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ORIGINAL ARTICLE

Identification of Changes in the Human Papilloma Virus 16 (HPV16) Genome During Early Dissemination of Cervical Cancer Cells May Complement Histological Diagnosis of Lymph Node Metastasis

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Abstract Cancer of the uterine cervix (CACX) is one of the most common carcinoma affecting women worldwide. During treatment, histologically lymph node (LN) metastasis and presence of HPV DNA in blood plasma act as a major prognostic factor. Despite the lack of apparent LN involvement, some early-invasive CACX patients have shown recurrences and poor survival. This is suggestive of undetected early dissemination of cancer cells characterized by presence of HPV DNA in histologically non-metastatic LNs which finally progresses into histologically visible metastasis. This present study investigated the status and origin of HPV genome during early dissemination by molecular analysis in primary tumor (PT), histologically non-metastatic pelvic lymph nodes (LNs) and blood plasma (BP) of same patient. First, CACX patients showing signs of early dissemination was identified by detection of HPV in PT (n = 22) and their corresponding histologically non-metastatic pelvic LNs (n = 45) and BP (n = 18) followed by typing of HPV16/18. This was followed by comparative analysis of the physical, copy number and methylation (enhancer/early/late) status of HPV16 genome present in LNs and BP with that of PT. Our study revealed for the first time that the HPV16 genome were frequently present in the integrated form though the copy number was low in both non-metastatic LNs and BP. However, the methylation pattern of PT was discordant with that of corresponding LNs and BP in majority of the cases. Critical assessment of HPV16 profiles established that the presence of hrHPV may be due to the early dissemination of PT cells having significant pathological implications.

Keywords Uterine cervical carcinoma · Disseminated tumor cells (DTCs) · Lymph node metastasis · Human papillomavirus (HPV) type 16 · HPV16 integration · HPV16 methylation

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Introduction

Cervical cancer (CACX) is the third most commonly diagnosed cancer and the fourth leading cause of cancer death among women globally. India alone accounts for 25% (67,500) of total cervical cancer deaths [1]. Though several etiological factors like multiple sexual partners, high parity, use of oral contraceptives and smoking have been associated with CACX, but high-risk human papillomavirus (hrHPV) infection is considered to be one of the principle etiopathological factor of the disease [2, 3].

Persistent hrHPV infection (primarily HPV16 and/or HPV18) promotes cellular transformation of normal cervical epithelial cells, which initially leads to pre-malignant lesions (cervical intraepithelial neoplasia, CIN) and if left untreated, it

finally develops into an invasive tumor [4]. During its gradual progression, early dissemination of cancer cells from the primary site may occur via local lymphatic and bloodstream [5]. So, it is generally practiced that during radical hysterectomy of early invasive tumors (FIGO IA-IIA), pelvic lymph node(s) (LNs) surrounding the cervical lesion are removed (lymphadenectomy) not only as a safety measure but also for histological examination of metastasis. Histologically metastasis-positive pelvic LN acts as an independent poor prognostic factor and may dictate the use of adjuvant therapy after surgery. However, recurrence is still reported in the early invasive CACX patients despite having histologically metastasis-negative pelvic LNs, suggestive of undetected early dissemination of tumor cells [6].

Following the first report by Lancaster et al. [7], a number of similar reports correlated presence of hrHPV with both histologically metastasis positive and negative pelvic LNs. So, hrHPV in pelvic LNs (irrespective of histological status) may pose as a potential risk factor for recurrence and poor prognosis in the early invasive CACX patients [6]. It appears that presence of hrHPV in histologically metastasis-negative pelvic LNs may indicate undetected early dissemination of cancer cells from the primary site which finally may develop into histologically metastasis-positive LNs. Additionally, other studies indicate that micro-invasion followed by intravascular filtration of circulating tumor cells (CTC) in blood may be responsible for the presence of hrHPV in blood plasma (BP) of CACX patients [8]. Few reports also associated the presence of hrHPV in BP of CACX patients with disease progression and recurrence [9–11]. Thus, presence of hrHPV in both histologically metastasis-negative pelvic LN(s) and corresponding BP may be interpreted as an early signature of metastasis of primary tumor cells.

