HPV DNA in plasma of patients with cervical carcinoma

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Abstract

Background: HPV DNA has been detected in metastatic tumour and HPV plasma viraemia may indicate a poor prognosis and a high risk for metastasis. Objective: Detection of HPV DNA in plasma of patients with cervical carcinoma. Study design: A cross-sectional study was done, wherein cervical biopsies and plasma samples were collected from 58 women with invasive cervical carcinoma, 10 women with cervical intraepithelial neoplasia (CIN) and 30 control women in the same age range. Polymerase chain reaction (PCR) was employed to detect the presence of HPV DNA. Samples positive for HPV DNA were typed by restriction fragment length polymorphism (RFLP). To confirm that the HPV sequence in plasma was identical to that in tissue, sequencing was done on all the paired plasma and tissue samples.

Results: All the 30 paired cervical tissue and plasma samples from the controls were negative for HPV DNA. HPV DNA was detectable in cervical tissues of 55 (94.8%) of 58 patients with invasive cervical carcinoma and in all 10 patients (100%) with CIN and in eight (11.8%) of the total 68 plasma samples from patients. All eight plasma samples were from women with invasive cervical carcinoma with three each in stages IIIB and IV and one each in stages IIB and IB, respectively. Of the eight positive samples, seven were typed as HPV-16 and 1 as HPV-58. HPV types detected in cervical tissue and plasma pairs from these eight patients correlated as revealed by RFLP and sequencing. A patient with stage IB cancer had detectable HPV DNA in the external iliac lymph node, removed at Wertheims hysterectomy, which was histopathologically free of tumour. The HPV type in the node, was the same as that present in the paired tissue and plasma sample.

Conclusions: HPV DNA is detectable in the plasma of patients with advanced cervical cancer.

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1. Introduction

Many epidemiological studies have identified a consistent association of human papillomavirus (HPV) infection with the development of cervical cancer (Clifford et al., 2003; Walboomers et al., 1999). Two HPV gene products, the E6 and E7 oncoproteins, inactivate the cellular tumour suppressor proteins, p53 and the retinoblastoma gene products pRb, respectively (Dyson et al., 1989; Werness et al., 1990) resulting in loss of cell cycle control (Werness et al., 1990). This points to an important role of HPV in cervical carcinogenesis.

The prognosis for patients with locally advanced carcinoma of the cervix is poor, particularly for those with bulky tumour and parametrial extension (Montana et al., 1986). Metastasis to distant sites and local recurrence, are major problems associated with the clinical management of patients with locally advanced cervical carcinoma. Hence identification of patients at risk for distant metastasis along with early detection of distant metastasis are very important for initiating prompt and appropriate therapy. Thus, reliable markers of tumour metastasis and their detection gains utmost significance.

Circulating tumour DNA has been identified in the serum and plasma of cancer patients (Amkre et al., 1999; Sidransky, 2000; Stroun et al., 2000). Tumour specific DNA has been identified in the serum or plasma of patients through specific genetic and epigenetic alterations found in the primary
tumour (Sanchez-Cespedes et al., 2000). The exact pathway through which the tumour DNA is released into the bloodstream still remains unclear, but the presence of circulating tumour DNA is likely to be a reflection of tumour load or metastasis and thus may have some prognostic value for cancer patients (Castells et al., 1999; Jen et al., 2000; Lo et al., 1999a; Sidransky, 2000).

Studies have documented the presence of viral sequences in the peripheral blood of certain groups of cancer patients. Epstein–Barr Virus (EBV) DNA has been detected in the serum and plasma samples of patients with nasopharyngeal carcinoma (Lo et al., 1999a,b) and typing results of concomitant tumour samples and sera have shown that, serum EBV DNA represents disseminated tumour DNA (Mutirangura et al., 1998).

HPV mRNA was detected in the peripheral blood of patients with advanced stage cervical cancer with metastasis who were also positive for HPV DNA in cervical cancer tissues. In the laboratory, the blood samples were centrifuged at 2000 rpm for 10 min at 4°C, and transported to the laboratory at −60°C. Concomitantly blood samples were obtained by venipuncture from all of these patients using anti-coagulant (EDTA) and transported to the laboratory at +4°C as for tissues. In the laboratory, the blood samples were centrifuged at 2000 rpm for 10 min at +4°C. Supernatant plasma was then stored as multiple aliquots of 200 μl at −60°C.

