Chapter 4

Genetic and epigenetic status of HPV16 in CaCx patients before and after therapy: prognostic implications
4.1 Introduction

The contribution of HPV16 in the development of CaCx is well established and much studied; whereas, its association in the disease prognosis has drawn substantial attention in last one decade [discussed in detail in section 1.9.1B and 1.10]. Such predictive inferences are primarily based on the prevalence of viral DNA in the patients’ plasma, before or after therapeutic interventions, or in the post-therapy cervical specimens (Figure 4.1). The micro-invasion and intravascular filtration of circulating tumor cells (CTC) in blood might be responsible for the presence of HPV in plasma, which carries the virus away from the primary tumor sites [Park Y et al., 2011]. Though, the prevalence of HPV16 in plasma has been associated with recurrence of the disease, the pattern of viral physical and methylation status between primary CaCx and corresponding plasma is not well studied (Figure 4.1) [Pornthanakasem W et al., 2001].

On the other hand, presence of HPV16, sometime in high copy number, in the cervical swabs of post-therapy patients has been associated with poor disease prognosis [Nagai Y et al., 2004; Badaracco G et al., 2010]. But the changes in genetic (physical status and copy number variation) and epigenetic (methylation of its enhancer and promoters sequences) status of HPV16 due to therapeutic interventions, if any, has not been studied in pre and post-therapy samples from the same CaCx patients (Figure 4.1). Similarly, the how the HPV16 profiles change from post-therapy cervix to the corresponding plasma is not known, though poor disease prognosis was observed with prevalence of the virus in post-therapy plasma (Figure 4.1) [Nagai Y et al., 2004]. Thus, to find out the importance of HPV16 genetic and epigenetic profiles in prognosis of the disease, it is pertinent to make a comparative analysis of these profiles in pre-therapy plasma along with post-therapy cervical swabs and corresponding plasma from the same CaCx patients.
Figure 4.1: Diagrammatic representation of prognostic importance of HPV16 profile in primary tumor site and in corresponding plasma of pre and post-therapy CaCx patients. Tick marks indicate previous findings reported by different investigators and the lacunae in understanding of the HPV16 profile have been indicated with asking marks.
4.2 Objectives

To assess the relevance of HPV16 genetic and epigenetic profile in disease prognosis, the analyses have been made under following aspects:

1. **Comparative analysis of HPV16 genetic and epigenetic profile between primary CaCx and corresponding plasma.**

2. **Comparative analysis of HPV16 genetic and epigenetic profile between post-therapy cervical swabs and corresponding pre-therapy tumors.**

3. **Comparative analysis of HPV16 genetic and epigenetic profile between cervical swabs and corresponding plasma of post-therapy patients.**

4. **Clinical importance of HPV16 profiles in disease prognosis**

4.3 Materials and Methods

4.3.1 Chemicals and Reagents

Apart from the chemicals and reagents mentioned in chapter 2.2A.1.1 and 3.3.1 following chemicals were used in the analysis during this study:

4.3.1.1 Fine chemicals used in the study

TaqMan® Universal PCR MasterMix (2X) (Applied Biosystems, Foster City, CA, USA)

4.3.1.2 Primers

Same primer sets were used in this study as mentioned previously in Table 3.1

4.3.2 Collection of samples and cell line

The samples collected for the analysis of this study are shown in Figure 4.2.

a) **Pre-therapy samples:** Among the 98 HPV16 positive primary CaCx biopsies, collected from hospital of Chittaranjan National Cancer Institute, Kolkata, India (as
mentioned previously in 3.3.2b), 5ml blood were collected from 46 (46/98) patients. These 46 patients constitute the pre-therapy sample cohort for the present study (Figure 4.2A).

The clinical features of the patients are summarized in Table 4.1. The mean age of the primary CaCx patients was 45.4 ± 9.97 years (range 29-70 years). Among these patients 14/46 was of Stage I-II and 32/46 was of Stage III-IV, according to FIGO classification. Nodal involvement was seen in 11/46 patients.