During the development of primary cervical tumor, hrHPV undergoes several crucial changes in the viral copy number, physical status (episomal/integrated/mixed) and DNA methylation profiles of its upstream enhancer regions and its early and late promoters (P97 and P670) [4, 11, 12]. Studies have shown that both copy number and integration status of the viral genome increases with the invasiveness of the cervical tumor [13–15]. Though methylation studies provided inconsistent results, but hypomethylation of the early promoter (P97) and hypermethylation of the late promoter (P670) and the enhancer region of the viral genome have been observed in CACX [12, 14]. However, to the best of our knowledge no comparative molecular analysis of hrHPV genome among PT, histologically metastasis-negative pelvic LN(s) and BP of same CACX patient was attempted so far. Such information in these samples may shed light on the status and origin of hrHPV genome detected in the histologically metastasis-negative pelvic LN(s) and BP of CACX patients.

Therefore, in the present study at first, detection of hrHPV was carried out in primary tumor samples, histologically metastatic-negative pelvic LNs and BP of same CACX (FIGO IA-IIA) patients. It was followed by comparative analysis of viral copy number, physical status and DNA methylation profiles of hrHPV genome. Our data not only highlighted the status of the hrHPV genome in both LNs and BP but further indicated that this presence of hrHPV may be due to the early dissemination of PT cells which may further act as supplement to the histopathological findings.

Material and Methods

Collection of Samples

Tissue samples of primary uterine cervical tumors (FIGO IA-IIA) and their corresponding pelvic LNs tissue along with 5 ml of blood were collected from hospital section of Chittaranjan National Cancer Institute, Kolkata after appropriate approval from Institutional Ethical Committee which is in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from patients participated in the study. The average age of the patients was 51 years. Three types of specimen i.e. primary tumor, adjacent non-metastatic lymph node (number varying from one to five) and blood plasma were obtained from each patient. Tissue samples were frozen immediately after collection at −80 °C until use. Histological examination (of Hematoxylin and Eosin stained cryosections) by experienced pathologists identified metastasis-negative LNs. Upon histological examination, it was also seen that most of the tumors were Moderately Differentiated Squamous Cell Carcinoma (MDSCC). While plasma was separated from the blood samples and stored at −80 °C until use.

DNA Isolation from Samples

To remove normal cells in the primary CACX biopsies, microdissection of cryosections (5 μm) was performed using surgical knives under a dissecting microscope (Leica MZ16, Germany) / laser capture microdissection (LCM) microscope (Zeiss Palm/Apotome, Germany) [11, 16]. DNA from the microdissected CACX samples (n = 22), metastasis-negative pelvic LN tissues (n = 45) and blood plasma (n = 18) were isolated by proteinase K digestion followed by phenol–chloroform extraction, according to standard procedures [17].

Detection of HPV Followed by Typing for HPV16 and HPV18

The presence of HPV was detected, at first, by polymerase chain reaction (PCR) using primers (MY09 and MY11) designed from the consensus L1 region of the HPV genome.

Typing of hrHPV (HPV16 and HPV18) was done in HPV positive samples by PCR using specific primers [11, 13, 15] from the E6 region of HPV16 and the LCR region of HPV18 genomes followed by Southern blot hybridisation using a P^{32} labelled HPV16 and HPV18 specific probes [11, 13, 15]. HPV16 and HPV18 plasmid was used as positive control in respective typing.

Analysis of Physical Status of HPV16 Genome

The physical status of HPV16 genome was determined by calculating the ratio of E2 gene to E6 gene copy number using multiplex PCR amplification. Three separate PCR reactions (E2A/E6; E2B/E6; and E2C/E6) were carried out using three separate primer sets [11, 14, 15], designed from the amino terminal (E2A), hinge region (E2B) and carboxyl terminal (E2C) representing the whole E2 gene was used. After 2% gel electrophoresis, intensity ratio of PCR amplicons were calculated in UV trans-illuminometer using Labe image software. A ratio of 1 indicated pure episomal form (range, 1.00 to 3.00); while 0 indicated integration and a value of less than 1 (ranging from 0.05 to 0.99) indicated the presence of both episomal and integrated forms (mixed). HPV16 plasmid was used as control for episomal form while SiHa as integration control [11, 14, 15].

