

# Cytopathology and More | Evidence emerging for HPV-negative cervical cancer



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**January 2014—Some studies indicate that nearly** all cervical cancers are high-risk human papillomavirus (hrHPV)-related.<sup>1</sup> Recent studies suggested hrHPV testing had a very high sensitivity<sup>2</sup>; therefore, the American Society for Colposcopy and Cervical Pathology recommended Pap cytology and hrHPV co-testing as the preferred screening method in women 30 or older.<sup>3,4</sup> However, a wide range of negative detection rates of hrHPV have been reported in cervical cancers by different HPV testing methods, including the HC2 test (Qiagen), the most widely used HPV assay in the United States. Furthermore, large-scale studies have provided solid evidence for the existence of HPV-negative cervical cancers, which are present in almost all types of cervical cancers, including squamous cell carcinoma, usual adenocarcinoma, unusual subtypes of adenocarcinoma, and other rare types of cervical cancers. Therefore, to maximize the detection of cervical cancers, both hrHPV-positive and hrHPV-negative, the best measures are to perform co-testing with cervical Pap cytology and FDA-approved hrHPV tests.

The fact that hrHPV DNA is present in almost all invasive carcinomas indicates that HPV-negative carcinoma is extremely rare, if it exists at all. However, recent studies have documented the existence of true HPV-negative cervical cancers.

## **HPV detection by PCR methods on FFPE materials**

Despite the many assays available for HPV DNA detection, efforts have been made recently to develop more hrHPV DNA detection methods that have much higher sensitivity and specificity. Impressive progress has been made in the past few years on HPV typing based on polymerase chain reaction methods. The majority of protocols use degenerate or consensus primers, or both, followed by an additional assay that allows the identification of specific HPV types.<sup>5,8</sup> The most commonly used PCR assays amplify HPV L1, E1, or E6/E7 regions.<sup>9,10</sup> (See “Cervical cancer and HPV,” page 44.) Using degenerate and/or consensus primers has the advantage of being able to detect a large spectrum of HPV types by a single PCR. A similar LiPA assay was also developed, which is a test to use PCR with SPF10 broad-spectrum primers followed by DNA enzyme immunoassay and genotyping with a reverse hybridization line probe assay.<sup>11</sup> Gheit, et al., described a reliable E7 PCR-based assay to detect a large spectrum of hrHPV types in formalin-fixed paraffin-embedded specimens. This assay combines the advantages of the multiplex PCR methods—that is, high sensitivity—with an array primer extension (APEX) assay, which offers the benefits of Sanger sequencing with the high-throughput potential of the microarray.<sup>12</sup>

## **True HPV-negative cervical cancers by PCR**

Giorgi, et al., reported a pooled analysis of the three largest case series in which HPV typing was performed on formalin-fixed paraffin-embedded slides from histologically confirmed invasive cervical cancer cases.<sup>13</sup> Three different PCR methods were used to detect HPV DNA in these studies:

- Central and Southern Italy study. A PCR assay based on GP5+/GP6+

primers was used to detect 12 hrHPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59).<sup>14</sup>

- Rome study. PCR amplimers of the HPV DNA-positive samples, as identified by DEIA, were analyzed by Line Probe Assay-LiPA25 (Laboratory Biomedical Products, Rijswijk, Netherlands), which can detect 25 different high-risk and low-risk HPV types (6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, 74).<sup>15</sup>
- Milan study. Multiplex PCR was performed using the Qiagen Multiplex PCR Kit, which detected 19 pairs of HPV-type specific primers (type 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82).<sup>(16)</sup> This study demonstrated that 24 (4.2 percent) cases of 574 invasive cervical cancers were true HPV negative.

