CHAPTER V

DISCUSSION AND CONCLUSION

The aim of this study was to determine if the CCNA1 promoter is methylated in CC and to elucidate how the epigenetic alteration occurs during multistep CC development.

In CC cell lines, the MSP showed nonmethylation in HeLa (S), methylation in SIHA and another HeLa cell line (HeLa (K)) (Figure 8). The methylation sample displayed low level of expression when we performed RTPCR in different PCR cycles, in contrast sample with unmethylation displayed high level of expression. Conclude that, the direction level of expression was reverse to the promoter methylation and CCNA1 methylation may be one mechanism that control expression. The absence of methylation in HeLa(S) might indicate a demethylation process that occurs under different cell culture and maintenance conditions. We also calculated the actual percentage of CCNA1 by calibration experiments. It is noteworthy that the correlation between measured and actual CCNA1 methylation percentages was not linear, but exponential, as nature of PCR methods.

The discovery of an inverse correlation between CCNA1 methylation and expression in CC lines suggested possibility of the same situation *in vivo*. Interestingly, even if methylation was detected, CCNA1 was expressed in malignancy-adjacent histologically normal cervical tissues. Moreover, an inverse correlation between the

methylation level and mRNA quantity was observed. CCNA1 expression in methylated malignancy-adjacent histologically normal cervical epithelium may be due to normal cell contamination or partial methylation at the promoter according to CC multistep progression. Whereas complete methylation could be observed in most cancer cells, partial and non-methylated CCNA1 was discovered in the adjacent epithelia (Figure 12).

Examination of CCNA1 methylation along multistep cervical carcinogenesis as shown in Figure 17 and Table 3. Although the percent of methylation was not change from normal to low SIL, but when we compared from normal to the advance step, we can found the frequency of methylation was increased when increasing pathological change to cancer. In this study, HPV16 infection was found in almost cases of low SIL, MIC and SCC lead us to hypothesized the correlation of HPV oncoproteins and cyclin A1 methylation status.

Furthermore, we also observed CCNA1 methylation in adenocarcinoma and adenosquamous cell carcinoma, the results showed 7 in 9 cases (77.8%) and 1 in 3 cases (33.3%) had methylation respectively. The percentage of adenocarcinoma and adenosquamous cell carcinoma are different from squamous cell carcinoma may be due to the insufficient number of patients who were analyzed or may be differences molecular mechanism in grandular carcinogenesis compared to squamous epithelia.

The experiment evaluating cervical tissue *in vivo* led to three conclusions. First, *CCNA1* methylation was exclusively associated with cervical carcinogenesis. Second,

the epigenetic alteration occurred earlier than morphological transformation of the cellular phenotype. Finally, methylation may play a role in this gene inactivation.

In conclusion, this study demonstrated that: (i) *CCNA1* promoter hypermethylation in HPV-associated squamous cell CC is unusually common; (ii) it is specific to CC; and (iii) the methylation is more common in invasive phenotypes compared to other histopathological stages during multistep carcinogenesis. This finding identifies both the interesting biology of CC and a potential clinical application of *CCNA1* methylation as an additional molecular marker for the early diagnosis of invasive CC.

Annual cytology screening has dramatically increased the effectiveness of early CC detection. Nonetheless, additional tests will help to improve the sensitivity and specificity of a single Papanicolaou smear for histological analysis. Recently, testing for oncogenic HPVs has been introduced to aid in the triage of women with atypical squamous cells of undetermined significance (ASCUS)(99). However, because the majority of patients with HPV-associated lesions do not progress to invasive cancer, several studies have attempted to add a panel of tumor suppressor gene methylations to improve the effectiveness of molecular cytological diagnosis(100,101). Since the frequency of *CCNA1* methylation is high and specific to invasive CC, this gene should be a good candidate to increase the coverage rate for early cancer detection.

