Does pretreatment human papillomavirus (HPV) titers predict radiation response and survival outcomes in cancer cervix?—A pilot study

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Abstract

Objective. To evaluate if pretreatment HPV titers in cancer cervix could predict radiation response and survival outcomes.

Methods. Twenty-one patients of cancer cervix were treated by radiotherapy (RT) alone. HPV titers were estimated using DNA Hybrid Capture II test. Loco-regional response at 1 month of RT — complete or partial response (CR and PR respectively) and survival outcomes — local disease-free (LDFS), disease-free (DFS) and overall (OS) survivals were evaluated against pre- and posttreatment HPV titers.

Results. Pretreatment HPV titers ranged from 0.81 to 3966.10 RLU/cut off (mean ± SD: 1264.39 ± 1148.22, median: 1129.98). Of the demographic features evaluated, mean HPV titers were significantly different only for patients achieving CR or PR at completion of RT (mean ± SD for CR vs. PR: 1616.31 ± 1146.86 vs. 384.57 ± 538.80, P = 0.022). HPV titers at end of RT ranged from 0.12 to 487.42 RLU/cut off (mean ± SD: 37.31 ± 108.60, median: 2.33). Patients with higher pretreatment HPV titers (>1000 RLU/cutoff) had a higher CR (P = 0.022) and better survival compared to those with ≤1000 RLU/cutoff (LDFS, P = 0.004; DFS, P = 0.005; OS, P = 0.012). At completion of RT, those having ≥99.5% fall in HPV had superior survival outcomes than those with <99.5% reduction (LDFS, P = 0.002; DFS, P = 0.002; OS, P = 0.004).

Conclusions. Higher pretreatment HPV titers (>1000 RLU/cutoff) could be considered as a predictor of radiotherapy response and survival in cancer cervix. A reduction in these titers to 99.5% of their baseline values at end of radiotherapy is also associated with better survival outcomes.

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Introduction

Cervical cancer is the third most common cancer in woman worldwide and is a major cause of mortality among women in developing countries [1]. The association of human papillomavirus (HPV) with cervical carcinogenesis is firmly established by both epidemiologic and experimental studies and found to be present in 99.7% of cervical cancers [2,3]. HPV test is thus being evaluated as a potential alternative or adjunctive to cervical cytology for the early detection of cervical cancer precursor and prevention of invasive cervical cancer.

Radiotherapy (RT) forms the primary modality of treatment in invasive cervical cancers. Surgery alone or in combination with chemoradiotherapy has also been a part of the treatment armamentarium. Treatment outcomes have been usually related to a number of prognostic factors, namely — molecular, immunohistochemical, biochemical, radiobiological, and clinical, although a consensus has yet to emerge [4]. Attempts to determine the prognostic significance of HPV DNA in patients with cervical cancer have yielded conflicting results, partly due to differences in the methodology of HPV detection used and partly to the treatments advocated [5–8].

Since HPV has been strongly implicated as an etiopathogenic agent in cancer cervix, it could be of interest to examine if the quantum of HPV infection could be an important determinant of treatment outcome following RT. This study was therefore carried out to explore if pretreatment HPV titers could predict the loco-regional response and major survival end point in patients of cervical cancer treated with RT.
Attempts have also been made to estimate the changes in these titers following RT and determine its influence on survival end points.

Materials and methods

Patient population

Twenty-one previously untreated and histopathologically confirmed patients of squamous cell carcinoma cervix (FIGO stages I–IVA) were enrolled for the study. Pretreatment work up included routine clinical, hematological, biochemical, and radiological investigations following which patients were staged as per FIGO guidelines. All patients were planned for 50 Gy of external radiation therapy (EXRT) delivered in 5 weeks at 2 Gy per fraction. This was followed 1 or 2 weeks later by three applications of high-dose-rate intracavitary brachytherapy of 6 Gy each at weekly intervals prescribed at point A. None of the patients were subjected to surgery or chemotherapy.

Collection of cervical samples for HPV estimates

Serial cervical smears were taken from the cervical growth before the start of EXRT and also at 1 month following the completion of RT. Cervical cells for HPV DNA testing were collected using the HPV detection kit-DNA Hybrid Capture II kit (HC II; Digene, Gaithersburg, MD, USA). This consisted of a cervical brush and one tube with 1 ml of specimen transport medium. The brush was rotated three times in full turns in the endocervical os and swabbed on the ectocervical epithelium and then placed in the specimen transport media by breaking the stem of the sampler. The specimens were stored at 4°C prior to HPV DNA testing. Each patient sample aliquot received unique identification number to ensure that the processing and testing were performed in a blinded manner at the laboratory.