2.1.2. Controls (n = 30)

This group comprised of 30 women undergoing hysterectomy for reasons unassociated with HPV disease enlisted in Table 1. Pap-smears were negative for malignant cells in all of the control women. A part of the cervical tissue from the transformation zone of the hysterectomized specimen was taken and transported to the virology laboratory along with concomitantly collected blood samples. Transportation and processing of the samples was performed in the same way as that for patients.

The age range of the control group was 37–62 years (mean: 45.3, S.D.: 6.26). The histopathological report for all of the women in this group was chronic cervicitis and without any area of cervical neoplasia.

2. Material and methods

2.1. Subjects

2.1.1. Patients (n = 68)

Cervical biopsy samples were obtained from women who had undergone biopsy or surgery for invasive cervical carcinoma or cervical intraepithelial neoplasia (CIN) in the collaborating unit of Obstetrics and Gynaecology Department at the Christian Medical College, Vellore during the time period June 2001–April 2003. Ethical clearance was obtained from the institutional Research Committee. Verbal consent was also obtained from the study patients after explaining the nature and consequences of the study.

The samples were collected from a total of 58 women with invasive cervical carcinoma and 10 women with CIN (one each diagnosed as CIN I and II, respectively and eight diagnosed as CIN III). Staging of cervical carcinoma was done by the examining clinician according to the International Federation of Gynaecology and Obstetrics (FIGO) criteria. Biopsied samples were cut into two: one piece sent for histopathology and the other transferred into a sterile nuclease free tube containing viral transport medium (VTM), transported to the virology laboratory immediately at +4°C in an ice bucket. Upon receipt of the samples in the laboratory, the tissue samples were cut into small fragments of approximately 25 mg and stored as multiple aliquots at −60°C.

Table 1. Presence of plasma HPV DNA in the patient and control groups

<table>
<thead>
<tr>
<th>Subject category</th>
<th>Clinical diagnosis</th>
<th>Total number of patients</th>
<th>Age mean ± S.D.</th>
<th>Number positive for HPV DNA in plasma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient group</td>
<td>Stage IV</td>
<td>3</td>
<td>47 ± 5.55</td>
<td>3 (100)</td>
</tr>
<tr>
<td></td>
<td>Stage IIIB</td>
<td>18</td>
<td>47.6 ± 7.66</td>
<td>1 (4.8)</td>
</tr>
<tr>
<td></td>
<td>Stage IIIB</td>
<td>21</td>
<td>40 ± 9.17</td>
<td>1 (4.8)</td>
</tr>
<tr>
<td></td>
<td>Stage IIIB</td>
<td>1</td>
<td>47.5 ± 6.36</td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>Fibroid/adenomyosis</td>
<td>10</td>
<td>45 ± 5.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prolapse uterine</td>
<td>20</td>
<td>46.7 ± 6.45</td>
<td></td>
</tr>
</tbody>
</table>
2.1.3. Detection and typing of HPV DNA

DNA was extracted from the tissue samples using the QIAamp DNA Mini Kit (QIAGEN Germany). Following DNA extraction, HPV DNA amplification was carried out using the consensus primers MY09/MY11 (Ting and Manos, 1990). To ensure specimen adequacy and integrity of the extraction process, amplification of the beta globin gene was also done concomitantly using specific primers (Baner et al., 1991). Each reaction mixture consisted of 10 μl of the extracted DNA, 4 mM MgCl₂, 200 μM dNTPs, 50 pmol each of the consensus primers (MY09/MY11), 5 pmol each of beta globin primers (PC04 and GH20) with 2.5 U of FastStart Taq polymerase (Roche Diagnostics, GmbH, Mannheim, Germany) in a final reaction volume of 100 μl.

The thermal cycling conditions consisted of a pre-polymerase chain reaction (PCR) denaturation at 94 °C for 6 min followed by 30 cycles each of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min 30 s followed by a post-PCR extension at 72 °C for 10 min. The 450 base pair sized amplified products were detected by ethidium bromide containing agarose gel electrophoresis.

DNA was also extracted from 200 μl of plasma using the QIAGEN DNA Blood Mini kit (QIAGEN, Germany). Following DNA extraction, amplification was done similar to the protocol for tissues with the total number of cycles for the PCR increased to 40.