![Flowchart showing distribution of pre and post-therapy samples](image)

**Figure 4.2**: Work plan in flow chart showing distribution of different pre and post-therapy sample cohort.

**b) Post-therapy samples**: After surgery of the tumor mass, all the above mentioned 46 patients received radical radiotherapy, applied in the whole pelvic region for 25 days (total dose of 50 Gy by external-beam irradiation) along with subcutaneous injection of IFNα (3 Million Units) thrice a week for 4 weeks. After completion of radiotherapy, 39 patients (39/46) returned for the follow-up visit (Figure 4.2B). From
these 39 post-therapy patients, cervical swabs were collected in 5ml of PBS along with 5ml of blood, between 9-18 months of their follow-up visit. Thus, these 39 CaCx patients constitute the post-therapy cohort in this study.

Biopsy samples were snap frozen immediately; the cervical cell suspensions were centrifuged at 5000 rpm for 10 minutes to isolate the cell pellets and plasma were isolated from the blood samples. All the samples were stored at -80°C until further use.

In all the cases, proper approval from Institutional Ethics Committee and written
informed consent from patients were obtained.

HPV16 containing SiHa cell line was procured from National Centre for Cell Sciences, Pune, India and was grown according to supplier's recommendations.

4.3.3 DNA isolation
DNA was isolated from the post-therapy cervical swabs and pre and post-therapy plasma by the standard procedure, as mentioned previously in chapter 2.2A.1.4.

4.3.4 HPV detection and typing
The prevalence of HPV and HPV16 were tested in the post-therapy cervical swabs and pre and post-therapy plasma by PCR using the MY 09/11 and HPV16 type specific primers, respectively (as indicated in Table 3.1), followed by Southern blot Hybridization by the same procedure, as mentioned previously in chapter 2.2A.1.5 and 2.2A.1.6.

4.3.5 Estimation of HPV16 Viral Load
Variation of HPV16 copy number was measured in primary CaCx biopsies and post-therapy cervical swabs from the same patient cohort to evaluate their possible correlations with presence of the virus in the plasma and disease prognosis.

Viral load was determined by Real-Time PCR (ABI Prism 7500, Applied Biosystems, USA) using TaqMan® Universal PCR MasterMix and HPV16 specific MGB probes (Applied Biosystems, USA). The reaction was carried out in a 15µl reaction volume having 7.5µl of 2x TaqMan® Universal PCR MasterMix, 0.75µl of the probe, 100 ng of template DNA. The cycling condition was as following: initial activation 95°C 10 min, followed by 40 cycles of 95°C 15 seconds, 60°C 1 min, plate read. SDS 7500 raw data was exported to Microsoft Excel for analysis. For standard calibration serial dilution of SiHa cell line was used in tenfold dilution series of 250 pg/µL, 25 pg/µL, 2.5 pg/µL, 250 fg/µL, 25 fg/µL, and 2.5 fg/µL [Hart KW et al., J Clinical Microbiology, 2001].
4.3.6 Analysis of Physical Status of HPV16

The physical status of the virus was determined in HPV16 positive post-therapy cervical swabs and pre and post-therapy plasma by multiplex-PCR, followed by validation through Real Time PCR in randomly selected 25 samples. The procedure has been described in detail in chapter 3.3.3 and the primer details have been mentioned in Table 3.1.

4.3.7 Analysis of methylation in HPV16 genome

The methylation status of enhancer (Enh), early promoter (p97) and late promoter (p670) regions of HPV16 in post-therapy cervical swabs and pre and post-therapy plasma were assessed by PCR-based Methylation Sensitive Restriction Analysis (MSRA), at first (as described previously in chapter 3.3.5). The validation of methylation analysis was done in 30 randomly selected samples by Methylation Specific PCR (MSP) (as described previously in chapter 3.3.5). The primer details have been mentioned in Table 3.1.