Testing for HPV DNA and threshold for test positivity:

HPV DNA Hybrid Capture II is an enzyme linked immunosorbent chemiluminescent assay using microplates and is based on sandwich hybridization of the denatured viral DNA with RNA probe containing 13 oncogenic HPV types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). The captured hybrids were then reacted with the second antibody conjugated to alkaline phosphatase and detected by a chemiluminescent dioxetane based substrate. Chemiluminescence emitted, while the substrate got cleaved by the alkaline phosphatase conjugate, was measured in the relative light units (RLU) by a microplate luminometer. The intensity of light emitted was proportional to the amount of target (HPV) DNA in the sample. The procedure was carried out as detailed by Lorincz [9].

The test samples were classified as positive if the ratio of the sample RLU and the positive control was equal to or greater than 1. The RLU/cutoff obtained was a relative estimate of the HPV viral titers, compared to that of the positive control. The positive control according to the manufacture’s protocol represents around 5000 copies of HPV DNA and semi-quantitatively equals to 1.0 pg/ml [10]. The RLU/cutoff estimates in this study could be considered as a “HPV relative titer index”, representing the ratio between RLU of the patient’s sample and that of the positive control.

Loco-regional response assessment

Loco-regional response assessments at the primary and parametria were carried out at weekly intervals during the course of RT by clinical examination including per speculum, per vaginal, and recto-vaginal examinations. At 1 month following the completion of EXRT and intracavitary brachytherapy, loco-regional response was categorized into complete or partial response as follows:

(a) Complete response (CR): those with complete regression of cervical tumor with smoothening of the parametrium. (b) Partial response (PR): those with presence of residual tumor or palpable nodularity on the cervix, palpable nodularity on the parametrium, or both.

Patients were followed up at regular intervals with clinical examinations consisting of per speculum, per vaginal, and recto-vaginal examinations. Pap smear, hematological, biochemical parameters, and other radiological investigations were carried out from time to time.

Statistical analysis

Independent Student’s two-tailed t test was performed to test the differences in means of the pretreatment HPV titer values for various patient demographic groups. Survival end points were calculated from the date of registration and those evaluated included local disease-free survival (LDFS), disease-free survival (DFS) and overall survival (OS). For univariate analysis, patients were grouped based on the median values of continuous variables — age (<55 vs. ≥55 years), hemoglobin (≤11 vs. >11 gm/dl), and overall treatment time (OTT) (≤58 vs. >58 days). For pretreatment HPV titers, patients were grouped at a cutoff value of 1000 RLU/cutoff while a cutoff value of 99.5% reduction from the corresponding base line HPV titers was considered for postradiotherapy HPV titers. Kaplan–Meier estimates were computed for LDFS, DFS, and OS and analyzed by log-rank test. Subjects who continued to be disease-free on follow-up were considered as censored for the purpose of corresponding survival endpoint. All survival analyses were based on “worst-case scenario”. Statistical calculations were performed using SPSS software package for windows, version 9.0 (SPSS Inc. Chicago, IL, USA).

Results

The demographic profiles of the patients are shown in Table 1. Most of the patients were postmenopausal and belonged to FIGO stage II with predominantly ulcero-proliferative tumors.

All patients had squamous cell carcinoma. The median EXRT doses and intracavitary doses were 50 Gy and 18 Gy in 3 fractions respectively. Of the 21 patients, 15 achieved a CR (71.4%) at 1 month following completion of RT.

Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>52.3 ± 8.5*</td>
</tr>
<tr>
<td>Menopausal status</td>
<td>t1.4</td>
</tr>
<tr>
<td>Prenomenopausal</td>
<td>t1.5</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>t1.6</td>
</tr>
<tr>
<td>Hemoglobin (gm/dl)</td>
<td>10.7 ± 1.8*</td>
</tr>
<tr>
<td>Type of lesion</td>
<td>t1.8</td>
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<tr>
<td>Proliferative</td>
<td>t1.9</td>
</tr>
<tr>
<td>Ulcero-proliferative</td>
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<tr>
<td>Infiltrative</td>
<td>t1.11</td>
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<tr>
<td>FIGO stage</td>
<td>t1.12</td>
</tr>
<tr>
<td>I</td>
<td>t1.13</td>
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<tr>
<td>II</td>
<td>t1.14</td>
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<tr>
<td>III</td>
<td>t1.15</td>
</tr>
<tr>
<td>IV</td>
<td>t1.16</td>
</tr>
<tr>
<td>Response at end of RT</td>
<td>t1.17</td>
</tr>
<tr>
<td>CR</td>
<td>t1.18</td>
</tr>
<tr>
<td>PR</td>
<td>t1.19</td>
</tr>
<tr>
<td>Overall treatment time (days)</td>
<td>60.3 ± 10.1*</td>
</tr>
<tr>
<td>Baseline HPV titer (RLU/cut off)</td>
<td>1264.39 ± 1148.21*</td>
</tr>
<tr>
<td>HPV titer at end of RT (RLU/cut off)</td>
<td>37.31 ± 108.60*</td>
</tr>
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* Mean ± standard deviation; RT: radiation therapy; CR: complete response; PR: partial response; RLU/cutoff: relative light units; SD: standard deviation.
The HPV titers showed a wide range among these patients, with values ranging from 0.81 to 3966.10 RLU/cut off (median: 1129 RLU/cut off). The mean HPV titers were quantified for each of the demographic groups, and it was evident that there was no significant difference in the mean HPV titers between the various groups, namely age, menstrual status, hemoglobin, gross tumor features, and FIGO stages (Table 2). However, only those who had achieved CR had a significantly higher HPV titer (range: 19.35 to 3966.10 RLU/cut off; mean ± SD: 1616.31 ± 1146.86; median: 1591.83) compared to those having PR (range: 0.81 to 1285.47 RLU/cut off; mean ± SD: 384.57 ± 538.80; median: 85.93) (P = 0.022).