Recently, Tjalma, et al., also reported large-scale data from 17 European countries in two parallel cross-sectional studies. A total of 3,162 cases with invasive cervical cancers were tested for HPV using the SPF10-DEIA/LiPA25 PCR system (SPF10- LiPA25). They found that 8.2 percent of cases of invasive cervical cancers were HPV negative.<sup>(17)</sup>

#### **True HPV-negative cervical squamous cell carcinoma and usual adenocarcinoma**

One large international retrospective cross-sectional study collected formalin-fixed paraffin-embedded histological samples of invasive cervical cancer from 38 countries.<sup>(18)</sup> HPV detection was done by PCR with SPF10 broad-spectrum primers followed by DNA enzyme immunoassay and genotyping with a reverse hybridization line probe assay (LiPA25), which detects 25 high- and low-risk HPV types. Sequencing was used to identify HPV type in HPV-positive samples that were not characterized by use of hybridization. A total of 10,575 cases of invasive cervical cancer were tested, and 1,598 (15 percent) of these were negative for HPV DNA. HPV DNA was not detected in 1,234 (13 percent) of 9,486 cases of squamous cell carcinoma, 290 (38 percent) of 760 cases of adenocarcinoma, 36 (19 percent) of 191 cases of adenosquamous cell carcinoma, and 38 (28 percent) of 138 histologically rare cases of invasive cervical cancer including undifferentiated, neuroendocrine, not otherwise specified, basal adenoid, and cystic adenoid carcinomas. The authors concluded that HPV negativity was largely attributable to technical artifacts (poor DNA quality and PCR inhibition) (60 percent of HPV-negative cases) as well as low viral load. However, the authors also cannot exclude the possibility that a small proportion of cases of invasive cervical cancer, especially in the group with adenocarcinomas, might arise independent of exposure to HPV. Otherwise it is difficult to explain why there exists a significant difference in HPV negative rates between cervical squamous cell carcinoma and adenocarcinomas (13 percent versus 38 percent).

#### **True HPV-negative unusual subtypes of cervical adenocarcinoma**

Invasive cervical adenocarcinoma has been increasing in incidence during the past few decades, particularly in younger women. Recent reports indicated that adenocarcinoma accounted for 20 to 25 percent of uterine cervical cancer compared with only five to 10 percent in the past.<sup>19</sup> The Surveillance Epidemiology and End Results database indicated that the age-adjusted incidence rate of cervical adenocarcinoma per 100,000 women increased from 1.34 in the 1970s to 1.73 in the 1990s, and the ratio of patients with adenocarcinoma versus squamous carcinoma doubled in this period.<sup>19</sup>

Pirog, et al., examined the prevalence of HPV in different histological subtypes of cervical adenocarcinoma and related tumors using FFPE tissue samples from 105 primary cervical adenocarcinomas and adenosquamous carcinomas.<sup>20</sup> Broad-spectrum HPV DNA amplification was performed using the short PCR fragment (SPF10) primer set, and the samples identified as positive for HPV DNA were genotyped with line probe assay (LiPA25). Their data

showed that HPV DNA was detected in 82 of 90 (91 percent) mucinous adenocarcinomas (endocervical, intestinal, and endometrioid types) and nine of nine adenosquamous carcinomas. It was not detected in nonmucinous cervical adenocarcinomas (four clear cell, one serous, and one mesonephric carcinoma) or minimal deviation adenocarcinomas (two cases). The data indicated that a few percent of usual endocervical adenocarcinomas and unusual subtypes of adenocarcinoma might be unrelated to HPV infection. The most common viral types detected in adenocarcinoma were HPV 16 (50 percent) and HPV 18 (40 percent).

In a population-based study of Korean women from 15 different institutes in Korea, testing for the status of HPV infection in adenocarcinoma of the uterine cervix, 432 cervical adenocarcinomas from 1997 to 2001 were examined.<sup>21</sup> HPV typing was performed with HPV DNA chip (82 cases) and PCR HPV typing (53 cases) by using FFPE archival tissue. The overall prevalence of HPV infection in cervical adenocarcinoma was 90 percent. The infection of HPV 16 and/or HPV 18 accounted for 78 percent of HPV-positive adenocarcinomas. They found that the minimal deviation adenocarcinoma was rarely associated with HPV infection, with HPV DNA detected in only one of four cases (25 percent).

Using Linear Array HPV genotyping, which can detect 37 HPV genotypes (18 high-risk types: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82; and 19 low-risk types: 6, 11, 40, 42, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, IS39, CP108), Houghton, et al., demonstrated that all three cases of minimal deviation adenocarcinomas were negative for HPV DNA.<sup>22</sup> In addition, they found that cervical adenocarcinoma subtypes of gastric (three cases), intestinal (three cases), mesonephric (two cases), clear cell (three cases), and hepatoid (one case) carcinoma were HPV negative.