Moreover, HPV16 physical status in randomly selected samples was validated by real time PCR using Power SYBR Green (Applied Biosystems) with the same primer sets and above controls [11, 14, 15].

Copy Number Analysis of HPV16 Genome

Copy number (viral load) of the HPV16 genome was determined in randomly selected HPV16 positive samples (Primary Tumor = 6; Lymph Node = 12; Blood Plasma = 6) using by the Power SYBR Green absolute real-time PCR method (Applied Biosystems) by HPV16 type specific primer [13]. The reaction was carried out in a 15 μl reaction volume having 7.5 μl of Power SYBR Green mix, 1 μl each of HPV16 specific primer set and 100 ng of template DNA. The cycling condition was as follows: initial activation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 1 min; plate read. Microsoft Excel was used to analyze SDS 7500 raw data. For standard calibration, serial dilution of HPV16 plasmid was used in the following dilution (10-fold) series: 500 pg/mL, 50 pg/mL, 5 pg/mL, 500 fg/mL, 50 fg/mL, 5 fg/mL, and 0.5 fg/mL; for negative calibration beta-Globin primers were used [13].

Methylation Analysis of HPV16 Genome

Methylation status of the LCR region of HPV16 genome consisting of early enhancer and early promoter (P97) were analyzed along with the late promoter (P670) by PCR based methylation sensitive restriction analysis (MSRA) using enzyme HpaII for digestion of P97 and P670 promoter, while HhaI enzyme for early enhancer region. The 445 bp fragment of β-3A Adaptin gene (K1) and 229 bp fragment of RARβ2 (K2) were used as digestion and integrity controls respectively [11, 14, 15].

Statistical Calculations

A $\chi 2$ analysis, with or without Yates' correction, was used to determine the association between various molecular analysis of HPV16 in different sample types. Fisher's exact test was also used where necessary. Probability values (p-value ≤ 0.05) were considered statistically significant. All statistical analyses were performed using the statistical program Epi Info 7 [11].

Results

Prevalence of hrHPV Types in Primary CACX, Lymph Node and Blood Plasma

At first, to identify CACX patients with early sign of dissemination, detection of HPV infection was performed in 22 cervical lesions along with corresponding 45 histologically metastasis-negative pelvic LNs and 18 BPs. All the 22 primary CACX tumors were HPV positive. In metastasis-negative pelvic LNs overall prevalence of HPV infection was 68.89% (31 out of 45). However, number of LNs adjacent to 22 primary tumors varied from 1 to 5, so we considered HPV positivity in at least one corresponding LN as a sign of early dissemination of PT cells. It was observed that 77.27% (17 out of 22) of primary tumor samples showed presence of HPV in at least one of their adjacent metastasis-negative LNs. While HPV prevalence was 72.22% (13 out of 18) in corresponding BP (Fig. 1).

Among these HPV positive cervical PTs, 68.18% (15 out of 22) were infected by HPV16 only, while 31.81% (7 out of 22) showed co-infection of both HPV16 and HPV18 (Fig. 2). As seen in case of PT, HPV16 was also the most prevalent subtype in both metastasis-negative pelvic LN (93.56%) and in BP (92.31%). But unlike PT, co-infection was found only in 6.45% (2 out of 31) and 7.7% (1 out of 13) of metastasis-negative pelvic LN and corresponding BP samples respectively (Fig. 2).

Hence, it appears that the HPV infection observed in histologically metastasis-negative pelvic LNs and BP may be due to early migration of primary cervical tumor cells. However, comparison of genetic and epigenetic status of the viral genome in LN and BP with that of PT may provide better insights.