4.3.8 Statistical Calculations

Chi-square analysis was used to determine association between genetic and epigenetic profile of HPV16 in different samples. Test of proportion and corresponding Z-values were calculated to assess the significant difference in epigenetic proportions between plasma and cervical lesions of the same patients, before and after therapy. Survival analysis was performed by Kaplan-Meier method in 39 pre-therapy CaCx samples and corresponding plasma, considering different clinicopathological features, viz. clinical stage, integration status, viral load, differential physical and methylation status of HPV16. These parameters were also analyzed in the post-therapy samples from the same patients (in 29 HPV16 positive cases only). The log-rank test was used to assess the differences in HPV16 genetic and epigenetic status with recurrence of the disease. Probability value (p-value) ≤0.05 was considered statistically significant. All statistical analyses were performed using statistical programs Epi Info 6.04, SPSS 10.0 (SPSS, Chicago, IL, USA) and online Z-Test calculator from www.socscistatistics.com.
Results

4.4.1 Comparative analysis of HPV16 genetic and epigenetic profile between primary CaCx and corresponding plasma

In chapter 3.4, the genetic and epigenetic status of HPV16 was analyzed in 98 primary CaCx patients (indicated in Table 3.2B). From this sample pool, the profiles of HPV16 in 46 patients’ have been retrieved in the present analysis (Table 4.2) to make a comparative analysis with their corresponding plasma. The median viral load of HPV16 found in these primary CaCx biopsies (N=46) was 2.35x10^{10} copies/100ng of DNA (range: 8.10x10^{13}-8.96x10^{5} copies/100 ng of DNA).

Figure 4.3: (A) Box-plot showing the comparative distribution of HPV16 copy number in pre-therapy CaCx samples. Group-A: Patients with HPV16 in pre-therapy CaCx and also in their plasma samples; Group-B: Patients with HPV16 in pre-therapy CaCx but not in the corresponding plasma. (B) Classification of post-therapy follow-up samples. Group-A and B referred to the same patients as described in figure 3A. (C) Box-plot showing the comparative distribution of HPV16 copy number in cervical swab of post-therapy patients contained the virus in their pre-therapy plasma (Group-A) and without the virus in their pre-therapy plasma (Group-B). Viral load has been represented as Log (HPV16 copies/100 ng gDNA).

HPV16 in plasma was detected in 29/46 (63%) of the CaCx patients (Table 4.2).
Table 4.2: The change of HPV16 physical and methylation status during progression of CaCx. Comparative HPV16 profile in pre-therapy CaCx biopsy samples (N=46) and plasma (N=46) with the corresponding post-therapy cervical swabs (N=39) and plasma (N=39) from the same patient cohort. Group-A refer to the patients having HPV16 in primary CaCx as well as in the plasma; in Group-B patients HPV16 was present only in primary CaCx but not in the plasma. E= Episomal, I= Integrated, M= Mixed/ Episomal + Integrated form of HPV 16. Enh= Early enhancer, P97= Early promoter, P670= Late promoter. Open boxes represent absence of methylation and filled boxes represent presence of methylation in the respective regions. LFU= Loss to Follow-Up, NA= Not Applicable.
Interestingly, the HPV16 viral load was significantly higher (p=0.03) in the tumor samples having HPV16 in their plasma (Group-A, N=29) (Figure 4.3A), compared to the patients without HPV16 in their plasma (Group-B, N=17). However, no significant differences in clinico-pathological features or in the physical status of HPV16 in CaCx biopsies were observed between these two groups (Table 4.1). Thus, higher viral load in the primary tumor seems to be the sole determinant of viral presence in the plasma.

On comparative analysis of HPV16 profile between tumor tissue and the corresponding plasma significant variations in physical and methylation status were observed. Difference in physical status of HPV16 between plasma and the corresponding tumor was noted in 48% (14/29) samples (Table 4.2, Pre-therapy samples). This discordance in HPV16 physical status was rendered either by the change of mixed form in CaCx biopsies to integrated form in plasma (7/14) or, by the change of integrated form in biopsy samples to mixed form in plasma (7/14).

