CHAPTER V

DISCUSSION

Cervical intraepithelial neoplasia (CIN) and cervical cancer remain important health problems for women in worldwide. Previous studies focused on the etiologic role of several sexually transmitted infections, including herpes simplex type-2 (HSV-2), *Chlamydia trachomatis, Trichomonas vaginalis* [8,9]. Since the mid 1970s, the accumulation of experimental, clinical, and epidemiologic evidences have indicated the role of some types of HPV in the pathogenesis of cervical neoplasia[23].

At present time, detection and typing of HPV in formalin fixed paraffin embedded tissue of CIN-III patients compared with control were done. PCR technique was used to amplify HPV-DNA in tissue samples because it is now believed to be the most sensitive method. Schadendorf, et al. (1991) [70] compared the PCR technique with immunohistochemistry (IHC) and *in situ* hybridization (ISH) for detecting HPV in paraffin embedded tissue and found that the order of sensitivity of the tests appears as PCR>ISH>IHC.

In this study, the L1 ORF was selected as a target for amplification using L1 consensus primers (MY09/MY11). L1 ORF encodes a major capsid protein and does not involve in cell transformation. Thus, interruption of this ORF does not occur in infected cell or even integrated in host genome[101]. Moreover, it is known to be conserve among HPV-type. The L1 primers were designed to amplify approximately 450 bp of L1 ORF [102]. The amplification region spans nucleotides 6722-7170 in HPV-6 and the corresponding region of the other genital HPV[102]. In addition, β-globin primers (PC04/GH20) were

added as internal control to confirm the proper amount of DNA for PCR reaction.

To determine the sensitivity of PCR system, the purified plasmid HPV-DNA (HPV-6,11,16,18, and 33) were serially diluted and amplified with L1 consensus primers and β-globin primers. Previous study by Poonnaniti, et al. (1996) [32] demonstrated the sensitivity of the test that at concentration of one pg plasmid HPV-16 in one μg of human DNA was the minimal amount to be detected, which was equivalent to one copy per cell. (the genome of HPV is about 10^6 times smaller than that of human diploid cell)[103]. It was demonstrated that the sensitivity in detection of HPV-DNA type 6,16,18 and 33 is the same (at least one pg) whereas at least one ng of plasmid HPV-DNA type 11was required (Figure 8).

The result showed that approximately 64% (64/100) of CIN-III patients and only 1% (1/100) of control group were positive for HPV-DNA by means of PCR and GE. Poonnaniti, et al. (1996) [32] demonstrated that DH of amplified products with non-isotopic oligolabelling probed (ECL system) increased the sensitivity of HPV-DNA detection 10 fold when compared to GE alone. Therefore, to increase the sensitivity of HPV-DNA detection, the amplified products were confirmed by DH with non-isotopic oligolabelling probes (ECL system). After DH, the percentage of HPV-DNA positive samples increased up to 72% (72/100) in CIN-III patients and 6% (6/100) in control group. This result gives a strong association between HPV infection and CIN-III (OR=40.28 ; 95% CI = 19.23-84.35). Olsen, et al. (1995)[15] also reported a very strong association between HPV and cervical dysplasia (adjusted OR=72.8 ; 95% CI = 26.7-191.9).

All positive samples were typed by DH using type-specific probes. The results revealed that among CIN-III HPV positive patients the most prevalence was HPV-16; 48.61% followed by HPV-18; 15.27%, HPV-6; 5.56%, HPV-33;5.56% and HPV-11; 2.7% (Table 7). In our study, single infection was found the most. Ten samples were double infection, i.e., HPV-16/18, 8.3% (6/72), HPV-6/16 2.7% (2/72) and HPV-16/33; 1.3% (1/72). Triple infection of HPV-16/18/33 was found only 1.3% (1/72). In addition, 37.5% (37/72) of CIN-III were untyped.

In our study, we used only 5 types-specific probes to type those HPV-positive samples. Therefore, we can not conclude definitely that those patients were truly single, double or triple infection. However, our results indicated that mixed infection among CIN-III with HPV-positive cases could be demonstrated. All of them (100%; 10/10) were mixed with high risk HPV types especially HPV-16 (Table 7). Among HPV-DNA positive control group, 2 cases (33.3%) were HPV-6 and only one (16.60) was HPV-18 whereas the remaining 3 cases were untyped. Comparing the percentage of HPV-DNA positive between CIN-III patients and control group was 72% VS 6% and statistic analysis of association between HPV and CIN-III was strongly indicated (χ^2 =91.55; $p<0.05$, Table 6).The relative risk (OR) of HPV-infection was 40 times in CIN-III group (OR=40.28, 95% CI= 19.23-84.35).

