CHAPTER VI

DISCUSSION

Cervical cancer is the second leading cause of cancer death in women worldwide. In Thailand, the incidence of CaCx ranks top among cancers in women. The development of CaCx was associated with the high-risk HPV infection. The E7 protein of high-risk HPV infection has been found to be important in malignant transformation. The HPV-specific immune responses were shown to protect against HPV-associated disease. Detailed study of HPV-specific T cell responses in Thai patients with CaCx may lead to development of the effective HPV vaccine.

In the present study, we detected HPV DNA by L1-PCR. The detection of HPV DNA using L1C1/C2 primer set giving PCR product size of 250 bp was relatively more efficiency sensitive than that using MY09/11 providing PCR product size of 450 bp (100% vs. 30%, Table 9). It is possible that the HPV DNA extracted from paraffin-embedded sample was fragmented and hence being difficult to amplify longer PCR product. In corresponding to previous observations (159-164), they demonstrated the formalin fixation, paraffin embedding, storage and DNA extraction process could cause DNA damage in the paraffin-embedded tissue. Not only HPV DNA detection but also HPV typing was also determined by RFLP. By using 3 restriction enzymes (*Hinf* I, *Hae* III, *Rsa* I), at least 9 HPV types (HPV-6, 11, 16, 18, 31, 33, 42, 52 and 58) could be identified (Table 12) (140).

Among 75 samples obtained from patients with CIN and CaCx, HPV DNA was able to be detected in 40 samples (62.67%). The prevalence of HPV infection in CIN and CaCx patients were 41.03% and 86.11%, respectively (Table 11). The presence of HPV infection was common in both CIN and CaCx cases (19, 20, 23-26, 69, 70, 74, 77, 165-171). When HPV typing was determined, HPV-18 (46.81%) was the most prevalent type followed by HPV-16 (25.53%), untyped (23.40%), mixed infection (10.64%), HPV-33 (6.38%) and HPV-11 (4.26%) (Table 11). Among CIN patients, HPV-16 and HPV-18 were found equal predominantly (43.75%) (Table 11). This finding was inagreement with

previous report in Thailand (HPV-16: 44.44%; HPV-18: 16.05%) (18) probably due to small number of samples. In contrast, the majority of HPV type in CaCx patients was HPV-18 (48.39%), followed by HPV-16 (16.13%) (Table 11). Our present observation disagreed to previous study in Thailand, Bhattarakosol, et al (1996) who found that HPV-16 was the most prevalence type (17). We found that the tissues of our study have histopathology of either adenaocarcinoma or squamous cell carcinoma whereas only squamous carcinoma tissues were recruited in the previous report. Recently HPV-18 is demonstrated to associate with adenocarcinoma, while HPV-16 is the most prevalent type in squamous cell carcinoma (19, 69, 72, 74, 90, 172, 173). However, we found that HPV-18 was the most prevalent type in both adenocarcinoma (50%) and squamous cell carcinoma (38.10%). Mixed HPV infection was commonly demonstrated in CIN and CaCx patients (17, 28, 69, 72-75, 77). Our results indicated that 6.25% of CIN and 12.90% of CaCx patients have mixed HPV infection (Table 11).

In order to analyse HPV-specific T cell responses by ELISpot assay in which overlapping peptide spanning E7 protein was needed for the T cell stimulation, the E7 amino acid sequences were acquired through sequencing of previously-defined HPV-16 DNA. HPV-16 E7 gene variation was found in 20% of samples. The hot spot of mutation in E7 was often restricted to amino acid 29 (A → G, N29S, Fig. 22A. and 22B.) which was consistent with previous studies (30-35, 51, 174-176). Indeed, this variation was found to be similar in Thailand, Korea, Indonesia and India (32, 35, 51, 53). The change of a single amino acid in E7 protein might have an effect in the biological properties. In recent study, the amino acid mutation at position 29 demonstrated the alteration of conformation and biological function (177, 178). It is possible that this mutation might effect have an effect on the important mechanism for malignant transformation. Indeed, amino acids 21 to 29 of the HPV-16 E7 protein have been shown to be the most critical sites for Rb binding (84, 85, 179).

