	Ref.		
E 350G	2	3	~	5 (6.67%)	Ref		
As	4	19	20	43 (58.11%)	0.010	10	(1.732-57.723)
AA	~	2	4	6 (8.11%)	~	~	~
Af-2	~	1	2	3 (4.05%)	~	~	~
Java135C	~	~	1	1 (1.35%)	~	~	~
Unknow	4	~	~	4 (5.41%)	~	~	~
Total	14	30	30	75 (100%)			

4. LCR polymorphism of HPV 16 As

19 HSIL cases and 20 SCC cases of HPV 16 As variant were selected for the study of LCR polymorphism. Furthermore, 10 of fresh cervical tissue biopsies from cervical cancer cases, which were confirmed HPV 16 As variant by HPV 16 E6 gene sequence were studied to compare the nucleic acid sequence variation of HPV 16 As variant LCR between FFPE and fresh cervical tissues. HPV 16 LCR in extracted

DNA from FFPE samples was amplified (spanning nt 7083-103) using four primer sets (LCR1, LCR2, LCR3 and LCR4) to solve the problem of DNA fragmentation in formalin fixation method. In addition, the forward LCR1 primer and the reverse LCR4 primer were used to amplify full length HPV 16 LCR in extracted DNA from fresh tissue samples as shown in the Table 7 and the position of four LCR primer sets overlapping as shown in the Figure 10. The LCR PCR products were purified and sequenced by Molecular Informatic Laboratory, Hong Kong. HPV 16 As LCR sequences were aligned and compared with HPV 16R reference sequence AY686584 by using the bioinformatic genomic tool Multalin sequence as shown for example in Figure 16.