In HNSCC, *CCNA1* promoter hypermethylation is inversely related to *TP53* mutation (15). Nonetheless, the frequency of *CCNA1* promoter hypermethylation in CC is

high, whereas the function of TP53 in CC is usually impaired as a consequence of protein degradation induced by binding of the viral E6 protein (102) . This observation may be due to either differences in tissue types or pathophysiological outcomes of *TP53* between mutations and diminution of the protein function subsequent to E6 binding. We prefer the latter hypothesis, since *TP53* and *CCNA1* have been shown to augment each other's expression (15,16). Consequently, the CCNA1 protein could help to increase physiologic TP53 to counter the function of E6, except for cases of *TP53* mutation. In other words, alterations of both *CCNA1* and *TP53* in HNSCC will be redundant. In contrast, in CC, a decrease in CCNA1 protein should prevent the increment of TP53 that would have compensated for the protein destruction by E6.

Multistep process analysis revealed that *CCNA1* methylation is remarkably specific for cervical carcinogenesis. The biological function of CCNA1 is to activate DNA breakage repair by mechanisms depending on CDK2 activity and Ku proteins (16). It is interesting to hypothesize why the genomic instability, triggered by impairment of the CCNA1 function, is crucial as an early event in CC development. Perhaps the rate of spontaneous mutations in cervical epithelial cells is too low to accumulate sufficient malignancy-transformation-dependent oncogene and tumor suppressor gene mutations if the cells possess fully functional CCNA1. Therefore, the frequency of invasive CC devoid of *CCNA1* methylation is limited.

Our work is the first paper to expressed CCNA1 promoter methylation in cervical cancer. Due to promoter methylation is an important mechanism for loss of function of

tumor suppressor genes in human that can eventually lead to loss of cell cycle control. There are numerous reports to demonstrating that tumor suppressor genes belonging to nearly every cancer pathway or function category have silenced or diminished their expression in CC. (Table 10) CCNA1 methylation have a high level (93.3%) compared to other TSG. So this gene should be a good candidate for many translational opportunities such as 1.Early detection by identified CCNA1 hypermethylation from cytological smears, 2.Prognostic or predictive by determined of CCNA1 hypermethylation from serum or plasma DNA, 3.Therapy by using DNA methylation inhibitors alone or as chemo- or radio- Sensitizers

In conclusion, aberrant methylation of cyclin A1 is strongly correlated with cervical carcinogenesis. A reasonable working hypothesis is that HPV E6 oncoprotein degrade P53 protein lead to cyclin A1 hypomethylation and activation. Finally, due to correlation with carcinogenesis. cylclin A1 methylation will beneficially as tumor marker and may represent a strong mechanism for the development of cervical cancer.

Table10. Tumor suppressor genes hypermethylated in cervical cancer

Gene	Rate	Function	Referrence
DcR1/DcR2	100%	Apoptosis	103
hTERT	57%	Apoptosis	104
p73	39%	Apoptosis	105
p16	8-42%	Cell-cycle	106-112
PTEN	58%	WNT-pathway	113
E-cadherin	28-80.5%	WNT-pathway	114-116
APC	11-94%	WNT-pathway	109,111,112
MGMT	5-81%	DNA repair	109,110,112,115
FANCF	30%	FA-RBAC pathway	117
BRACI	6.1%	FA-RBAC pathway	109
hMLHI	5%	Mismatch repair	110
RASSFIA	0-45%	Negative ras-effector	115,118-120
DAPK	45-100%	Metastasis/cell death	109,111,112,115
TSLCI	58-65%	Tumor suppressor	121,122
FHIT	11-88%	DNA repair cell death	109-112,115
HICI	18-45%	Transcription factor	109,111
RARβ	33-66%	Cell differentiation	109,112,123,124
TIMP2/TIMP3	47%/1-10%	Tissue inhibitor MTs	115,125,126
Caveolin-I	6%	Caveolar membrane	127
ER α	25%	Steroid hormone receptor	112