The HPV-specific T cell responses demonstrated in our study were relatively low when compared to the T cell responses observed in other viruses as Human immuno deficiency virus (HIV) and cytomegalovirus (CMV). The discrepancies in the magnitude of T cell responses could be explained by the difference in the natural history of these virus infections. Indeed, unlike HIV and CMV infections which are disseminated

throughout the body, HPV infection is localised in ano-genital organ. Being localised in one area is unlikely to efficiently prime and stimulate HPV-specific T cell response. In the present study, we attempted to demonstrate HPV-E7-specific CD8+ T cell responses in CIN and CaCx patients by *ex vivo* peptide-based IFN-γ ELISpot assay (Table 13). We designed overlapping peptides that based on our sequence from HPV-16 E7 gene. The overlapping peptides with length of 20 aa overlapping by 10 aa were designed. Overlapping peptides have been used successfully in other ELISpot assays for HPV-specific CD8+ T cell responses to HPV-16 E6 and E7 (187). We observed no *ex vivo* HPV-E7-specific CD8+ T cell responses either in CIN (Table 15 and Fig. 23) or CaCx patients (Table 18 and Fig. 29). Similar to our observation, Beverley, et al. (1994) found that it was difficult to generate HPV E6 and E7-specific CTL in human; and it has been suggested that they might be absent or present at low frequency (180). Moreover, two recent studies demonstrated that only low levels of HPV-specific CTL responses could be detected in CIN III and CaCx patients (120, 181). So, we augmented HPV-specific T cell by culturing PBMC with pooled overlapping peptides to increase sensitivity of the ELISpot assay as previous described for detecting T cell responses against *Mycobacterium tuberculosis* (158). As expected, HPV-E7-specific CD8+ T cell responses were detected in our study. Only one patient in the study who had HPV-16 infection showed CD8+ T cell responses, whereas other patients who had infected with other types of HPV were negative in ELISpot assay (Table 14 and 17). This finding may suggested that the designed HPV-16 overlapping peptides showed non cross-reactivity with other HPV types.

In this study, HPV-E7-specific CD8+ T cell responses were more readily detectable in CIN patients [3 out of 11 (27.27%)] than in CaCx patients [1 out of 10 (10%)] by cultured ELISpot assay. The magnitude of T cell responses to E7 overlapping peptides in CIN patients (median = 1,340 SFU/10^6cells) were more than the T cell responses in CaCx patients (median = 188 SFU/10^6cells). Our results were similar to previous report (37) which demonstrated that HPV-16 E7-specific CTL were detected predominantly in women with CIN (37).

There were two CIN patients (AA and YP) who had demonstrable HPV-specific T cell response had no detectable HPV infection by PCR. One explanation for this is that

HPV infection are known to be transient infection (39, 79, 100, 184-186), it is also possible that these patients could clear the HPV infection by their HPV-specific immune responses. Interestingly, there was one CaCx patient who had T cell response against E7 peptide, and indeed this patient had no HPV infection by PCR. It is likely that the T cell response in this patient might help clear the HPV infection. However, the biopsy material which was removed from uninfected area could not be excluded.

Our study demonstrated that the HPV-specific T cell responses are greater in CIN patients than in CaCx patients. This information imply that HPV-specific T cell may have protective role in the natural history of cervical cancer. However, our study focused on the T cell response against HPV E7 protein which might represent only a fraction of total HPV-specific T cell responses. Detailed study of HPV-specific T cell responses against other oncogenic proteins is therefore needed to complete the gobla picture of immune response to HPV infection. Information obtained from this study may further support the development of HPV peptide-based vaccines or antigen-specific adoptive immunotherapy for the prevention and treatment of cervical carcinoma.