Cervical tissue and plasma samples positive for HPV DNA were typed by restriction fragment length polymorphism (RFLP) using a set of seven different restriction endonucleases BamHI, DdeI, HaeIII, HinfI, PstI, RsaI and Sau3AI (Roche Diagnostics, GmbH, Mannheim, Germany). Digested products were detected by electrophoresis through an ethidium bromide containing agarose gel electrophoresis. The molecular weights of the bands obtained were analyzed with the aid of a gel documentation system (Gel Doc 2000, BioRad).

Interpretation of the HPV type was done by comparison of the digestion pattern obtained with a previous publication (Bernard et al., 1994). Standard precautions to avoid false positive signals while performing the PCR assays were followed (Kwok and Higuchi, 1989).

2.2. DNA sequencing

To validate RFLP findings, DNA sequencing was performed on all of the plasma samples positive for HPV DNA along with the paired cervical tissue samples. Amplified products generated using the MY09/MY11 primers were subjected to cycle sequencing with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Sequencing reactions were run on the ABI Prism 310 Genetic Analyser (PE Applied Biosystems, Foster City, Calif.). Viral sequences thus obtained were analyzed using sequencing analysis software and sequencing navigator. Study sequences were compared with GenBank sequences using the Basic Local Alignment Search Tool (BLAST) programme. The identity score between the HPV DNA sequences present in tissue and plasma, was obtained using the CLUSTAL W (1.82) Multiple Sequence Alignment Software (European Bio Informatics Institute).

3. Results

HPV DNA was not detectable in both cervical tissue and paired plasma of all 30 control subjects. In the patient group, HPV DNA was detectable in the cervical tissues of 55 (94.8%) of the 58 patients with invasive cervical carcinoma and in all of the 10 patients (100%) with CIN. The HPV types detected in the cervical tissues were types 16, 18, 31, 33, 35, 45, and 58. Of the total of 68 matching plasma samples from patients, eight (11.8%) samples were positive for HPV DNA. All of the eight samples were from patients with invasive cervical cancer and none from cases of CIN. The clinical stage of cervical cancer in each of these patients is given in Table 1. All three patients with invasive carcinoma had cervical HPV types 16, 18, and 45.

<table>
<thead>
<tr>
<th>Age</th>
<th>Parity</th>
<th>Presentation</th>
<th>Stage</th>
<th>Histology</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>7</td>
<td>Vaginal discharge; postmenarcheal node</td>
<td>IV</td>
<td>Moderately differentiated adeno carcinoma</td>
<td>Anuria after 1 year</td>
</tr>
<tr>
<td>40</td>
<td></td>
<td>Vesicovaginal fistula; inguinal nodes</td>
<td>IV</td>
<td>Moderately differentiated squamous carcinoma</td>
<td>Death after 2 weeks</td>
</tr>
<tr>
<td>46</td>
<td>Not known</td>
<td>Vaginal discharge; polypoid</td>
<td>IV</td>
<td>Poorly differentiated squamous carcinoma</td>
<td>Well 3 months after chemotherapy</td>
</tr>
<tr>
<td>38</td>
<td>4</td>
<td>Vaginal discharge; polypoid</td>
<td>IIIIB</td>
<td>Well differentiated squamous carcinoma</td>
<td>Residual disease after radiotherapy; Hysterectomy elsewhere</td>
</tr>
<tr>
<td>45</td>
<td>3</td>
<td>Vaginal discharge</td>
<td>IIIIB</td>
<td>Poorly differentiated squamous carcinoma</td>
<td>Defeated and lost to follow up</td>
</tr>
<tr>
<td>42</td>
<td>4</td>
<td>Metrorrhagia</td>
<td>IIIIB</td>
<td>Poorly differentiated squamous carcinoma</td>
<td>Lost to follow up after 5 months</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>Vaginal discharge</td>
<td>IIIIB</td>
<td>Poorly differentiated squamous carcinoma</td>
<td>Disease free 10 months after Wertheims hysterectomy</td>
</tr>
<tr>
<td>60</td>
<td>6</td>
<td>Post menopausal bleeding</td>
<td>IIIB</td>
<td>Moderately differentiated squamous carcinoma</td>
<td>Anuria after 1 year</td>
</tr>
</tbody>
</table>

Table 2 Characteristics of patients with HPV viraemia
cervical carcinoma who were negative for HPV in cervical tissue were also negative for plasma HPV DNA.

Of the eight plasma samples, seven were typed as HPV-16 and one as HPV-58 by RFLP of all of which were identical to the type present in the paired cervical tissue sample. DNA sequencing performed on all the paired cervical tissue and plasma samples, confirmed the types identified by RFLP.