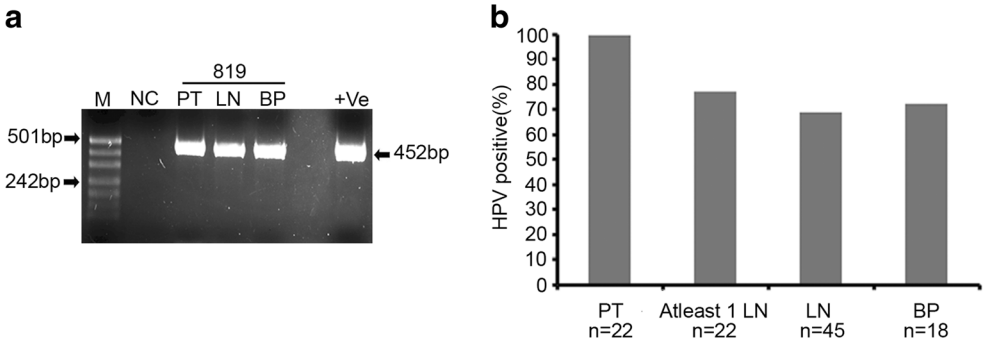

Fig. 1 Detection of HPV using primers (MY09 and MY11) designed from the consensus L1 region of HPV genome. **a** Representative image showing the primary tumor (marked PT) corresponding non-metastatic lymph node (marked LN) and blood plasma (marked BP) of same CACX patient (Sample ID 819) were all HPV positive. pUC19 plasmid digested with HpaII marker was used as DNA marker (M). HPV16 plasmid was used as positive control (marked as +VE). NC is non-template control. **b** Distribution of HPV positivity across PT ($n = 22$), total LN ($n = 45$) and BP ($n = 18$). To identify early dissemination, HPV positivity in atleast one LN sample ($n = 22$) was considered separately

Physical Status of HPV16 Genome

Physical status of HPV16 was first analyzed in 22 primary tumor samples. The integrated form was the most frequent 72.7% (16/22) while, 27.3% (6/22) of PT showed mixed status (Fig. 3).

Similarly, it was observed that metastasis-negative pelvic LNs mostly harbor the integrated form 83.9% (26/31), followed by the mixed form 12.9% (4/31). In case of BP, 92.3% (17/18) of the samples have shown presence of integrated form (Fig. 3).

Copy Number Analysis of HPV16 Genome

Viral copy number and the integration status of the genome were reported previously to be associated with the overexpression of oncoproteins E6 and E7 and enhance HPV16 virulence [18, 19]. Copy number analysis in PT samples showed high (median = 669,570.87) copy number of HPV16. Whereas, low viral copy number was observed in case of metastasis-negative LNs (median = 16.51) and BP (median = 1.96).