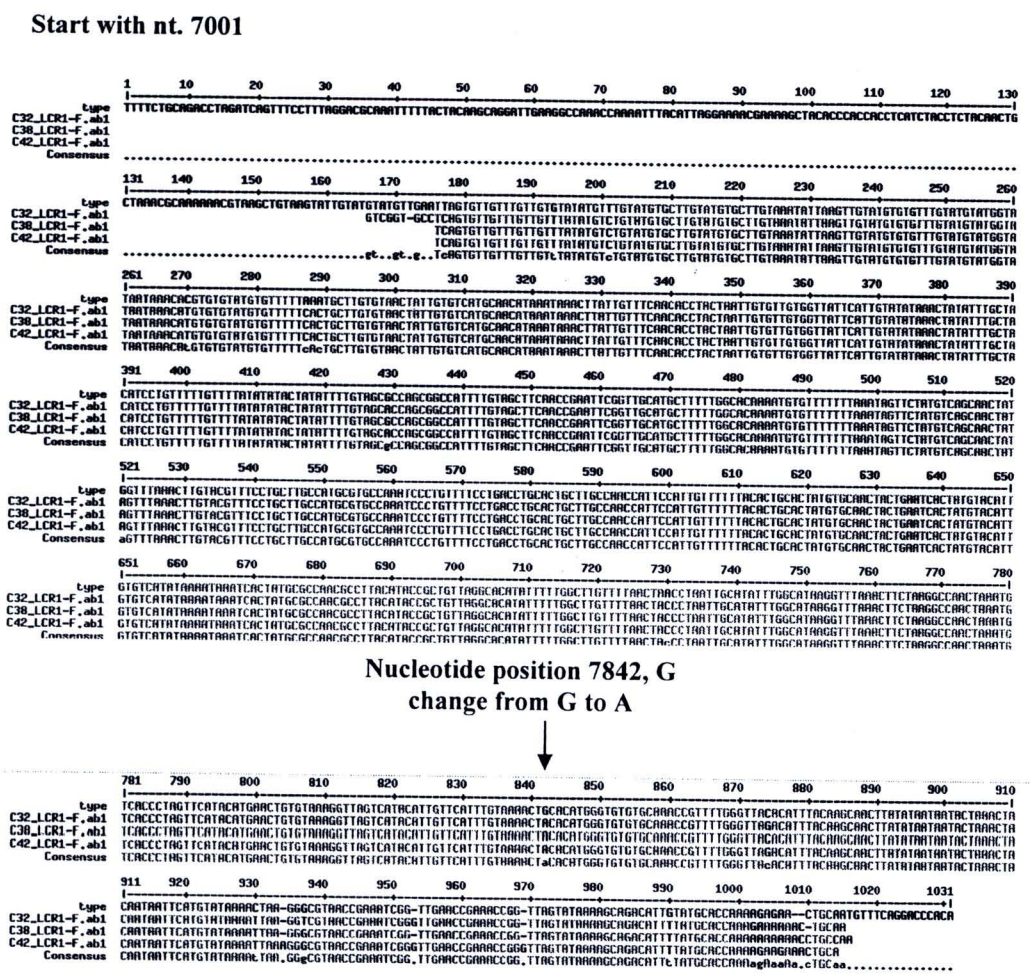

Figure 16 Nucleotide alignment of HPV 16 As variant LCR compared with HPV 16 reference sequence AY686584.

For the results of HPV 16 LCR amplification from 49 HPV 16 As variants, all of cervical cancer biopsy cases (10 cases (100%) were amplified and sequenced but 16 of 19 (84.21%) HSIL, 17 of 20 (85%) SCC in FFPE were amplified and sequenced. The LCR sequence variations were compared between FFPE and fresh tissue biopsy samples

Thirty-two nucleic acid sequence variations spanning nucleotide position 7083-103 were detected. Twenty-two positions of nucleic acid sequence variation corresponded to the previously reports [10, 56, 64-67]. that consisted of 7175A>C, 7177T>C, 7193G>T, 7201T>C, 7270C>T, 7287A>C, 7289A>C,T, 7339A>C,T, 7485A>C, 7489G>A, 7521G>A, 7623G insertion with C, 7730A>C, 7781T>C, 7791T deletion, 7802C>A, 7813T insertion with G, 7842G>A, 7868G>A, 7886C>G, 24C>T, 81G>T. Ten positions of nucleotide variation have not been reported yet including 7218T>A, 7384C>T, 7429G>A, 7430C>T, 7617C insertion with A, 7844A>C, 7874C>G, 28G insertion with A, 46T insertion with A, 61T insertion with A. Moreover, this study found triallelic change at position 7289, 7339 (A>C,T) and 7842 (G>A,T). Frequency of nucleic acid sequence variation in each position was shown in Table 12.

Table 12 Nucleotide sequence variation in HPV 16 As variant LCR compared with HPV 16 LCR reference sequence (AY686584 that are available through the GeneBank database).

	Nucleotide Position	Variation	Transcriptional factor binding sites	HSIL n = 16	SCC n = 27	Total n= 43 (%)
a	7175	A>C		16	27	43 (100%)
	7177	T>G		16	27	43 (100%)
	7193	G>T	TEF-1	16	27	43 (100%)
	7201	T>C		16	27	43 (100%)
	7270	C>T		15	26	41 (95.35%)
	7287	A>C		16	27	43 (100%)
	7289	A>C,T		16	25	41 (95.35%)
	7339	A>C,T		3	~	3 (6.98%)
	7485	A>C	GRE-1	~	1	1 (2.33%)
	7489	G>A	GRE-1	~	1	1 (2.33%)
	7521	G>A		16	27	43 (100%)
	7623	G insert with C		~	1	1 (2.33%)
	7730	A>C		16	27	43 (100%)
	7781	T>C	YY-1	1	3	4 (9.30%)
	7791	T deletion		~	1	1 (2.33%)
	7802	C>A		~	1	1 (2.33%)
	7813	T insert with G		~	1	1 (2.33%)
	7842	G>A,T	YY-1, SP-1,OCT-1	16	27	43 (100%)
	7868	G>A		1	~	1 (2.33%)
	7886	C>G		1	~	1 (2.33%)
	24	C>T	E2BS-3	16	27	43 (100%)
	81	G>T		16	27	43 (100%)
	7218	T>A		1	1	2 (4.65%)
b	7384	T>G		~	2	2 (4.65%)
	7429	G>A		10	8	18 (41.46%)
	7430	C>T		~	2	2 (4.65%)
	7617	C insert with A		~	1	1 (2.33%)
	7844	A>C		1	1	2 (4.65%)
	7874	C>G		9	8	16 (37.21%)
	28	C insert with A		~	2	2 (4.65%)
	46	T insert with G		~	2	2 (4.65%)
	61	T insert with G		~	2	2 (4.65%)