The alignment score for the HPV types identified in the paired set of samples ranged from 95 to 99 as given by the CLUSTAL W software.

Seven of the eight patients with HPV positivity in plasma presented with advanced stage cervical cancer; three (37.5%) with stage IV cancer, three (37.5%) with stage IIIB cancer and one stage IIB (12.5%). All of these patients were referred to the radiation oncology unit in the same hospital. The characteristics of all the 8 patients with HPV viraemia is given in Table 2.

4. Discussion

In this study, HPV DNA was detectable in the plasma of a small subset of patients with HPV positive invasive cervical carcinoma. The same HPV type was identified in both the cervical biopsy and the concomitant plasma sample as revealed by RFLP and DNA sequencing. HPV DNA was not detected in any of the plasma samples from controls or from patients with CIN, thereby confirming the specificity of plasma HPV DNA detection. As seen in Table 1, the presence of HPV DNA in plasma is directly related to the stage of the cancer with seven of the eight patients presenting with advanced cancer (all three patients with stage IV cancer in this study had HPV viraemia). On the other hand, none of the patients with CIN had HPV DNA in plasma.

The presence of circulating plasma HPV DNA has been correlated with metastasis in both HPV-associated cervical (Pornthanakesam et al., 2001) and head and neck cancer (Capone et al., 2000). At the time that this report was written, with the exception of two patients (both in stage IV cancer), metastasis was not documented in the remaining six of the eight patients positive for plasma HPV DNA. Of the two patients with metastases, one revealed metastases to the left supraclavicular lymph node and the other to the inguinal lymph nodes. Among the remaining patients who could be followed up, one patient in stage IIIB cancer did not reveal metastasis. The single patient with stage IB cancer who had HPV DNA in one of the pelvic lymph nodes removed during Wertheims hysterectomy (which was free of tumor histopathologically) did not have any evidence of metastases even after 10 months following hysterectomy. The absence of clinical evidence of metastases in patients who could be followed up and the inability to follow up the other patients precluded our ability to predict whether HPV plasma viraemia in our study correlated with metastases.

HPV DNA plasma positivity has been found to correlate with poor prognosis (Pornthanakesam et al., 2001). As seen in the present study, detection of HPV DNA in plasma of patients with advanced stage cervical cancer would not be additionally informative as such patients obviously have a poor prognosis. On the other hand, plasma HPV DNA positivity is of significance when detected in patients with early stage cancer. It is these patients who would need careful monitoring. In our study, patients in early stage invasive cancer were too few in number (one each in stages IB and IIB, respectively). The patient with stage IIB cancer was lost to follow up while the patient with stage IB cancer was disease free 10 months following Wertheims hysterectomy. Thus, the prognostic value of plasma HPV DNA detection could not be substantiated due to low numbers and the cross-sectional nature of this study. This report, however, represents a feasibility study suggesting that HPV DNA is detectable in plasma of patients with cervical cancer further adding to previously existing knowledge.

It is to be noted that all the control women in the present study were negative for HPV DNA though previous studies have shown that high-risk HPV types are detectable in cytologically normal young women (Bauer et al., 1991; Chan et al., 2002; Deacon et al., 2000; Hildesheim et al., 2001). This discrepancy could be attributed to the following reasons. This control population represented predominantly older women (mean age ± S.D.: 45.3 ± 6.26) who were presumably less sexually active and with a normal cervical cytology. Studies have shown that the prevalence of high-risk HPV infection in such women declines with increasing age (Chan et al., 2002; Herrero et al., 2000; Jacobs et al., 2000). This could be related in part to the decreased sexual activity of the older women making them less prone to infection with the high-risk HPV types. Additionally, as all women in this age group had negative Pap smears with no specific cervical pathology except for a diagnosis of cervicitis (cytology, courtesy Department of Pathology) it is unlikely that they could be harbouring high-risk HPV types. Though the patient group also consisted of women in more or less the same age group, these were women with obvious cervical pathology. Cervical neoplasia in these women would have been related to the persistent presence of high-risk HPV types detected in them as shown earlier (Ho et al., 1998). In the absence of any other discernible risk factors between the patient and the control group it could be suggested that the presence of high-risk HPV types in the patients could have contributed to cervical neoplasia in them.