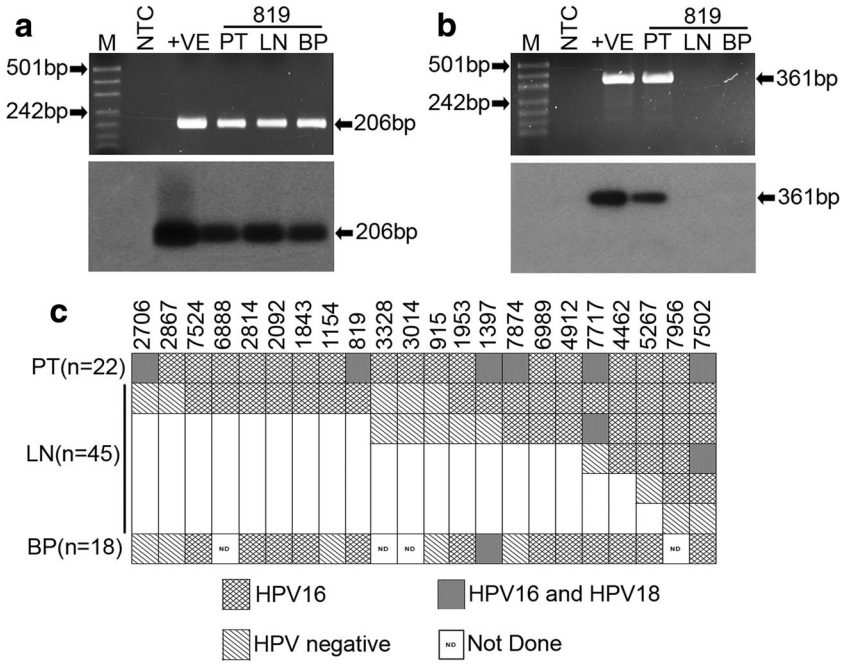

Fig. 2 Detection of hrHPV types (HPV16/18) in HPV positive samples by type-specific PCR followed by Southern hybridization using specific probes. **a** Representative gel picture showed CACX patient (Sample ID 819) had HPV16 infection in PT and corresponding LN and BP as well. Here HPV16 plasmid was used as positive control (marked as +VE). Corresponding southern blot was shown below. **b** The representative figure depicted that CACX patient (Sample ID 819) had also HPV18 infection in PT but not in corresponding LN and BP. pUC19 plasmid digested with HpaII marker was used as DNA marker (M) and NTC was non-template control. **c** Schematic representation showing HPV types in PT and corresponding LN and BP of CACX patients

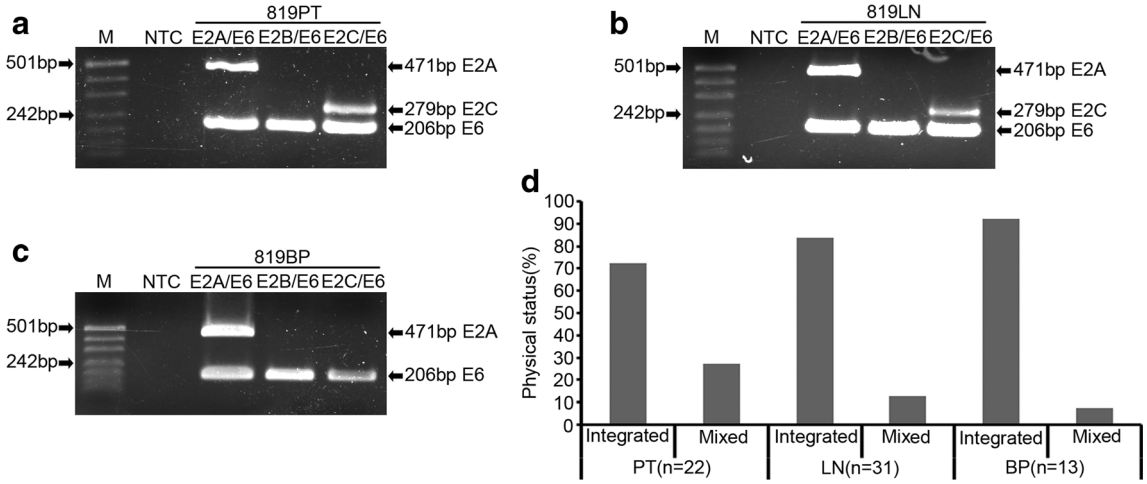

Fig. 3 Physical status of HPV16 genome was analyzed using separate multiplex PCR of E2A/E6, E2B/E6 and E2C/E6. **a** Representative figure showed PT of CACX patient (Sample ID 819) had integrated HPV genome and integration occurred at E2B site. **b** Corresponding LN of the same patient (Sample ID 819) had also integrated genome where integration was observed at E2B region. **c** In case of corresponding BP of same patient (Sample ID 819) integration of HPV genome occurred at both E2B and E2C site. pUC19 plasmid digested with HpaII marker was used as DNA marker (M) and NTC was non-template control. **d** Distribution of physical status of HPV genome across PT, LN and BP of CACX patients

Promoter Methylation Analysis of HPV16 Genome

Expression of HPV16 oncoproteins E6 and E7 were reported to be dependent on the promoter methylation status of the enhancer, early promoter (P97) while late proteins are dependent on late promoter (P670). So, at first promoter methylation analysis was done in PT which showed comparable methylation of 40.9% (9/22) and 36.4% (8/22) in enhancer region and in early promoter (P97) respectively. While considerable high methylation of 68.2% (15/22) in late promoter (P670) was also observed (Fig. 4).