The postradiotherapy HPV titers estimated at 1 month ranged from 0.12 to 487 RLU/cut off (mean ± SD: 37.31 ± 108.60; median: 2.33). This amounted to around 500 times fall in the HPV titers following radiotherapy and the paired difference of means between the pretreatment and post-RT HPV titers were significant (P < 0.001). In terms of percentage reduction in HPV [computed as (Pretreatment HPV titers – Posttreatment HPV titers / Pretreatment HPV titers) × 100], the change in the HPV titers ranged from −99.9% to +373.7% (mean ± SD: −71.2% ± 103.3%; median: −99.5%).

### Pretreatment HPV titers and survival endpoints

Median follow-up was 29 months (range 2–50 months). Survival outcomes were compared for various demographic groups and HPV titers. The survival end points — LDFS, DFS, and OS were not significantly different for patient groups, namely age, menstrual status, hemoglobin, and gross tumor features. Patients in stages I and II had a better OS (P = 0.036). However, those attaining CR with RT and patients with pretreatment HPV titers of >1000 RLU/cut off had a significantly better survival for all survival end points (CR vs. PR, LDFS: P = 0.0004; DFS: P = 0.0002; OS: P = 0.001); (HPV > 1000 vs. ≤1000 RLU/cut off, LDFS: P = 0.004; DFS: P = 0.005; OS: P = 0.012) (Table 3).

### Postradiotherapy HPV titers and survival endpoints

Since a median fall of 99.5% in HPV titers was observed following RT, patients were grouped into 2 categories — those with fall of ≥99.5% vs. <99.5% fall of their pretreatment HPV titers. All the survival end points were significantly better for those demonstrating a fall of ≥99.5% in their HPV titers compared to their pretreatment values (LDFS: P = 0.002; DFS: P = 0.002 and OS: P = 0.004). None of the patients who had a fall of ≥99.5% had any failure in any of these end points during the period of follow-up ranging from 18 months to 50 months.

### Discussion

Human papillomavirus infection represents the most important risk factor for developing cervical cancers. The strong relationship between HPV and human cervical carcinoma is suggested by several lines of evidence: (a) HPV DNA was...
found in more than 90% of such tumors, (b) vast majority of HPV positive tumor biopsies and nearly all HPV containing cell lines derived from cervical carcinoma reveal specific transcripts originating uniformly from E6 and E7 proteins suggesting of persisting HPV DNA, (c) transforming activity of E6, E7 has been demonstrated clearly in vitro, and their level of expression has been shown to be linked specifically to the mitotic activity of HPV infected cervical tumors, and (d) continued expression of E6 and E7 regions of the viral genomes has been shown to be necessary for maintaining the malignant phenotype [11].

Variety of molecular methods exists for use of detection and quantification of HPV DNA namely, direct probe methods (southern blot), signal amplification (Hybrid Capture II), and target amplification (polymerase chain reaction (PCR) technology). The direct probe techniques offer the least sensitivity for detecting a specific DNA sequence, whereas the high sensitivity is achieved by target amplification [12]. The HPV DNA Hybrid Capture II (HC) assay is the only HPV test with Food and Drug Administration (FDA) approval at this time that detects a group of 13 cancers associated HPV types and has demonstrated good reproducibility [13,14]. The sensitivity of HPV testing by HC II for detection of cervical intraepithelial neoplasia (CIN) II and III varied from 62 to 97%, specificity from 41 to 92% and positive predictive value from 6.7 to 13.7% [10,15–18] and is equivocal to PCR assays [14,19].

Although the involvement of HPV in the development of cervical cancer has been firmly established, however, its prognostic significance in patients presenting with HPV positive and HPV negative cervical cancer has yielded conflicting results. A good correlation between presence of HPV and outcomes has been demonstrated by several workers [5,6,8,20]. Others have failed to document such a correlation [7,21–25]. These could be as a result of varied methodologies which have been adopted over a period of time to test for HPV and also impact of treatment delivered to the patients.

