Comprehensive analysis of Human Papillomavirus and Chlamydia trachomatis in in-situ and invasive cervical adenocarcinoma

Gynecologic Oncology 2009; 114: 390-4
Abstract

Objective: *Chlamydia trachomatis* (Ct) has been implicated as a co-factor in cervical carcinogenesis. The goal of the current study was to investigate if Ct may play a role in pathogenesis of cervical adenocarcinoma and, specifically, if there is a co-infection between Ct and Human Papillomavirus (HPV) in cervical adenocarcinomas. The second goal of the study was to determine the distribution of HPV genotypes in most recent cases of in-situ and invasive cervical adenocarcinomas.

Methods: Biopsies of 71 cervical adenocarcinomas (31 in-situ and 40 invasive) were tested for the presence of Ct using two novel PCR assays. In addition, all cases were tested for HPV using SPF10-PCR assay and genotyped using LIPA25 test.

Results: None of the cases were found to be positive for Ct using two independent PCR assays. All lesions, however, were positive for HPV with the exception of a case of minimal deviation adenocarcinoma. Overall, 94.2% of cases were positive for either HPV16 (n=44, 62.8%) or HPV18 (n=20, 28.5%), or both (n=2, 2.8%). Other single HPV types included HPV45 (n=3, 4.2%) and HPV35 (n=1, 1.4%).

Conclusion: The study demonstrated lack of co-infection between *Chlamydia trachomatis* and *Human Papillomavirus* in-situ and invasive adenocarcinoma of the uterine cervix. The role of Ct as a carcinogenetic co-factor may be restricted to cervical squamous cell carcinomas. Accounting for type cross-protection, currently available HPV vaccines are likely to prevent close to 100% of HPV-positive cervical adenocarcinomas.

Introduction

The incidence of invasive squamous cell carcinoma has decreased in the developed countries thanks to effective screening programs; however, the incidence of cervical adenocarcinoma has been increasing in the recent years. This trend has been observed in both the industrialized and developing countries [1,2,3]. A recent study from Canada reported a 40% increase in the incidence of cervical adenocarcinoma between 1970 and 1996, which coincided with a 51% drop in the incidence of cervical squamous cell carcinoma during the same period [3]. These observations bring cervical adenocarcinoma into the focus of research on tumor pathogenesis and prevention.

The role of HPV as an etiologic factor of cervical adenocarcinoma has been firmly established, however, only few studies have analysed and compared distribution of HPV genotypes in endocervical adenocarcinoma in-situ (AIS) and invasive adenocarcinoma. For many decades detection of HPV in cervical adenocarcinomas posed a technical difficulty due to a low viral load in these lesions. Only recently, thoroughly designed broad-spectrum PCR primers facilitated amplification of HPV DNA with high sensitivity and allowed wide-spectrum HPV genotyping [4-7]. With the raising incidence of cervical adenocarcinoma, it appears that the screening programs are not offering adequate protection against this type of malignancy and HPV vaccination could provide a prophylactic alternative. Reliable data on HPV genotype spectrum in cervical adenocarcinomas is necessary to assess the potential benefit of the vaccines. In our institution we have conducted a study of HPV prevalence in archival cases of cervical adenocarcinoma collected between 1978 and 1998 [8]. Introduction of Gardasil and Cervarix vaccines prompted us to re-analyse HPV genotype distribution in cases of invasive and in-situ cervical adenocarcinoma collected between 1999 and 2008 to assess HPV genotype spectrum in most recent cancer cases.

Results of several recent studies suggested that *Chlamydia trachomatis* (Ct) might be a cofactor in pathogenesis of cervical squamous cell carcinoma. In this study we were interested in detection of Ct in cervical adenocarcinoma, since such finding could result in additional preventive strategy for this subtype of cervical malignancy.

Reports from several multicenter studies have shown that detection of Ct antibodies in serum correlates with a higher risk for development of squamous cell carcinoma of the cervix (SCC) [9-13]. The antibody titer analysis implied that an active, chronic infection with Ct was responsible for the increased risk [10]. In addition, an association between SCC and Ct detected by PCR in cytological material has been described [13]. These studies suggested that Ct
might play a role of a co-factor in cervical carcinogenesis. Two possible mechanisms were proposed. Ct may be facilitating Human Papillomavirus (HPV) infection by damaging the mucosal barrier. Alternatively, Ct may support HPV persistence by interfering with immune response and viral clearance [14,15]. Synchronous co-infection between Ct and HPV in cervical squamous neoplasia has been reported [16], and cases with co-infection showed altered expression of some of cell cycle and immune response markers [16].