In corresponding BP, comparable overall methylation frequency of enhancer (38.5%), early promoter (30.7%) and late promoter (76.9%) were observed, while concordance was seen in 5 out of 13 samples (Fig. 4).

On the other hand, in case of metastasis-negative pelvic LNs, only early (41.93%) and late (70.96%) promoters showed similar overall methylation frequency when compared with PT. But in the enhancer region there is an increase in methylation frequency (58.06%) in LN samples than PT (Fig. 4).

Discussion

The present study first aimed to analyze the prevalence of HPV in PT (FIGO IA-IIA) along with corresponding histologically metastasis-negative pelvic LNs and BP. It was followed by further comparative analysis of the genetic and epigenetic status of hrHPV genome in these samples for better diagnosis and therapeutic intervention.

As dissemination of solid tumors may occur through local lymphatic and bloodstream so, at first detection of tumor dissemination was carried out in early-invasive (FIGO IA-IIA) CACX patients. It was identified by analysis of HPV prevalence in PT (100%) followed by histologically metastasis-negative pelvic LNs (77.27%) and BP (72.22%) of same CACX patients. For the first time our study reported HPV prevalence in PT, histologically metastasis-negative pelvic LNs and BP in same CACX patients. A recent meta-analysis reported prevalence of HPV in non-metastatic pelvic and/or para-aortic LN to be 39% (258/915) [6]. We reported here higher prevalence of HPV in histologically metastasis-negative pelvic LN. It seems that the use of fresh-frozen tissues and sensitivity of PCR based detection method may be the reason for higher prevalence of HPV infection in our study.

Further comparative analysis of hrHPV subtype showed that only HPV16 infection was most frequently present in PT (68.18%) and their corresponding LNs (93.56%) and BP (92.31%). Like several previous reports [6, 11], we also observed here that the hrHPV type detected in the PT was similar to the type found in adjacent metastasis-negative pelvic LNs and BP indicating primary cervical lesion as the origin of hrHPV positive cells. Interestingly, we also report here that PT samples (31.81%) showing co-infection of both HPV16 and HPV18 have often shown infection by HPV16 only in their corresponding LNs and BP. Though further detailed study needs to be done but it seems that in heterogeneous cell population of these PTs HPV16 genome containing tumor cells may have disseminated early due to high virulence. In addition, we further provided a complete comparative overview of viral physical, copy number and methylation status in PT and corresponding histologically metastasis-negative pelvic LNs and BP of CACX patients to further highlight the early events in the process of dissemination (Fig. 5).