To the best of our knowledge, there has been no study reported to correlate the outcomes to radiotherapy with the pre- and postradiotherapy HPV titers. The present pilot study was therefore designed to explore the possibility of assessing if the pretreatment HPV titers as estimated by the DNA HC II test could predict the outcomes in cancer cervix that have been uniformly treated with radiotherapy with a standard treatment protocol. As already stated, the RLU/cutoff values estimated represent the “HPV relative titer index” for the test samples.

Of the predictors evaluated in this study, it was apparent that patients who went on to achieve CR at 1 month following RT had a significantly higher pretreatment base line HPV titers (\(P = 0.022\)) (Table 2). The survival end points evaluated also showed that those achieving CR had a significantly better long-term LDFS, DFS, and OS. On a multivariate analysis too (data not shown), the extent of response to radiotherapy (CR or PR) had emerged as the only significant predictor for all the survival end points. Thus, since there was a significant difference in the pretreatment HPV titers of patients who went on to achieve CR or PR, the pretreatment HPV titers could be considered as an important marker for the likelihood of a favorable response to RT. This was also apparent in patients with a higher pretreatment HPV titers (>1000 RLU/cut off) who went on to have a better survival outcome (Table 3 and Figs. 1–3). Pretreatment HPV titers could therefore be considered as a key predictor in patients of cancer cervix.

The changes in the HPV titers following radiotherapy have been quite impressive with a median fall of 99.5% from the baseline values. This was also translated in a superior survival, with none of the patients who had a reduction in HPV to at least 99.5% or more failing during the follow-up (Table 3, Figs. 4 to 6).

Looking at the etiopathogenesis of cancer cervix by HPV, the E6 and E7 proteins of oncogenic HPV types temporarily degrade p53 and inactivate pRb (retinoblastoma gene) tumor suppressor genes and consequently initiate malignant transformation [7,26]. The inactivation of these tumor suppressor genes could get reverted into its wild form, once the HPV virus load is reduced by RT. It has been shown in vitro that genotoxic treatment could reduce E6/E7 expression and induce apoptosis in HPV positive cancer cell lines [27]. Radiation thus could decrease the capacity of E6/E7 to interfere with p53/pRb and other host proteins rendering HPV positive cancers more...
responsive to radiation therapy. It has been also reported that irradiation could also enhance the local tumor immunogenicity as a consequence of upregulation of the E6/E7 and major histocompatibility complex Class I on HPV positive cells, leading to favorable outcomes [26]. On the contrary, patients with a low pretreatment HPV titers or negative HPV could have the neoplastic process triggered by serial mutations of the various proto-oncogene leading to oncogenic activation or mutation of the p53 [8,27]. These malignant transformations might render the tumor more aggressive and virulent leading to failures with radiotherapy alone [28].

The results of this pilot study indicate that patients with high initial HPV titers could be favorably treated with radiotherapy alone. Chemotherapy in HPV positive tumors could be ineffective since in vitro studies have been reported these to be chemoresistant, while HPV negative cells were found to be chemosensitive [29]. A combined chemoradiotherapy approach

Fig. 2. Survival plots for various end points in patients with HPV titers ≤1000 or >1000 RLU/ cutoff. (a) Local disease-free survival (median survival for ≤1000 RLU/cutoff vs. >1000 RLU/cutoff: Not reached vs. 0 months, log-rank $P < 0.001$) (b) disease-free survival (median survival for ≤1000 RLU/cutoff vs. >1000 RLU/cutoff: Not reached vs. 0 months, log-rank $P < 0.001$) (c) Overall survival (median survival for ≤1000 RLU/cutoff vs. >1000 RLU/cutoff: Not reached vs. 12 months, log-rank $P = 0.001$).

Fig. 3. Survival plots for various end points in patients with decrease in HPV titers of either ≥ or <99.5% from their pretreatment values. (a) Local disease-free survival (median survivals for ≥ or <99.5%: Not reached vs. 20 months, log-rank $P = 0.002$) (b) disease-free survival (median survivals for ≥ or <99.5%: Not reached vs. 17 months, log-rank $P = 0.002$) (c) Overall survival (median survivals for ≥ or <99.5%: Not reached vs. 20 months, log-rank $P = 0.002$).
could therefore be selectively advocated for HPV negative
tumors or those having low pretreatment HPV titers. This could
have a bearing on cost effective therapeutic options, especially
in developing countries having a high incidence of cancer
cervix.

Even though this pilot study conducted with a small sample
size of 21 cervix cancer patients shows a promising role of HPV
titers as an important prognosticator, it would be worthwhile to
further evaluate and confirm its role in a larger patient group.

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