Likewise, in the enhancer region, dissimilarity in methylation pattern was seen between tumor and corresponding plasma in 34.4% (10/29) samples (Table 4.2, Pre-therapy samples). The discordance was rendered by unmethylation of the enhancer in 5/10 plasma samples and de novo methylation in rest of the 5/10 plasma samples, compared to the corresponding CaCx samples. In the early promoter region, dissimilarity in methylation pattern was seen between tumor and respective plasma in 48.3% (14/29) samples. The discordance was due to unmethylation of the early promoter in 14 plasma samples, compared to the corresponding tumor tissue samples. Statistically, the proportion of patients having unmethylated early promoter either in plasma (14/29, 48%; Z=4.47 & 4.84, p<0.01) or both in tumor and corresponding plasma samples (14/29, 48%; Z=4.47 & 4.84, p<0.01) were significantly higher than the proportion of patients with methylated early promoter either only in plasma (0/31, 0%) or in both tumor tissue and plasma (1/29, 3.4%). On the other hand, in the late promoter region, dissimilar methylation pattern between tumor and corresponding plasma was seen in 31% (9/29) samples. Such discordance was rendered by unmethylation of the late promoter in 6/9 plasma samples and de novo methylation in 3/9 plasma samples, compared to the corresponding tumor tissue. However, the proportion of patients having methylated late promoter in both tumor and
corresponding plasma (17/29, 58.6%) were significantly higher than methylated late promoter only in plasma (3/29, 10.3%; Z=3.74, p<0.01) or only in tumor (6/29, 20.6%; Z=2.0, p<0.05) or unmethylated late promoter both in tumor and plasma (3/29, 10.3%; Z=3.4, p<0.01).

Thus, in pre-therapy patients, prevalence of integrated or mixed form of HPV16 with unmethylated early and late promoter was evident in the plasma as compared to the tumor samples.

4.4.2 Comparative analysis of HPV16 genetic and epigenetic profile between post-therapy cervical swabs and corresponding pre-therapy tumors

In the post-therapy cohort, cervical swabs were taken from 22/29 patients of Group-A and 17/17 patients of Group-B, after therapy (Figure 4.3B). Presence of HPV16 was seen in 16/22 (73%) cervical swabs from Group-A and 13/17 (76%) cervical swabs from Group-B with median viral load of 3.4x10^7 copies/100ng of DNA (range: 1.2x10^8-2.8x10^5 copies/100 ng of DNA) and 4.6x10^6 copies/100ng of DNA (range: 3.5x10^8-1.3x10^5 copies/100 ng of DNA), respectively. Interestingly, integrated or mixed form of HPV16 was found in 22/29 (75.8%) and 7/29 (24.1%) post-therapy swabs, respectively. Methylation of enhancer region was scarcely present in 2/29 (6.8%) samples, but the methylation of early and late promoters was seen in 13/29 (44.8%) and 7/29 (24.1%) samples, respectively.

On comparing the HPV16 positive post-therapy cervical swabs (N=29) to their respective pre-therapy tumors, difference in physical status of HPV16 was seen in 6/29 (21%) samples with selection of integrated/mixed form of the virus in post-therapy swabs (Table 4.2). Contextually, integration was predominant in the post-therapy swabs (22/29, 76%), than the primary tumors (18/29, 62%). Compared to the pre-therapy tumors, dissimilarity in methylation pattern was seen in 8/29 (27.6%) post-therapy cervical swabs for the enhancer region due to unmethylation in 7/8 and de novo methylation in 1/8 samples. In the early promoter region dissimilarity of methylation was found in 14/29 (48.25%) cervical swabs compared to the corresponding pre-therapy tumors. This was due to unmethylation in 6/14 and de novo
methylation in 8/14 cervical swabs. For the late promoter region, dissimilarity of methylation was found in 20/29 (68.9%) cervical swabs with unmethylation in 18/20 and de novo methylation in 2/20 samples, compared to the corresponding primary tumors. Statistically, the proportion of post-therapy patients with unmethylation of enhancer (6/29, 20.6%) or late promoter (18/29, 62%) in the cervical swabs were significantly higher (Z=2.0, p<0.05 and Z=4.4, p<0.01, respectively) than the proportion with methylated enhancer (1/29, 3.4%) or late promoter (2/29, 6.8%). However, no such significant difference has been seen in methylation status of early promoter between pre and post-therapy samples.