The endocervix (together with the squamocolumnar junction) is the major site of Ct infection in the lower genital tract. Given this localization, we hypothesized that Ct infection may play a role in initiation of malignant transformation of the glandular endocervical epithelium via outlined above mechanisms related to HPV co-infection. In addition, Ct may play a role in progression from adenocarcinomas in-situ to invasive adenocarcinomas by evoking the inflammatory response that damages the mucosal barrier including the basement membrane.

For this study, we used three groups of neoplastic glandular lesions representing the chronological steps of tumor evolution: 1) adenocarcinoma in-situ, representing a recent malignant transformation; 2) concurrent AIS and invasive adenocarcinoma, representing a recent onset of the invasive process; and 3) pure invasive adenocarcinoma, representing a longstanding invasive phase in which the in-situ component has been obliterated. Ct testing was performed using a previously described highly sensitive PCR/genotyping assay [17] and an in-house PCR, developed specifically for formalin-fixed paraffin-embedded material. In addition, HPV genotyping was performed on all cases using the very sensitive version 1 SPF10-LiPA25 system.

Methods

Samples

The study included 31 cases of adenocarcinoma in-situ (28 endocervical and 3 intestinal subtype) and 40 cases of invasive adenocarcinoma (33 endocervical, 4 endometrioid, 2 intestinal subtype, 1 minimal deviation adenocarcinoma = adenoma malignum) which consisted of 23 cases of invasive adenocarcinoma with concurrent AIS and 17 cases of pure invasive adenocarcinoma. The cases were re-reviewed and diagnoses were confirmed using standard histologic criteria [18]. The presence of coexisting HSIL lesion was recorded. All cases were retrieved from the files of the Pathology Department at the Weill Medical College of Cornell University New York, NY. Institutional review board approval was obtained for this study.

DNA preparation

Genomic DNA was prepared from two to three 4μm sections from each case using standard methods. Briefly, the slides were deparaffinized and tissue was scraped with a sterile blade. The DNA was released from the samples using 100 μl proteinase K solution (1mg/ml) for 16 h at 56°C. Following heat inactivation at 95°C for 10 minutes 10 μl of the supernatant was used for PCR. Appropriate positive and negative controls were incorporated during DNA preparation and subsequent testing. The negative controls allowed monitoring for possible tissue contamination. To ensure adequate DNA preparation, PCR amplification of a 132 bp fragment of the β-globin gene was performed in a separate reaction. All 71 cases of adenocarcinoma showed positive amplification of β-globin gene and were used for subsequent Chlamydia and HPV testing.

Chlamydia trachomatis detection

Testing for Ct was performed using the Detection and genoTyping (Ct-DT) assay (Labo Biomedical Products BV, Rijswijk, The Netherlands), as described previously [17]. It is a PCR-based assay in which a 157/160 bp fragment of the OmpA gene and a 241 bp fragment of cryptic plasmid are amplified. The test is sensitive for the detection of all 19 Ct serovars. After amplification, differentiation between serovars A, B/Ba, C, D/Da, E, F, G/Ga, H, J/1a, J, K, L1/L2/L2a, L3 can be performed. The differentiation is based on the OmpA gene variation. In addition, a novel in-house Small Fragment (SF-CT) PCR assay was developed to amplify two targets: a 157/160 bp fragment of the OmpA gene (primers as described previously [17]) and a shorter, 89 bp fragment of the Ct cryptic plasmid. The forward and reverse primer sequences for cryptic plasmid amplification were 5’gggagaaagaatgtagctgtg’3 and 5’caagtctcaaggaggttaagc’3. This second PCR was developed specifically for formalin-fixed paraffin-embedded cases. The use of biotinylated reverse primers allowed the capture of the amplimers onto streptavidin-coated microtiter plates. Subsequently, the amplimers were detected using digoxigenin-labeled probes. The SF-CT PCR was validated on cervical swab samples and had the same sensitivity as the Ct-DT assay. All PCR reactions were carried out in parallel with negative controls (water) and positive controls consisting of Ct serovar L2/434-B reference strain DNA. A case of colon adenocarcinoma metastatic to the cervix was used as a negative tissue control.