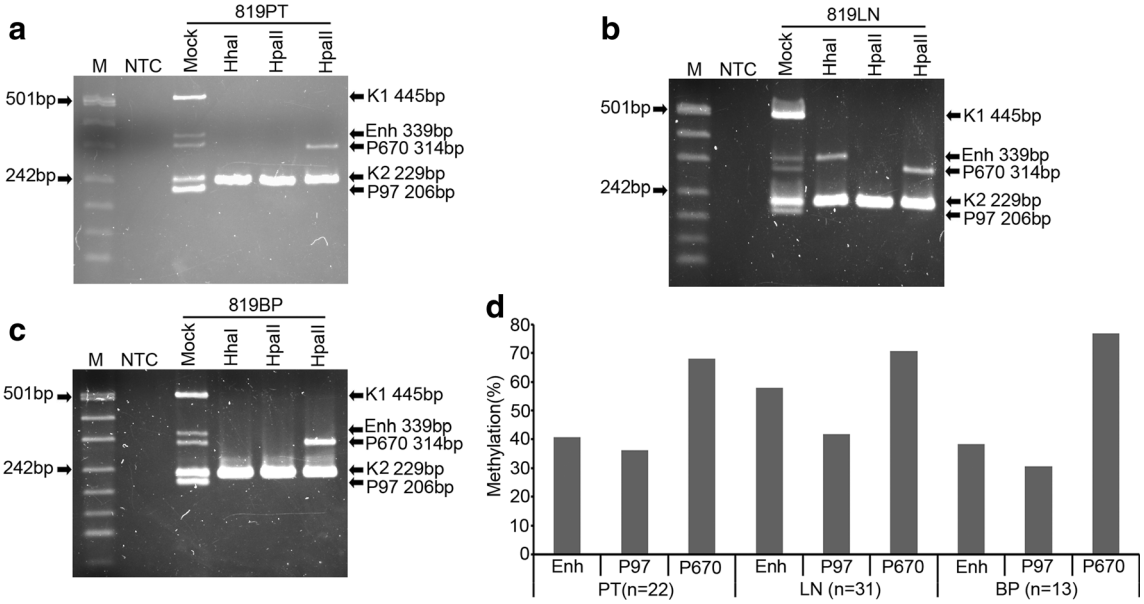

Fig. 4 Methylation analysis of Enhancer (Enh), Early promoter (P97) and Late promoter (P670) of HPV16 genome using methylation sensitive restriction analysis (MSRA). **a** Representative agarose gel picture showing HPV16 genome in PT of patient (Sample ID 819) to be unmethylated at the Enh (digested with HhaI) and P97 (digested with HpaII) while methylated at P670 (digested with HpaII). Mock = PT digested with no enzyme. **b** Corresponding LN of same patient showed methylation at the Enh and P670 and unmethylation at the P97 region. **c** Methylation at the P670 site and no methylation at P97 and Enh sites were observed in case of BP of same patient. pUC19 plasmid digested with HpaII marker was used as DNA marker (M) and NTC was non-template control. **d** Distribution of promoter methylation status of Enh, P97 and P670 across PT, LN and BP samples of CACX patients