a; Nucletide sequence variation previously reported

b ; Novel nucleotide sequence variation firstly found in this study

TEF-1; Transcription factor binding site, GRE-1; Glucocorticoid response element-1, GRE-2; Glucocorticoid response element-2, NF-1; Nuclear factor-1, SP-1; *trans*-acting transcription factor-1, Oct-1; Octamer binding transcription factor, YY-1; Yin-Yang factor, E2BS; E2 binding site.

5. Transcriptional activity of HPV 16 As LCR

HPV 16 As LCR with nucleic acid sequence variation from cervical cancer cases and HPV 16 LCR of plasmid reference were used. Their full length LCR containing p97 promotor of oncogene E6 and E7 was amplified using the forward LCR1 primer and the reverse LCR4 primer as shown in the Table 7. The amplified LCR was purified and ligated into the pDRIVE cloning vector (Qiagen PCR Cloning Kit,Quigen), then subcloned into a promoterless luciferase reporter vector, pGL3 basic vector (Promega). Each of the constructed pGL3-LCR vectors was transiently transfected into C33A cell line for measurement of the LCR activity by luciferase assay. The result of pDRIVE cloning vector containing HPV 16 LCR construction and promoterless luciferase expression vector (pGL3 basic vector) containing HPV 16 LCR construction as shown in the Figure 18 and Figure 19, respectively.

Table 13 showed the LCR nucleotide sequence of the HPV 16 E prototype and the 2 selected HPV 16As variants which selected for LCR transcriptional analysis. These two cases were different on LCR nucleic acid sequence located proximal to the p97 promoter of E6/E7 oncogenes (no.36 nt 24C>T, 81G>T represent as LCR As variant and no.42 nt 7874C>G, 24C>T, 28G insertion with A, 26T insertion with G, 61T insertion with G and 81G>T represent as LCR As lineage).

Table 13 Two HPV 16 As variant samples and LCR nucleotide sequence variation, which selected for analysis of HPV 16 As LCR transcriptional activity compared with HPV 16 LCR prototype.

HPV 16 VARIANT/ nt. position	7171	7193	7201	7227	7228	7229	7429	7521	7730	7842	7874	2244	2288	4646	6161	8181
LCR E Prototype*	T	G	T	C	A	A	G	G	A	G	C	C	G	T	T	G
LCR As variant **	C	T	C	T	C	C	–	A	C	A	–	T	–	–	–	T
LCR As lineage ***	C	T	C	T	C	C	A	A	C	A	G	T	A^	G^	G^	T

*LCR E prototype: the reference sequence AY686584 HPV 16 complete genome.

**LCR As variant: the sequence related to previously studies in other continent.

**LCR As lineage: the novel nucleotide sequence variation reported in this study.

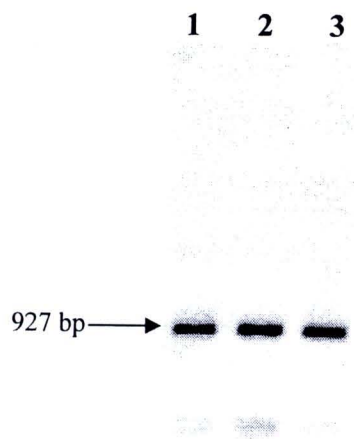

Figure 17 HPV 16 reference plasmid and two HPV 16 As variant LCR amplification. PCR products were tested on an ethidium bromide stained 0.7% agarose gel electrophoresis. Lane 1; HPV 16 LCR reference plasmid, Lane 2; HPV 16 As variant, Lane 3; HPV 16 As lineage and Lane 4; blank.