4.4.3 Comparative analysis of HPV16 genetic and epigenetic profile between cervical swabs and corresponding plasma of post-therapy patients

Among the HPV16 positive post-therapy patients, presence of the virus in plasma was seen in 14/16 (88%) in Group-A and 10/13 (77%) in Group-B (Figure 4.3B). Strikingly, plasma of these Group-B patients was HPV16 free in the pre-therapy condition. In the post-therapy cervical swabs, the viral load of Group-A and Group-B patients was found to be comparable (p=0.26), although at pre-therapy it was significantly higher in Group-A patients (Figure 4.3A vs. C). This observation further justifies the association of higher viral load in cervix and presence of HPV16 in the plasma.

Among the HPV16 positive plasma samples (N=24, 14 samples from Group-A and 10 samples from Group-B), difference in physical status was seen in 5/24 (20.8%) samples than the corresponding cervical swabs (Table 4.2, Post-therapy samples). Among them, change of HPV16 physical status from mixed in cervical swabs to integrated in the corresponding plasma was seen in 4/5 samples. No difference in methylation pattern of enhancer region was noted between plasma and corresponding cervical swabs. In the early promoter region, dissimilarity of methylation status was seen in 11/24 (45.8%) samples due to unmethylation of the early promoter in 6/11 plasma samples and de novo methylation in 5/11 plasma samples. Statistically no significant differences in the proportions of early promoter methylation between
cervical swabs and corresponding plasma were observed (i.e. cervical swab+/plasma+ vs. cervical swab+/plasma- vs. cervical swab-/plasma+ vs. cervical swab-/plasma-; p>0.05) in post-therapy patients. In the late promoter region, dissimilarity of methylation pattern was seen in 9/24 (47%) plasma samples, of which 8/9 showed de novo methylation and 1/9 showed unmethylation, compared to the status in corresponding cervical swabs. Statistically, the proportion of patients having methylated late promoter only in the plasma (8/24, 33.3%) was significantly higher than proportion with unmethylated late promoter in the plasma (1/24, 4.1%; Z=2.6, p<0.01).

**4.4.4 Clinical importance of HPV16 profile in disease prognosis**

Clinical outcome of the 39 patients was investigated for a follow-up period upto 18 months. Recurrence of the disease was found in 12 (12/39, 31%) patients with loco-regional metastasis (N=3/12) or metastasis in distant organs (N=9/12) (Table 4.2). Log rank test revealed poor prognosis in patients with high viral load (>10^{11} copies/100 ng gDNA) in primary CaCx biopsies and differential methylation status of early and late promoters between the primary CaCx biopsies and corresponding plasma (Figure 4.4A and B).

Similarly, in the post-therapy samples, recurrence of the disease was associated with high viral load (>10^6 copies /100 ng gDNA) in the cervical swabs and differential methylation status of early and late promoters between the post-therapy cervical swabs and corresponding plasma (Figure 4.4C and D).

Most importantly, recurrence with metastatic lesions in distant organs was associated with unmethylated early promoter and methylated late promoter in both pre-therapy (p<0.01) and post-therapy plasma (p<0.05), compared to the patients with loco-regional recurrences.
4.4 Kaplan-Meier survival analysis curves showing cumulative recurrence of CaCx patients with higher viral load in primary tumor tissue (A) and by the presence of differential promoter methylation status of HPV16 between primary CaCx and the corresponding plasma (B). Recurrence of the disease was also associated with higher viral load in the post-therapy cervical swabs (C) and by presence of differential promoter methylation status of HPV16 between post-therapy cervical swab and the corresponding plasma (D). Survival time (in months) was defined as date of surgery to the date of follow-up with known recurrence.