Consistently, Kusanagi, et al., performed PCR with HPV genotype-specific primers, which cover 16 HPV genotypes (6, 11, 16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66) and showed absence of hrHPV detection in seven cases of endocervical adenocarcinoma with gastric morphology and phenotype.<sup>23</sup>

A retrospective study of invasive cervical carcinoma from the cases collected from Jikei University School of Medicine in Tokyo, Japan (1990 to 2005), and Memorial Sloan-Kettering Cancer Center in New York, NY (1992 to 2006), was conducted to detect HPV DNA from 26 cases of unusual subtypes of cervical cancer: clear cell carcinoma (n=9), gastric type adenocarcinoma (n=11), minimal deviation adenocarcinoma (n=3), mesonephric adenocarcinoma (n=1), serous adenocarcinoma (n=1), and malignant mixed Müllerian tumor (n=1).<sup>24</sup> HPV DNA detection was done by SPF10 PCR and LiPA<sup>25</sup>. All three cases of minimal deviation adenocarcinoma were negative for HPV DNA. Furthermore, HPV DNA was not detected in any of nine cases of clear cell carcinoma or in single cases of mesonephric adenocarcinoma and malignant mixed Müllerian tumor. One case of serous adenocarcinoma was positive for HPV type 16.

Recently, Kenny, et al., examined HPV DNA in seven cervical mesonephric adenocarcinomas. HPV detection was done by Linear Array HPV genotyping analysis, which can detect 37 HPV types: 18 high risk (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82) and 19 low risk (6, 11, 40, 42, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, IS39, CP108). They found that all cases were HPV negative.<sup>(25)</sup>

Table 1 summarizes the results of negative HPV detection in unusual types of cervical cancers.

In summary, about 90 percent of cervical adenocarcinomas are hrHPV-positive based on the aforementioned studies. Compared with cervical squamous cell carcinoma, cervical adenocarcinoma has a slightly lower hrHPV detection rate. Similar to cervical squamous cell carcinoma, types 16 and 18 are still the most commonly isolated HPV types; however, HPV 18 is proportionally more common in cervical adenocarcinoma than in cervical squamous carcinomas. Most of the unusual subtypes of invasive cervical carcinomas, such as minimal deviation adenocarcinoma, gastric-type, intestinal-type, mesonephric, and clear cell, are not related predominantly to HPV.<sup>26</sup>

### **Negative HPV test in Pap cytology specimens from patients with cervical cancer**

A variable percentage of cervical cancer patients had negative HPV testing in Pap cytology specimens. Data from Kaiser Permanente showed that 18 (37 percent) of 49 cervical squamous cell carcinoma patients and six (22

percent) of 27 cervical adenocarcinoma patients had a baseline negative HPV test result five years before a histological diagnosis of cervical cancer.<sup>2</sup> A multicenter data collection of prior hrHPV testing results of 58 invasive cervical carcinomas diagnosed in 2012 from 16 U.S. institutions presented at the 61st annual ASC scientific meeting demonstrated that among 44 patients within one year of tissue diagnosis of cervical cancer, four of 45 HPV tests (8.9 percent) were negative. The negative HPV detection rates were 22.7 percent and 25 percent one to three years and three to five years, respectively, before a histological diagnosis of cervical cancer. The negative hrHPV rate may be higher in patients with adenocarcinoma than in patients with squamous carcinoma.<sup>27</sup>

Our other study indicated that four of 29 patients (14 percent) had HPV-negative results more than four months to three years before histopathologic diagnoses of cervical glandular neoplasia, including three cases of AIS and one case of invasive adenocarcinoma.<sup>28</sup>

Among FDA-approved HPV tests, HC2 is considered a gold standard test. Any newly developed HPV test should meet the same validation criteria as HC2, such as selectivity, specificity, accuracy, precision, detection limit, and quantization limit. However, studies from different countries such as Slovenia, China, and Korea have demonstrated that HC2 can have negative results in cervical cytology specimens from patients with cervical cancer, and the HC2-negative rates ranged from 6.6 to 12.6 percent.