Human Papillomavirus detection and typing

Broad-spectrum HPV DNA amplification and mucosal HPV genotyping was performed using the SPF10-LiPA25 system (SPF10 HPV LiPA, version 1; manufactured by Labo Bio-Medical Products, Rijswijk, The Netherlands) as described
The results of HPV genotyping are presented in Table 1. From a total of 70 combined cases of AIS and invasive adenocarcinomas, 94.2% were positive for either HPV16 (n=44, 62.8%) or HPV18 (n=20, 28.5%), or both (n=2, 2.8%). Of the other single HPV types detected, HPV45 accounted for 3 cases (4.2%) and HPV35 was found in one case (1.4%).

The ratio of HPV16 to HPV18 showed a gradual shift from marked HPV16 predominance in AIS towards more equal distribution in pure invasive adenocarcinoma. HPV16:18 ratio was equal to 71:26, 65:35 and 56:38 in AIS, AIS+invasive adenocarcinoma and pure invasive adenocarcinoma, respectively.

Multiple HPV infection in AIS (22.6%) was twice as common as in invasive adenocarcinomas (10.2%).

Of all the cases positive for HPV16, as much as 63% (29 of 46) had a coexisting HSIL, while only 32% (7 of 22) of adenocarcinomas positive for HPV18 had a concurrent HSIL (chi-square test = .015).

The majority of AIS cases (n=24, 77.4%) coexisted with high grade squamous intraepithelial lesions (HSIL). HSIL was significantly less common in cases of AIS+invasive adenocarcinomas (n=9, 39.1%) and pure invasive adenocarcinomas (n=4, 25.0%) (chi-square test = .0002).

Results

**Chlamydia trachomatis detection**

*Chlamydia trachomatis* DNA was not detected in any of the 71 samples of endocervical adenocarcinoma with neither Ct-DT nor SF-CT PCR assays. Figure 1 shows agarose gel with results of positive control amplification in SF-CT PCR using serial dilutions of isolated Ct serovar L2/434-B reference strain DNA. Lane 3 contains 10 µl of amplified, undiluted, isolated reference strain DNA (V0) and lanes 4 to 12 contain 10 µl of amplified Ct reference strain DNA in 0.5 log serial dilution (V1 to V9, respectively). Amplification was positive until V5 for both the cryptic plasmid and OmpA VS2 amplicons. The result of this dilution series was identical to the result of amplification using Ct-DT PCR with the same dilution series (data not shown), indicating that both assays have comparable sensitivity.

**Human Papillomavirus detection and typing**

All cases of adenocarcinoma were positive for HPV with the exception of a case of minimal deviation adenocarcinoma, which was expected to test negative and was excluded from further analysis.
contains 10-20 copies of the cryptic plasmid and only single copies of chromosomal genes. PCR assays amplifying sequences from cryptic plasmid are expected to have at least 10-fold higher sensitivity than PCR assays amplifying equally long sequences from Ct chromosome. A weakness of this study was that the new SF-CT PCR test was validated on cervical cytology samples and not on paraffin embedded tissue. Nevertheless, the sensitivity of a PCR reaction is mainly determined by the availability of target molecules, the absence/presence of PCR inhibiting factors and the length of the generated amplicon. In our study the presence of DNA and the absence of PCR inhibiting factors was verified with β-globin control PCR reaction in which the β-globin amplicon (132 bp) was longer than the Ct amplicon (89 bp). In addition, Ct control reactions using isolated serovar L2/434-B reference strain resulted in positive amplification in both Ct-DT and SF-CT PCR.

Despite the use of two novel, sensitive Ct detection assays we did not detect Ct DNA in any of the 71 cases of cervical adenocarcinoma. Recently, Zereu et al [19] reported a similar result in 67 cases of cervical adenocarcinoma using a less sensitive PCR assay in which a 281 bp amplicon was generated from the Ct major outer membrane protein (MOMP) gene.

Previous epidemiological studies suggested a role for Ct as a co-factor in cervical carcinogenesis [9-13] demonstrating that Ct antibodies were associated with a higher risk of SCC. These findings were corroborated by the results of a cytological study reporting a correlation between early Ct infection and subsequent development of cervical SCC [13]. Synchronous co-infection between Ct and HPV in squamous neoplasia and alteration of cell cycle and immune response markers has been reported [16]. If Ct is indeed a co-factor in cervical carcinogenesis, such role may be limited to squamous cell carcinoma.