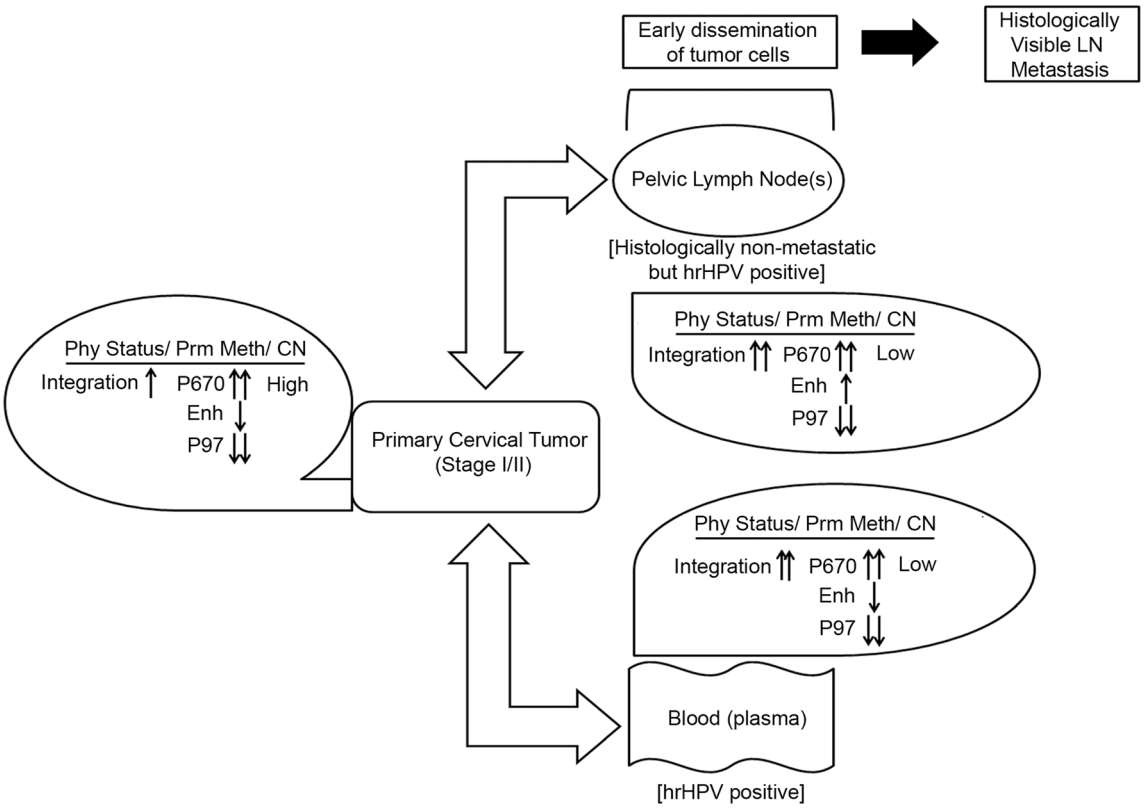

Fig. 5 The schematic diagram summarizes the current study results. Here the Physical status (Phy Status), Promoter Methylation pattern (Prm Meth) and Copy number (CN) of HPV16 genome in Primary cervical tumor (PT) and corresponding HPV positive histologically non-metastatic Lymph nodes (LNs) and Blood Plasma (BP) during early dissemination of tumor cells was shown

The physical state of the HPV16 genome in tumor cells may be in the integrated form or it may be in the mixed (integrated and episomal) form. It is well documented that E2 protein may inhibit the transcription of two important HPV16 oncoproteins E6 and E7 from their major promoters and so the loss of functional E2 gene may provide selective advantages during progression of PT. Concordant with previous studies [11, 13, 15], we also report here that in PT, the frequently encountered hrHPV subtype HPV16 resides in the integrated form. For the first time our present investigation revealed that the HPV16 genome detected either in pelvic non-metastatic LNs or in BP also harbors the integrated form. Here also the viral form showed concordance between PT with that of LNs and BP.

It was previously reported that high copy number of hrHPV genome in PT enhances HPV virulence [11, 13, 18, 19]. So, here we compared for the first time the copy number of the HPV16 genome in pelvic LNs and BP with respect to PT of same patient. A very low copy number of HPV16 genome was observed in histologically metastasis-negative pelvic LNs and BP though PT samples showed very high copy number of HPV16. It appears that in case of early-invasive (FIGO IA-IIA) PT, only few cancers cells have disseminated to adjacent pelvic LNs and BP leading to low copy number of the viral genome.

In CACX patients, it was already reported that hypomethylation of the HPV16 LCR region consisting of early promoter (P97) and enhancer play a crucial role in increased expression of HPV16 E6/E7 oncoproteins other than integrated genome status and high copy number [11, 15]. Moreover, late promoter (P670) responsible for transcription of capsid proteins L1 and L2 reported to have high frequency of methylation in CACX [14]. Concordant with previous reports, our study also revealed hrHPV16 genome residing in PT of early-invasive patients showed low methylation frequency of LCR (early promoter/enhancer) and high frequency of methylation in the late promoter. Some LNs and BPs showed differential methylation pattern than that of respective PT. This might be due to the differences in the disseminated tumor cells in different LNs and BP and also probably microenvironment where the tumor resides. Furthermore, statistical analysis (data not shown) showed no significant clinical correlation between viral genomic and epigenetic status with that of disease recurrence and overall survival. This might be due to the early stage of tumor cell dissemination and as progression to visible metastasis and disease recurrence depends on several parameters primarily individual patient's physiology and awareness.

Conclusion

It is well understood from our study that disseminated tumor cells in LNs and BP originated from the primary cervical tumors. Distinct genetic and epigenetic profiles of the viral genome residing in different microenvironments were also observed which paved the way for further expression based studies. Nonetheless, knowledge accrued from the present study may complement the histological diagnosis leading to better therapeutic intervention.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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