In the UK ARTISTIC clinical trial (A Randomised Trial in Screening To Improve Cytology), HC2-negative results were documented in three of 12 patients (25 percent) who were diagnosed with invasive cervical carcinoma during two screening rounds.<sup>29</sup> Poljak, et al., of Slovenia studied the presence of high-risk HPV by using real-time PCR and HC2 assays on 95 cases of archived routine cervical specimens, collected from the same number of women with histologically confirmed cervical carcinoma. Their comparative evaluation of HPV status by these two methods suggested the HC2-negative rate was about 12.6 percent.<sup>30</sup> A similar HC2-negative rate was reported by Wu, et al., of Guangdong province, China, with a 10.1 percent HC2-negative rate in patients with invasive cervical cancer,<sup>31</sup> though a slightly lower rate of five percent of HC2-negative testing in invasive cervical cancer was reported from Zhejiang province, China.<sup>32</sup> A study from Korea also demonstrated a similar range of HC2-negative results: 5.2 percent in cervical squamous cell carcinoma and 11.6 percent in cervical invasive adenocarcinoma.<sup>33</sup>

Australia has a unique, successful National Cervical Screening Program, which has been in place for 20 years. Farnsworth reviewed the program's cytological screening data. HC2 is the most commonly used HPV test in New South Wales, but the type of assay used in these reports was not known with certainty. The percentage of negative HPV testing within 30 months preceding the histologic diagnosis was eight percent for cervical squamous cell carcinoma and up to 80 percent for cervical adenocarcinoma.<sup>34</sup>

These studies indicate that HC2, a widely used assay to detect HPV in cytological specimens in the U.S., has a wide range of negative test results for hrHPV in cervical cytological specimens from patients with cervical cancers. The question behind these negative results is whether they are true negatives or false-negatives. Recently, we studied 287 patients with cervical squamous cell carcinoma and collected their hrHPV test results before the histological diagnosis. Among 31 cases of squamous cell carcinoma with liquid-based cytology and hrHPV co-test results less than one year before squamous cell carcinoma diagnosis, three patients (9.7 percent) had HC2-negative HPV results. PCR with GP5+/GP6+ consensus primers was performed on FFPE tissues, and HPV DNA was detected in all three cases.<sup>35</sup> Naryshkin and Austin reported a similar case with false-negative HPV detection.<sup>36</sup> These data indicate that at least a certain percentage of HC2-negative cases are false-negatives. The plausible explanations for false-negative HPV testing include, among others, 1) detection assay does not cover specific HPV type; 2) low titer or low copy number of HPV; 3) presence of inhibitors in specimen; 4) limitation of analytic sensitivity; and 5) inadequacy of specimen.<sup>37</sup>

True HPV-negative cervical cancers also account for a small portion of cervical cancers that test negative for HPV. As mentioned, data based on sensitive PCR tests using formalin-fixed paraffin-embedded materials have proved that the existence of true HPV-negative invasive cervical cancer is undebatable. It implies that true HPV-negative cervical cancers are derived in a hrHPV-independent pathway. This pathway could be identical to hrHPV-independent p53 pathway of vulvar intraepithelial neoplasia and squamous cell carcinoma, though they have been

much less well studied, and the molecular mechanisms involved in this pathway have not yet been fully elucidated.<sup>38</sup>

Finally, rapidly developing type II cervical cancer might also account for a very small portion of these cervical cancers that test HPV negative, since HPV testing might be performed out of the HPV infection period.<sup>39</sup>

## Conclusion

In summary, emerging evidence strongly supports the concept of HPV-negative cervical cancers. HrHPV-negative cervical cancers are present in almost all types of cervical cancers: squamous cell carcinoma, usual adenocarcinoma, unusual subtypes of adenocarcinoma, and other rare types of cervical cancers. Therefore, to maximize the detection of cervical cancer, co-testing with cervical Pap cytology and an FDA-approved hrHPV test is strongly recommended in the newly updated screening guidelines for the prevention and early detection of cervical cancer.<sup>3,4</sup> However, the current guideline with co-testing at five-year screening intervals needs to be evaluated further by additional historical data collection, since there seems to be a considerable number of negative results for both Pap and hrHPV testing within the period of three to five years before invasive cancer diagnosis.□

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