In the current study HPV was detected in all cases of cervical adenocarcinoma, excluding a single case of minimal deviation adenocarcinoma. Minimal deviation adenocarcinoma (“adenoma malignum”), has been recently identified as a malignancy not related to HPV infection but associated with a rare condition of lobular endocervical glandular hyperplasia/pyloric gland metaplasia [20, 21]. Adenoma malignum is a rare tumor, accounting for approximately 1-2% of adenocarcinomas.

HPV16 and HPV18 were found in 93.5% cases of adenocarcinoma in-situ and 94.8% cases of invasive adenocarcinoma. Only two previous studies analyzed and compared HPV type distribution in AIS and invasive adenocarcinomas: a multicenter study from the Netherlands by Zielinski et al [22, 23] and our previous multicenter study (Pirog et al [8]). The analysis in other publications was limited to either invasive adenocarcinomas (Castellsague et al [24], An et al [25]) or to detection of HPV16 and 18 only (Tawfik et al [26], Riethdorf et al...
[27], Anciaux et al [28]). Older studies were also hampered by low sensitivity assays [28,29]. The summary of previously reported results is outlined in Table 2.

As compared to our earlier study we have observed a higher rate of HPV positivity in invasive adenocarcinomas (97.5% versus 80.8% [8]) and we ascribe it to higher diagnostic accuracy and exclusion of tumors spreading to the cervix from endometrial cavity or other primary sites, like the gastrointestinal tract. In a follow up of the cases from our previous study [8] we have identified three HPV-negative adenocarcinomas which were initially misclassified as primary cervical malignancies but were in fact the metastases from an endometrial, colonic and primary peritoneal carcinoma, respectively. In all 3 cases the cervical mass/tumor was initially the only finding and the primary malignancy was discovered subsequently, within the following year.

The overall HPV positivity in invasive cervical adenocarcinomas reported in the most recent studies exceeds 90% and is close to 100% for cervical adenocarcinomas in-situ. In studies that analyzed the spectrum of HPV genotypes, HPV16 and HPV18 together accounted for 75.7% to 94.8% of cases, with an average of 89.8% [8, 22, 24, 25 and current study]. While the percentage of combined HPV16 and HPV18-positive cases showed relative consistency between the studies, the percentage of individual HPV types showed striking variation, without a clear geographical pattern. And thus the ratio of HPV16:18 ranged from marked HPV16 predominance (Tawfik et al [26], North Africa & South America in Castellsague et al [24]) to marked HPV18 predominance (Zielinski et al [22], South East Asia in Castellsague et al [24]) and some studies showed flip of the ratio between AIS and INV (Riethdorf et al [27], Anciaux et al [28]). One would expect that if AIS is a precursor of invasive adenocarcinomas then these two types of lesions should have similar HPV type distribution within the cases drawn from the same population. In our current study the ratio of HPV16 to HPV18 showed a gradual shift from marked HPV16 predominance in AIS (71:26) towards more equal distribution in pure invasive adenocarcinomas (56:38). A similar trend was observed in our previous study. We hypothesized that the relative decrease of HPV16 prevalence in invasive lesions in our study might be related to either a faster progression of HPV18-positive AIS, or elimination of some of HPV16-positive AIS via either a spontaneous regression, or an earlier excision. This latter explanation may be plausible. Since Pap test has relatively higher sensitivity for detection of HSIL than AIS [30], AIS coexisting with HSIL may be detected and excised earlier than AIS without coexisting HSIL. In our study, 61% of HPV16-positive adenocarcinomas had coexisting HSIL, compared to only 33% cases positive for HPV18. More efficient detection and excision of AIS concurrent with HSIL may

