CHAPER V
DISCUSSION

The epidemiological studies and molecular virology indicated that HPV infection have been consistently associated with cervical cancer because more than 99% of cervical cancers cases contain HPV DNA [2, 68]. Infection with HPV types is different distributed in both cervical lesion grades and geographical region. HPV 16, the HR- HPV conferring for invasive cervical cancer is the most prevalent type found more than 50% of cervical cancer cases in all countries of the world [48]. In addition, HPV 16 intratypic variant is also the most extensively studies, because the different HPV variants have altered biochemical and biological properties and represent an additional risk factor in the development of squamous intraepithelial lesions and invasive carcinoma [51].

Archival FFPE c ervical tissues are important resources for HPV detection to further elucidate the role of HPV in the pathogenesis and clinical implication of cervical carcinoma [69] and HPV DNA has generally been found to be 46.9% to 100% of archival cervical carcinoma specimens using PCR-based methods [70-72].

This present study investigated the prevalence of HPV infection and HPV genotype distribution in FFPE samples with cervical grade lesion from women in 4 regions of Thailand, histologically diagnosed as normal, LSIL, HSIL and SCC. HPV 16 variant, LCR nucleic acid sequence variation of HPV 16 As variant was also investigated. Moreover, the association between LCR transcriptional activities of the HPV 16 As variant in cervical cancer was evaluated.

In our study, HPV DNA detection in all of 614 qualified FFPE samples from Thai women using GP5+/GP6+ consensus primers found that HPV DNA prevalence was 91.19% of normal, 84.62% of LSIL, 90.08% of HSIL and 93.33% of SCC cases. We found the highest prevalence of HPV DNA in SCC cases, which was comparable to previous data on worldwide studies of HPV DNA in cervical carcinomas including: 83–89% [73], >95% [74], and 99.7% [2] This prevalence of HPV infection in cervical cancer was comparable to other parts reported of Thailand, i.e., Southern

Thailand (95.3%) [75] Central Thailand (68%,-82%, 86.3%) [76-78] , Northeast Thailand (86.7%) [79] and Northern Thailand (96.9%) [72]. These differences in the detection rates are most likely related to techniques used, population behaviors and environmental factors. The HPV DNA prevalence was decreased with the degree of disease severity and corresponded to that found worldwide such as 83.5%-84.2% in HSIL [80, 81] and 71.1-94% in LSIL [80, 82].

In the normal cases of FFPE sample in this study, the frequency of HPV detection (91.19%) was much greater that reported worldwide. It has been reported that HPV prevalence in women with no intraepithelial lesion can vary among populations. In population based studies, the HPV frequency was 25.6% in Sub-Saharian Africa,8.7% in Asia, 14.3% in South America, 5.2% in Europe [83], 12.4% in the State of Morelos in Mexico [84] and 40.9% in State of Guerrero in Southern Mexico [80]. These variation may be explained with several reason such as the inclusion of women referred from public health centers has advantages for high participation but also has caveats, such as selection bias, On the other hand, HPV detection methods may give temporal variations of HPV prevalence such as the highest of HPV being in the years 2005 (51.6%) and 2006 (56.2%), which combination techniques were started. Shew, M., L. et al suggested that the highest prevalence rate of HPV DNA came from the PCR method that is the most sensitive assay method. HPV prevalence rates are as high as 80% in adolescent and of these infection, 60%-75% are caused by high-risk viral type [85]. For estimating HPV prevalence in 591 cases of Honduran women with normal cytology using the SPF10 LiPA25, the overall HPV prevalence was found 51% and suggested that these result come from more sensitive PCR detection system. In our study, PCR based method using GP5+/6+ primers were used and autonested PCR was performed to confirm HPV-DNA in some cases. These methods might provide high HPV prevalence in normal samples. In addition, age group difference, HPV prevalence declines with age, however, in Asia, it is variable; in Korea, Thailand, and Vietnam, it declines with age; but in India, prevalence remains high and very similar in all age groups [86]. Furthermore, cultural factors can be related such as lack of education, early start of sexually activity and multiparity [87]. This represent of HPV DNA in NIL cases confirmed the existence of the non-productive or latent phase of HPV infection [88].