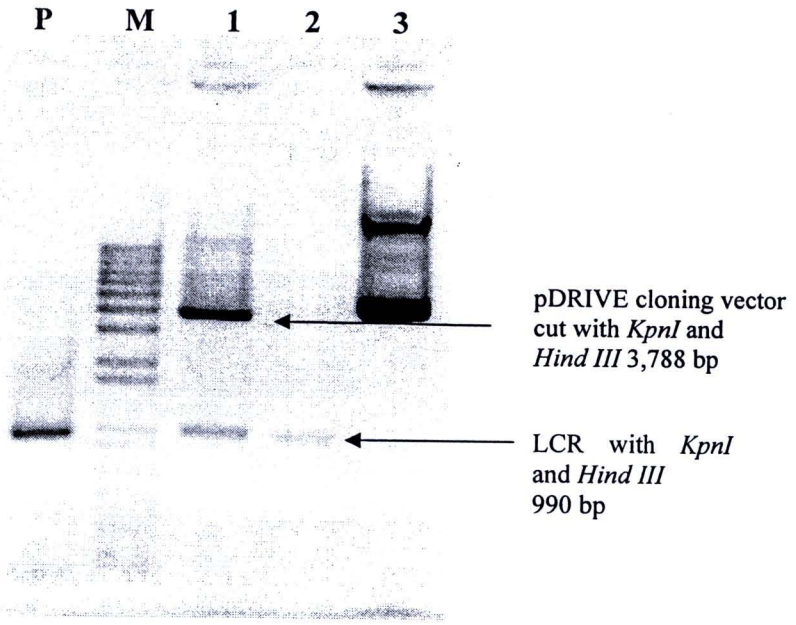

Figure 18 The pDRIVE cloning vector containing HPV 16 LCR digested with restriction endonuclease *KpnI* and *Hind III* (NEB) restriction enzymes. Lane P; LCR positive, Lane M; 1Kb plus marker, Lane 1: pDRIVE cloning vector containing LCR cut with *KpnI* and *Hind III*, Lane 2 Purified HPV 16 LCR with *KpnI* and *Hind III* and Lane 3; Uncut pDRIVE cloning vector containing LCR.