4.5 Discussion

This study was aimed to elucidate the changes in genetic and epigenetic profile of HPV16 in CaCx patients to assess their prognostic implication and their clinical relevance in prediction of recurrences. For this, comparison of HPV16 physical status, viral copy number variation and methylation profile of the enhancer, early and late promoter regions have been made between the pre-therapy and post-therapy groups from a single patient cohort. The genetic/epigenetic profile of the virus was also correlated with prognosis of the disease.

Our data indicates the prevalence of HPV16 in plasma of CaCx patients having significantly higher viral load in the tumor. On comparative analysis of HPV16 profile between pre-therapy CaCx samples and corresponding plasma, considerable
differences in physical form (14/29) was observed. In addition, hypomethylation of early promoter (14/29) in plasma compared to the corresponding CaCx was favored; however, late promoter was hypermethylated in CaCx and corresponding plasma. The CTC was considered as repository of the virus in plasma of CaCx patients which originates due to micro-invasion and early dissemination of tumor cells into bloodstream, making the primary tumor vulnerable to bad prognosis [Marusyk A et al., 2012; Gray JW, 2003]. The presence of intra-tumor heterogeneity or change in the profile of HPV16 during its migration through CTC could be responsible for such heterogeneity between primary tumor and corresponding plasma. Though the existence of integrated HPV16 in plasma of CaCx patients was reported previously [Pornthanakasem W et al., 2001], here we have showed the association of worst prognosis with the high viral copy number in biopsy samples and the heterogeneous methylation pattern of early promoter and late promoter between tumor tissue and corresponding plasma.

In comparison to the primary CaCx samples, the integration frequency of HPV16 has significantly increased in post-therapy cervix. The methylation of the early promoter did not change significantly; but significant increase in unmethylation of the enhancer and late promoter regions were observed in post-therapy cervical swabs. Thus, integration and hypomethylation of HPV16 genome are associated with persistence of the virus in cervix, after therapy.

Unlike in pre-therapy CaCx patients, the physical status of HPV16 did not change significantly between post-therapy swabs and corresponding plasma. However, significant increase in hypomethylation of early promoter and hypermethylation of late promoter was observed in post-therapy plasma than the corresponding swabs.

Importantly, recurrence of the disease (especially in distant organs) was significantly associated with unmethylation of early promoter and de novo methylation of late promoters of HPV16 in plasma, compared to the cervix. Thus, it seems that selection of the integrated clone in the post-therapy plasma hypomethylation of p97 renders more virulent property to the CTC to metastasize.

Our previous study revealed the association of HPV16 prevalence in post-therapy plasma and higher viral load in the corresponding cervical swabs with poor disease
prognosis [Singh RK et al., 2006]. Although the prevalence of HPV16 in mixed form in pre-therapy CaCx and respective post-therapy samples was reported by Badaracco et al. [Badaracco G, 2010], the physical and methylation profile in post-therapy plasma has not been reported earlier.

It seems that increased genetic and epigenetic heterogeneity (i.e. increased integration and promoter hypomethylation) sustains the HPV16 in cervix as well as in CTC, despite therapeutic interventions and are more selected to recur and metastasize. After selection of integrated clones in circulation further heterogeneity is contributed through change of methylation in the promoters that facilitate the disease to recur in distant organs. Thus, profiling of HPV16 physical and methylation status in plasma of primary CaCx patients could be used as early diagnostic marker for metastatic disease. Furthermore, its profile in post-therapy patients might be important for better planning of treatment regime to reduce disease recurrence.

### 4.6 Conclusion

1. Prevalence of HPV16 in pre/post-therapy plasma was significantly associated with high viral-load in the corresponding primary tumor site of cervix.

2. In pre-therapy plasma, prevalence of integrated/mixed form of HPV16 was comparatively higher with significantly increased hypomethylation of p97 and hypermethylation of p670, than the corresponding CaCx.

3. Integrated form of HPV16 was more persistent in cervix, after therapy, with hypomethylation of the early enhancer and p670.

4. In post-therapy plasma, the physical status of HPV16 was similar to its corresponding, but with hypomethylation of p97 and hypermethylation of p670.

5. Patients with differential methylation of HPV16 promoters in plasma and higher viral-load showed poor prognosis with metastasis in distant organs.