The distribution of HPV genotype is generally consistent across cervical cancer in all regions of the world. From a comprehensive meta-analysis of 85 studies published up to February 2002 (including those in the aforementioned IARC series), comprised of 10,058 cervical cancer cases, the eight most common types including HPV 16, 18, 33, 45, 31, 58, 52 and 35 were accounted for 90% of cases [48]. The determination of HPV genotype distribution in our study found 20 out of 37 HPV genotypes by RLBH assay were identified in Thai women population. The five most common of HR-HPV infection included HPV 16 (86%), HPV 18 (67.27%), HPV 58 (6.36%), HPV 45 (4.36%) and HPV 52 (2.91%), which correspond well to previous data on the eight most common HPV types found in cervical cancer case, especially in Asia, the relative importance of three HR-HPV types 16, 18 and 58 are comparable. In addition, the prevalent of LR-HPV types (HPV 6, 11, 40, 43, 70 and 82) were identified mostly in normal and LSIL case and decreased or not found when stage of the disease is increased (data not shown).

HPV DNA detection on FFPE samples and HPV genotype distribution were determined using SPF10 primers set for PCR and line probe assay. In Indonesia, Schellekens, M.C. *et al*, 2004 studies on the prevalence of HPV in 74 cervical cancer specimens.HPV DNA was detected in 96% of the samples and 12 different HPV types was characterized. HPV 16 was the most common (44%) found in this study [89].

In Thai women population, HPV DNA detection on FFPE was reported. HPV prevalence and HPV genotyping from cases of invasive squamous cell carcinoma of the cervix in Northern Thai women were studied and reported that HPV DNA was detected in 96 of 99 cases (96.9%) and HPV 16 and HPV 18 were the most common subtypes, detected in 62/96 cases (64.4%)[72]. In Northeast Thailand, Chopjitt *et al* [16] studied in FFPE samples of CIN II-III and SCC, HPV DNA were found in 97.3% of CIN II—III, and 100% of SCC, the prevalence of HPV 16 was found frequently in SCC (75%) cases.

In this study, the most common is HPV 16 infection (86%) and its prevalence was increased with a severe stage of the disease 74.13% of LSIL cases, 87.16% of HSIL cases and 91.96% of SCC cases. However, multiple genotypes has been reported to occur in HPV positive cases [90]. A frequency of multiple infections ranging from 11.5% in Turin, Italy, [91] to 42.4% in Ho ChiMinh City, Vietnam. However, recent

studies from northern and southern Italy reported much higher frequencies of 30–50% [92, 93]. A Danish study from 2002 reported the frequencies of multiple HPV infections to be 12–36% depending on the severity of the disease [94], while another study from 2008 reported a frequency of multiple HPV infections of 59% among HPV positive women aged 20–29 years [95]. Previous studies in Korean women population of the distribution and prevalence of HPV genotypes in routine Pap smear of 2,470 cases determined using DNA Chip by Hwang et al,2004 reported that multiple infections were identified in about 20% of the HPV-positive cases and the percentage of multiple HPV infection was significantly decreased from LSIL (25.9%) to HSIL (13.6%); $P < 0.001$ [96]. Among cervical specimens from more than 1000 Danish women, HPV type distribution and the frequency of single and multiple HPV types for specimens from 113 women who underwent conization and were diagnosed with cervical intraepithelial neoplasia grade II or worse (CIN2+) were studied using microarray technology. They found that 49% of the HPV-positive patients were infected with multiple HPV types. Women younger than 30 years of age had a higher frequency of multiple infections (61%) than women older than 30 years (39%). They concluded that cervical infection with multiple HPV types is common among women in all age groups and among women with or without the diagnosis of CIN2+ [97].

Our study detected three patterns of HPV infection that were single, double (two HPV types) and triple+ (more than three HPV types) infection. This study found that HPV single infection was increased according to disease severity, 12.19% of normal cases, 30.77% of LSIL, 34.86% of HSIL cases and mostly detected in 37.5% of SCC cases, where as HPV double infections and triple infections decreased when disease severity was higher. Our study confirmed the results from previous data on the percentage of multiple HPV infections in Korean population between LSIL and HSIL cases.

In previous report, HPV 16 is the most common found, therefore, the epidemiologic studies of HPV 16 distribution with different grade lesion from Thai women in 4 regions of Thailand were evaluated. This result confirmed that HPV 16 was highest prevalence in different grade lesions in 4 regions of Thailand. The percentage of HPV 16 prevalence was also increased according to disease severity 106 of 143 (74%) LSIL cases, 95 of 109 (87.16%) HSIL cases and 103 of 211

(91.96%) of SCC cases. In Northeast region, HPV 16 detection was 20 of 38 (52.63%) LSIL cases, 21 of 30 (72.41%) HSIL cases and 25 of 28 (83.33%) of SCC cases, which were corresponded to the results of HPV 16 represent in FFPE samples from Northeast, Thailand by Chopjitt *et al* studies [16] Previous studies of HPV 16 prevalence in South (Hat-Yai) of Thailand in patient of SCC using Southern blot analysis found the most common types were HPV 16 (60% of HPV positive). HPV 16 and HPV 18 were the most common subtypes, detected in 62/96 cervical cancer cases (64.4%) of Northern Thai women [72]. The studies from Tungsinnunkong *et al* [88] reported that HPV 16 was included in 13 HR-HPV types. Thus the high prevalence of HPV 16 was associated in cervical cancer patient in all region of Thailand.