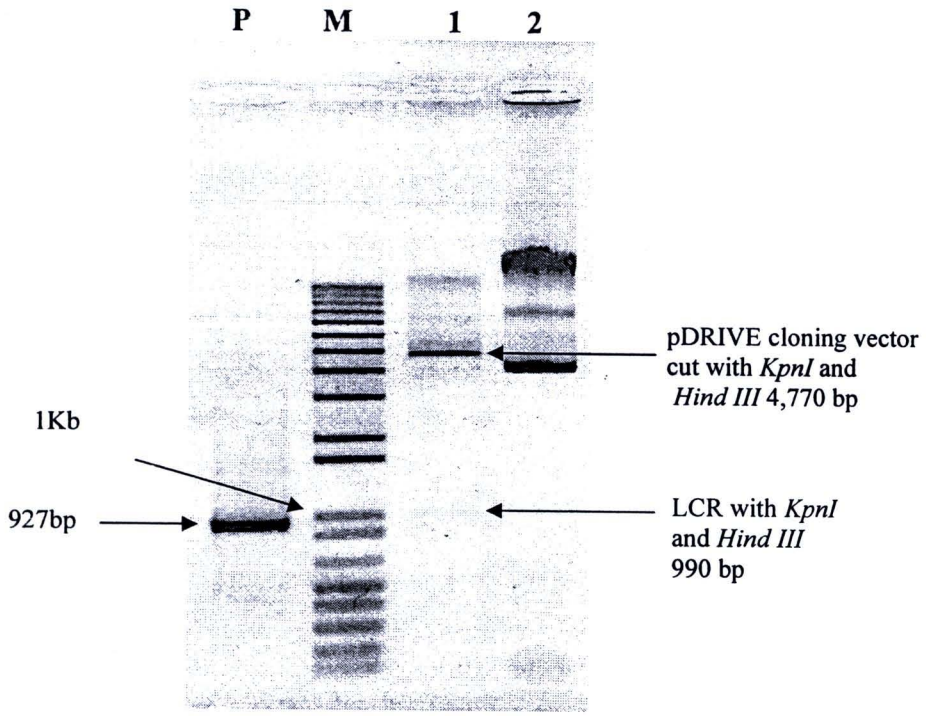

Figure 19 The promoterless luciferase expression vector (pGL3 basic vector) containing HPV 16 LCR digested with restriction endonuclease *KpnI* and *Hind III* (NEB) restriction enzymes. Lane P; LCR positive, Lane M; 1Kb plus marker, Lane 1: pGL3 basic vector containing LCR cut with *KpnI* and *Hind III* and Lane 2; Uncut pGL3 basic vector containing LCR.

The results indicated that both of HPV 16 As LCR showed higher transcriptional activity than prototype, especially in the case of HPV 16 As lineage with novel variation at proximal to promoter p97 significantly showed the highest transcriptional activity (P = 0.000; 95% CI 26.294-42) with 35-fold of the E prototype (P = 0.000; 95% CI 19.482-35.773) and 3-fold of HPV 16 As variant. Whereas HPV 16 As variant with previously reported variation had the activity 10 folds higher than the prototype but no statistical significance (P = 0.110). The LCR transcriptional activities are shown in Figure 20.

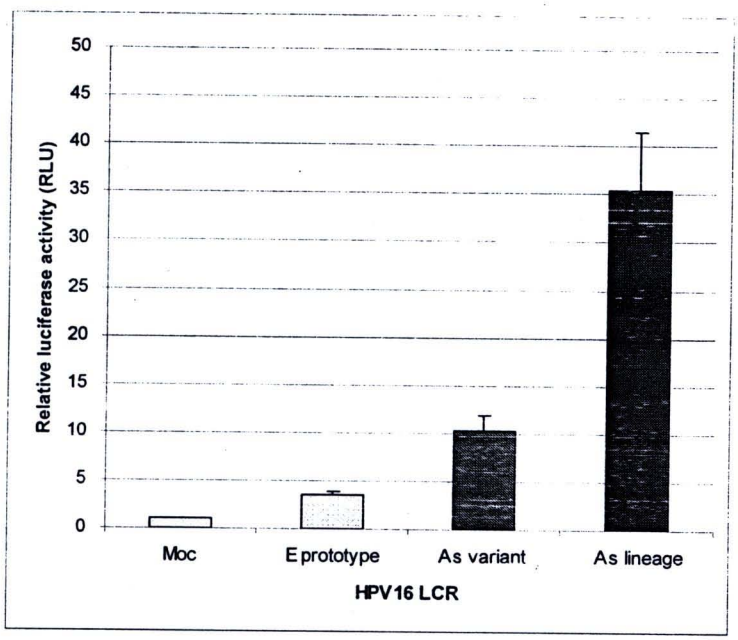

Figure 20 LCR transcriptional activity of the HPV 16 E prototype, HPV 16 As variant and HPV 16 As lineage. The presented data represent averages of three independent experiments, with error bars indicating SD.
MOC: non-infected C33A cells line, E prototype: HPV 16 E protype LCR, As variant: HPV 16 As variant LCR previously reported and As lineage: HPV 16 As variant LCR with novel nucleotide variation.