The mean age of CIN-III patients in this study was 39.3 years and 75% of CIN-III patients with age below or equal to 39 years were HPV-DNA positive. Morrison, et al.(1991)[94] and Schiffman, (1992)[104]. reported decreasing prevalence of HPV infecton with increasing age. Most studies showed the peak prevalence of HPV infection is between the ages of 20 and 25 years[16,75,105]. It is believed that if the infection occurred in young age, the enhance or possibility to develop high grade dysplasia (CIN-II-III) and

progression to cervical cancer will be high. As the study of Koutsky, et al. (1992)[75] 2 year follow-up of HPV-positive patients with normal cytology, 39% turned to high grade dysplasia. Moreover, Schiffman also found that 63% of cytologically negative, HPV-DNA positive women developed cytological changes suggestive of HPV infection during an intensive 4 year follow-up[26]. It probably suggested that Thai women might get HPV infection in young age below 35 years old. Bhattarakosol, et al. (1996)[22] reported the 82% of Thai cervical cancer patients were HPV infection and HPV-16 is the dominant type (42.7%) followed by HPV-18 (20.7%), HPV-33 (3.6%) and 6.1% were double infection between HPV-16/18. If cervical cancer was developed from CIN precancerous lesion which associated with HPV infection, the percentage of HPV positive in CIN-III patients should be equal to cervical cancer patients. Our present data showed slightly lower than that expected. It is not known what role of each HPV type plays in progression of the disease in the case of a mixed infection. Although, the percentages of both studies were different, the results indicated the same pattern of HPV-type infection.

According to recently report of HPV in cervical cancer in worldwide, HPV-16 is the most prevalence type in Europe, North America, Central and South America, Africa, and Southeast Asia including Thailand. In contrast, HPV-18 is predominant type in Indonesia[17]. Walker, et al. (1989)[106] showed that HPV-18 is especially associated with adenocarcinoma but in some studies its prevalence is at least equal to or greater than HPV-16[107].

This study also showed that about 2.7% of CIN-III patients positive for each HPV-6 and HPV-11. The low risk HPV types are rarely associated with cervical cancer but, most commonly detected in external condylomata and low grade CIN[26]. HPV-6,11 may be found in about 15% of such minor sub clinical lesion, and mixed infection with both low risk and high risk group

occurred in another 2-10%[26]. As previously reported by Nimmanahaeminda, et al (1994)[108] HPV-6/11 was detected in approximately 1.72% of carcinoma *in situ* and invasive squamous cell carcinoma. In the control group, this study showed that approximately 6% were positive for HPV-DNA. Previous study of Siripanyaphinyo, et al.[109] revealed the prevalence of HPV-DNA by PCR were 5 % in Thai normal women. Si, et al. (1991)[110] also reported that the rate of HPV detection in Chinese women increased from 8.3% in normal cervical epithelium to 20% in chronic cervicitis. Our results revealed only 6% HPV positive in chronic cervicitis.

In addition, 38% (30/78) of HPV-DNA positive patients were untyped because this study used only 5 type specific probes (HPV-6,11,16,18, and 33). HPV-L1 consensus primers used for PCR amplification could be amplified at least 25 types of genital HPV[102]. Therefore, the unclassified type may be either type out of those 5 types or new type. More than 70 types of HPV have been described and more than 35 types associated with anogenital diseases[23]. Therefore, the negative samples can not be excluded for positively of having HPV-Infection. On the other hand it is possible that there is another causes apart from HPV infection for some of the CIN and cervical cancer. Crook, et al. (1992)[111] reported that HPV-DNA negative cervical cancer showed evidence of somatic mutation within p53 tumor suppressor gene and suggested that expression of a mutant p53 protein contributes to the development of HPV-negative tumors. Nevertheless, the role of p53 mutation in development of cervical cancer is controversial at present. Kurvinen, et al. (1994)[112] have studied the stage of the p53 gene in HPV-positive and HPV-negative genital precancerous lesion and carcinomas, and revealed that no mutations were detected in any specimens, including HPV-negative cases, suggesting that the functional inactivation of p53 is not invariably required for the induction of malignant transformation in the genital tract.

Our results in accordance with the numerous data found in literature seem to confirm the hypothesis that certain HPV-types greatly involved in the development of CIN and cervical cancer and confirm the pattern of distribution of HPV-types in Thai patients. HPV detection and typing may have future applications either in the management of women with mild cytological abnormalities, improving the sensitivity of cytology for high-grade precancerous lesion, or as an alternative screening test and for prognostic prediction of cervical cancer. As cervical cancer is the major cancer in Thai women, it would be ideal to develop a vaccine to prevent this disease. Vaccine development cannot take place unless it is known which HPV-types are present in our communities.