Given the most prevalence of HPV 16 in cervical squamous intraepithelial lesion and cervical cancer cases, HPV 16 intratypic variant is the most mainly extensive studied. It has been proposed that HPV 16 variant most likely differ in their ability to alter biological properties and characterized for risk factor in development of squamous intraepithelial lesions and invasive carcinoma [51]. In this study, we determined the HPV 16 variant in HPV 16 E6 gene using three primer sets spanning nt 83-559 of HPV 16 E6 gene. Six different HPV 16 variants were identified as E prototype (16.22%), E variant (6.67%), As (58.11%), AA (8.11%), Af-2 (4.05%), Java-135C (1.35%) and unidentified (5.45%). The detected rate of HPV 16 As variant increase according to the lesion of severity, 4 of 14 (28.57%) normal-LSIL cases, 19 of 30 (63.33%) HSIL cases and 20 of 30 (66.67%) SCC cases. This result showed the associated risk of women infected with HPV 16 As variant in HSIL and SCC cases with odds ratio 4.318 (95% CI 1.090 – 17.112) comparing with European and non-European variant. Comparing with European branch (E prototype and E variant) moreover, this result showed the associated risk with odd ratio 10 (95% CI 1.732 – 57.723) when comparing with European branch (E prototype and E variant). Our result supported the data of HPV 16 variants have significant geographical distribution. The HPV 16 As variant was the most common variant found in this study that corresponded well to previous reports on HPV 16 variant, which mostly found in Asia and Southeast Asian population and it is rare or absent in other continents [4, 52, 98]. Previous studies on HPV 16 variant in Khon Kaen, Thailand found HPV 16 As (58.7%) in all of sample and showed a risk association in 73.9% (OR 7.292; 95% CI

1.311—40.542) of SCC and 57.1% of cervical intraepithelial neoplasia II-III. Our studies confirmed the strong association of HPV 16 As variant with cervical cancer development in Thai women population. Many studies have shown that infection with HPV 16 E variant were found to have a lower risk progression to cervical cancer than those caused by other variants and sequence variation on HPV variants may influence the event of HPV persistence and progression to cervical intraepithelial neoplasia and cervical carcinoma [46, 99, 100].

Previous data suggested that LCR has been found to be the most variable region and nucleic sequence variation in this regions may have an impact on the oncogenicity of the virus because HPV 16 LCR, which regulates viral transcription contains specific binding sites for viral and cellular transcriptional factor [9]. Our studies determined the full length of HPV 16 As variant LCR nucleic acid sequence variation from FFPE samples, 19 HSIL cases and 20 SCC cases of HPV 16 As variant and confirmed the LCR nucleic acid sequence variation in 10 cases of fresh cervical tissue with HPV 16 As variant. This study found that LCR sequence variations from FFPE sample and fresh biopsies cases are comparable. Thirty-three nucleic acid sequence variation spanning nucleotide position 7083-103 were detected in this studies compared with prototype (Reference sequence AY686584 that are available through the GeneBank database). Results of HPV 16 As LCR sequence variation at position 7842 (G>A) was found 39 of 43 (90.69%), while the remaining case had nucleotide sequence variation at the same position (7842) but change from G>T. These studies corresponded well with previous report on variation at position 7842 is specific for HPV 16 Asian variant [8, 55]. The most common nucleic sequence variation position were 7175 (A>C), 7177(T>G), 7193 (G>T), 7201 (T>C), 7287 (A>C), 7521 (G>A), 7730 (A>C), 24 (C>T) and 81 (G>T), which were found 100% in this study. These variations may be a characteristic feature of LCR of HPV 16 As variant in Thai women. The similar variations were reported in studies on Korean women. The sequence variation and the transcriptional activity of LCR in HPV 16 E7 variants in cervical cancer were investigated. In cases of HPV 16 As variant with nucleotide sequence variation at position 7842 (G>A), there were nucleotide position change at position 7175 (A>C), 7177(T>G), 7193 (G>T), 7201 (T>C), 7287 (A>C), 7521 (G>A), 7730 (A>C), 24 (C>T) [56].