It has been shown that prevalence of low-risk and uncharacterized risk HPV types is higher in cytologically normal older women (Chan et al., 2002; Herrero et al., 2000). If some of these controls were harbouring such HPV types, it is possible that the primers employed in this study (MY primers) failed to amplify the viral DNA. Decreased amplification efficiency of the low-risk HPV types by the MY primers has been previously shown (Gravitt et al., 2000).

In contrast to a previous study, where the HPV type present in the paired tissue and plasma did not correlate in all the viremic samples (Dong et al., 2002), in our study...
a 100% concordance was seen. The use of the consensus MY09/MY11 primers for the detection of HPV DNA in plasma of cervical cancer patients has not been applied so far. Plasma HPV DNA detection by the use of these consensus primers, has the additional advantage in that, it could help in the detection of many HPV types thereby avoiding the usage of type specific primers. Usage of type-specific primers will require multiple sets of amplification for each sample. The use of type-specific primers for only the predominant HPV types 16 and 18 as in a previous study (Dong et al., 2002) will allow for the detection of only these HPV types in plasma, while other HPV types probably remain undetected. Such an approach could result in an underestimation of the true HPV DNA prevalence in plasma. This was also alluded to by the researchers of the above study (Dong et al., 2002).

Speculatively, HPV DNA in plasma could be due to various factors, such as the presence of blood borne virus particles, necrosis or apoptosis of cells resulting in release of viral DNA into the bloodstream (Capone et al., 2000) or presence of virus in nontumour cervical cells or non cervical cells (Tsong et al., 1999). The first possibility wherein plasma HPV DNA could be due to presence of blood borne virus particles is unlikely in this study as the presence of circulating blood borne HPV virions have not been shown to exist (Tsong et al., 1999). The second possibility of tumour necrosis has been suggested as a general mechanism for the presence of tumor DNA in plasma, based on the finding that increased amount of DNA is present in plasma of patients with large tumors and metastases (Stroun et al., 2000). But in the case of HPV viremia there are two observations not in favour of tumor necrosis. One is that, HPV viremia has been reported even in early stage cervical cancers (Porathnakasem et al., 2001). Secondly, it has been seen that serum HPV DNA levels actually decrease following primary treatment (Widschwendter et al., 2003) suggesting that tumor necrosis may be an unlikely cause of HPV viremia.

The third possibility for the presence of plasma HPV DNA being due to apoptosis of cells could result in the cervical cells becoming bereft of their membrane integrity resulting in HPV DNA release into the bloodstream. However, it is to be noted that apoptosis is a property that is lost by the proliferating cancer cells. Proliferating cervical cancer cells harbouring high-risk HPV DNA also express the E6 oncoprotein. One of the prime functions of the E6 protein is degradation of the tumor suppressor protein, p53 (Werness et al., 1990). p53 is a cell-cycle regulatory protein that promotes the apoptosis in cells that have undergone irreparable DNA injury (Howley et al., 2001). Thus, the degradation of p53 by the HPV E6 protein prevents apoptosis of cells resulting in the accumulation of cells with genomic abnormalities (Howley et al., 2001) contributing to progression of cervical neoplasia. As a result of this phenomenon, it seems unlikely that apoptosis could result in release of HPV DNA into blood. The fourth possibility, wherein plasma HPV DNA could be due to presence of HPV DNA in nontumour cervical cells or non cervical cells is not likely in our study as the same HPV sequences were obtained from all of the eight cancerous cervical tissue/plasma pairs. This strengthens the fact that the HPV DNA in plasma originated from the cervical tissues of these patients.

Multiple variables, such as the immune status of the host and invasive capacity of the tumour play a very important role in the establishment of a successful metastasis (Fidler, 1990). It is possible that in patients with plasma positivity but no evidence of metastasis, the metastatic process may be delayed or prevented by the individual’s host immunity (Capone et al., 2000). Thus, the presence of HPV DNA does not necessarily indicate that metastases will develop but can rather serve as an indicator of high risk for metastasis and poor prognosis.

In conclusion, in this study we report the detection of HPV DNA in the plasma of patients with HPV associated cervical carcinoma. Similarity in the HPV types detected in cervical tissue and plasma samples strengthens the conclusion that the circulating HPV DNA is only from the HPV present in the cervical tissue. As the plasma samples drawn in this present study, represent a single time point during the patient’s disease process, longitudinal studies may throw more light on the role of HPV plasma detection as a predictor of metastasis and poor prognosis.

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