### Table 2 Comparison of the results of HPV DNA genotyping in AIS and invasive adenocarcinomas.

<table>
<thead>
<tr>
<th>Publication</th>
<th>lesion N</th>
<th>HPV+</th>
<th>HPV 16+18</th>
<th>Ratio</th>
<th>OTHER SINGLE HPV</th>
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<tbody>
<tr>
<td>Quint et al- current study</td>
<td>AIS 31</td>
<td>100</td>
<td>93.5</td>
<td>71.26</td>
<td>6.4 [HPV45,35]</td>
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<tr>
<td>USA</td>
<td>INV 40</td>
<td>97.5</td>
<td>94.9</td>
<td>62.36</td>
<td>5.1 [HPV45]</td>
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<tr>
<td>Zielinski et al 2003 [22]</td>
<td>AIS 65</td>
<td>100</td>
<td>93.8</td>
<td>40.68</td>
<td>6.1 [HPV45,31]</td>
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<tr>
<td>Holland</td>
<td>INV 77</td>
<td>93.5</td>
<td>88.8</td>
<td>35.58</td>
<td>11.1 [HPV45,39]</td>
</tr>
<tr>
<td>Pirog et al 2000 [8]</td>
<td>AIS 23</td>
<td>100</td>
<td>91.3</td>
<td>65.30</td>
<td>8.6 [HPV45,35]</td>
</tr>
<tr>
<td>USA &amp; Poland</td>
<td>INV 73</td>
<td>80.8</td>
<td>86.4</td>
<td>44.44</td>
<td>13.5 [HPV45,52]</td>
</tr>
</tbody>
</table>

### Studies including only invasive adenocarcinomas

<table>
<thead>
<tr>
<th>Publication</th>
<th>lesion N</th>
<th>HPV+</th>
<th>HPV 16+18</th>
<th>Ratio</th>
<th>OTHER SINGLE HPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castellsague et al 2006 [24]</td>
<td>combined samples from three continents</td>
<td>INV 157</td>
<td>92.9</td>
<td>85.6</td>
<td>52.39</td>
</tr>
<tr>
<td>North Africa</td>
<td>INV 23</td>
<td>95.6</td>
<td>86.4</td>
<td>73.14</td>
<td></td>
</tr>
<tr>
<td>South America</td>
<td>INV 50</td>
<td>92.0</td>
<td>83.7</td>
<td>72.20</td>
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<tr>
<td>South East Asia</td>
<td>INV 84</td>
<td>92.8</td>
<td>86.5</td>
<td>35.58</td>
<td></td>
</tr>
<tr>
<td>An et al 2005 [25]</td>
<td>INV 113</td>
<td>88.5</td>
<td>75.7</td>
<td>48.35</td>
<td>24.2 [HPV33,45,59]</td>
</tr>
<tr>
<td>South Korea</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</table>

### Studies limited to detection of HPV16 and HPV18

<table>
<thead>
<tr>
<th>Publication</th>
<th>lesion N</th>
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<th>HPV 16+18</th>
<th>Ratio</th>
<th>OTHER SINGLE HPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tawfik et al 2006 [26]</td>
<td>AIS 20</td>
<td>75.0</td>
<td>66.53</td>
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<tr>
<td>Scotland</td>
<td>INV 97</td>
<td>57.7</td>
<td>75.48</td>
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<tr>
<td>Riethdorf et al 2000 [27]</td>
<td>AIS 33</td>
<td>87.8</td>
<td>79.31</td>
<td>not tested</td>
<td></td>
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<tr>
<td>Germany &amp; USA</td>
<td>INV 54</td>
<td>85.1</td>
<td>48.65</td>
<td>not tested</td>
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<tr>
<td>Anciaux et al 1997 [28]</td>
<td>AIS 21</td>
<td>76.1</td>
<td>38.63</td>
<td>not tested</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>INV 20</td>
<td>55.0</td>
<td>73.27</td>
<td>not tested</td>
<td></td>
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</tbody>
</table>

AIS = adenocarcinoma in-situ
INV= invasive adenocarcinoma
a data for adenosquamous carcinomas not included
b combined data for cervical adenocarcinomas and adenosquamous carcinomas
manifest in a high rate of HPV16-positive AIS, and a lower rate of HPV16-positive invasive adenocarcinomas. HPV45 and HPV35 were other single HPV genotypes identified in cervical adenocarcinomas in our study. As Cervarix vaccine targeting HPV16 and HPV18 has been shown to offer a cross-protection against HPV45 and HPV31 [31, 32], it is expected that vaccination programs may also prevent non-HPV16/18 positive cervical adenocarcinomas.

In conclusion, the current study demonstrated lack of co-infection between Human Papillomavirus and *Chlamydia trachomatis* in adenocarcinoma in-situ and invasive adenocarcinoma of the uterine cervix. HPV16 and HPV18 were detected in 94.2% of the cases. Accounting for type cross-protection, currently available HPV vaccines are likely to prevent close to 100% of HPV-positive cervical adenocarcinomas.

Disclosure/Conflict of Interest:
The authors declare no competing interests and no financial interests.

References