Previous data reported on HPV 16 LCR nucleotide variation at position 7193 (G>T) and 7521 (G>A), which are TEF-1 and YY-1 binding site, respectively. These mutations have been found to be commonly distributed in the majority of cervical cancer patients worldwide [9, 23, 57]. The mutation of YY-1 binding site was intensively studied and the result demonstrated that mutations of YY-1 binding site have an enhancing the p97 promoter activity on the expression of viral oncoprotein. Moreover, Dong *et al*, [101] studied about evidence of YY-1 represses promoter activity by competing with SP1 for DNA binding at the promoter proximal YY-1 binding motif in HPV 16 LCR. They suggested that mutations of YY-1 binding site increased 3-6 folds of p97 activity and competition between SP-1 and YY-1 for DNA binding played a major role in YY-1 repression mediated by the binding site at position 7840-7848 [101, 102]. Studies from Veress *et al* [10] suggested that HPV 16 LCR with nucleic acid sequence variation at the position 7193G>T, 7233A>C, 7339A>T, 7394C>T, 7395A>C, 7485A>G, 7489G>A, 7521G>A, 7669C>T, 7689C>A, 7729A>C, 7743T>G, 7764C>T, 7786C>T, 7886C>G increased ~ 1.7-fold of the HPV 16 LCR transcriptional activity [66]. In our studies, most of HPV 16 LCR variation including at position 7193G>T, 7339A>T, 7485A>G, 7489G>A, 7521G>A, 7729A>C, and 7886C>G were corresponded to Veress, G studies and these variation may affect the LCR transcriptional activity of HPV 16 As variant found in Thai women.

Ten positions of nucleic acid sequence variation in this study have not been reported including the positions 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. These variations were firstly reported in our studies. Most of them were associated with YY-1 and TEF-1 binding site. Especially at the position 7429G>A, 7874C>G, 28G insertion with A, 46T insertion with A and 61T insertion with A , they located close to E2 binding site #4 (nt 7453-7464) , E2 binding site #3 (nt 7860-7871), E2 binding site #2 (nt 35-46) and E2 binding site #1 (nt 50-61),respectively [65]. Variations at these novel positions of LCR of HPV 16As variant may have the crucial role for transcriptional modulation of HPV 16 E6 and E7 oncogenes on p97 promoter by E2 gene.

HPV 16 As variant LCR transcriptional activity was evaluated for the association of LCR transcriptional activity of the HPV 16 As variant with an oncogenic potential in cervical cancer development by using luciferase assay. LCR transcriptional analysis of HPV 16 As variant was compared with HPV 16 E prototype reference. Two different cases of LCR nucleic acid sequence variation including HPV 16 As variant of which sequence related to the previous studies in other continents [4, 52] and HPV 16 As lineage of which nucleotide variation firstly reported in this study (novel variation: nt 7429G>A, 28G insertion with A, 46T insertion with A and 61T insertion with A) from fresh cervical tissue biopsy were selected. The HPV 16 LCR exhibited different in promoter activity. The transcriptional activity of HPV 16 As variant LCR exhibited higher than the HPV 16 E prototype. Interestingly, HPV 16 As LCR 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-fold higher than the E prototype, however it is not statistical significant (P = 0.110). These results suggested that oncogenic potential of HPV 16 As variant in Thai women population could be influenced by sequence variation in the HPV 16 LCR regulatory region. Previous studies by Vagress *et al* studies on transcriptional activity of LCR full length of HPV 16 variants using luciferase tests in HeLa cells, the results showed that HPV 16 AA variant had higher transcriptional activity with approximately about 20-fold and 28-fold than HPV 16 E variants and E prototype ,respectively [10]. Chen *et al.* studied about mutation in the LCR and their functional consequences in oral cancer. They found that promoter activity of the mutated HPV 16 LCR was significantly higher than wide type. They suggested that mutations in the LCR of HPVs in oral cancer could lead to increased expression of E6/E7 oncoproteins, which might contribute to the carcinogenic process [19].

In conclusion, this study demonstrated prevalence of HPV infection and HPV genotypes distribution in FFPE cervical samples with different cervical grade lesion from women in 4 regions of Thailand. The most common HPV genotype found in this study was HPV 16 and HPV 16 As variant was frequently found in Thai women and showed a risk association for cervical cancer in Thai women. Several nucleic acid

sequence variations were found in LCR of the HPV 16 As variant, which showed the specific characteristic patterns in Thai women. Transcriptional analysis of LCR explores the oncogenic potential of the HPV 16 As variant with different LCR polymorphisms, contributes to understanding of molecular mechanisms involved in HPV 16 oncogenicity and demonstrates the correlation between HPV 16 infection and progression to cervical cancer in Thai women population. These may important to provide information for HPV vaccine design strategies and cervical cancer control.