Usefulness of DNA Ploidy Measurement on Liquid-Based Smears Showing Conflicting Results Between Cytology and High-Risk Human Papillomavirus Typing

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Abstract

To improve the positive predictive value (PPV) for high-risk human papillomavirus (HR-HPV) in primary screening, DNA ploidy was measured on the same liquid-based sample by image cytometry in 984 cases showing discrepancies between cytology and HR-HPV testing. Of the conflicting results, 14.5% corresponded to a cytologic lesion (from atypical squamous cells of undetermined significance to high-grade squamous intraepithelial lesion [HSIL]) without HPV detected, and 85.5% of smears were within normal limits but revealed an HR-HPV infection. A suspect DNA profile was associated significantly with a lesion. In 497 patients who underwent repeated HPV testing, a normal DNA profile at the first smear predicted the clearance of HPV infection (sensitivity, 81.5%; specificity, 45.4%; PPV, 69%; negative predictive value, 62.4%). In persistent HR-HPV infection, a suspect DNA profile at the first smear increased the PPV from 10.8% to 22.7% for the detection of a histologically proven HSIL with a sensitivity of 95.2%. DNA ploidy can be used to select smears with high risk of HSIL, especially in cases of persistent HR-HPV infection.

At present, screening of cervical cancer is based on the detection of cytomorphologically abnormal cells. Detection has been improved by the use of liquid-based cytology methods. These methods offer some advantages in comparison with conventional Papanicolaou (Pap) smears. In particular, they reduce problems related to sampling errors1 and increase the proportion of satisfactory examinations according to the Bethesda System2 and the number of positive diagnoses3 (high-grade squamous intraepithelial lesion [HSIL], low-grade squamous intraepithelial lesion [LSIL], and atypical squamous cells of undetermined significance [ASCUS]). Numerous studies have reported that liquid-based cytology is more accurate than conventional cytology and has the potential to optimize the effectiveness of primary cervical cancer screening.4-7 However, even if the number of false-negative results has decreased with the use of liquid-based cytology, the sensitivity of cervical cancer screening still can be improved with the development of new approaches such as high-risk human papillomavirus (HR-HPV) testing and DNA ploidy measurement, which can be done on the same sample.

It is well established that HR-HPV infection is a necessary but not sufficient condition for cervical carcinogenesis.8-11 Indeed, HR-HPV types have been detected in 99.7% of invasive cervical carcinomas.10 Moreover, HR-HPV infections are associated with a relative risk of between 8 and 11 for the development of SIL,12 and essentially LSILs containing HR-HPV progress to HSIL.13 Because of this, the use of HR-HPV testing in primary screening is proposed by some authors. A recent study of 7,932 females demonstrated that the sensitivity of HPV testing for detecting a histologically proven HSIL was dramatically higher than that of conventional and liquid-based cytology.14 However, in the
experience of Clavel et al, the specificity and positive predictive value of HPV testing for detecting HSIL remained low at 85.6% and 9.3%, respectively. The high incidence (about 10% in our study) of HR-HPV infection in females with normal smears is largely responsible for that. Thus, the use of additional biomarkers would be of great help to select more specifically the smears of patients in whom HSIL could develop.

It is well established that lesions in which cervical cells have an aneuploid DNA profile are more likely to persist or progress than those with diploid or polyploid profiles. Indeed, Lorenzato et al reported that DNA ploidy measurement by image cytometry on conventional cervical smears positive for HR-HPV could help detect females at high risk for the development of HSIL. Nevertheless, the major bias in that previous work was that the 3 variables (cytology, HPV testing, and DNA measurement) were evaluated on 3 different samples. This could explain some discrepancies observed between results obtained with conventional cytology and DNA measurement. Because the cells in suspension in the preservation medium represent a general representative cell population, the lack of reproducibility between smears for cytology, DNA measurement, and HPV testing could be overcome by the use of liquid-based cytology.

Cytologic abnormalities suggestive of a lesion in a smear with a concomitant HR-HPV infection generally are confirmed by the histologic examination. The major problems concern the management of cervical smears within normal limits with HR-HPV infection (about 10% in our experience) and cervical lesions without detectable HR-HPV. Thus, our aim was to study the usefulness of DNA image cytometry in such cases with conflicting results between cytology and HR-HPV testing, using a unique sample.

Materials and Methods

The study concerned a total of 7,949 liquid-based cytology specimens submitted for HR-HPV testing with the Hybrid Capture II (HC-II; Digene, Gaithersburg, MD) test. Of these, results for a total of 984 females with a median age of 39 years (range, 15-87 years) showed conflicting results for the 2 tests and underwent a DNA ploidy analysis.

This population included females undergoing their triennial routine screening in the Department of Obstetrics and Gynecology of the University Hospital of Reims, Reims, France. All were informed of the aim of the study and gave their consent.

Cytology

The 7,949 females underwent a cervical scrape with a Cervexbrush (Rovers Medical Devices, Oss, the Netherlands) at the first examination. Samples were prepared for liquid-based cytology with the ThinPrep technique (Cytyc, Boxborough, MA), and 4 mL of the sample was used for HPV testing. Smears were classified according to the Bethesda System for reporting cervical diagnosis: within normal limits, with ASCUS, with atypical squamous cells suggestive of HSIL, and suggestive of LSIL and HSIL. All smears were examined by 2 independent cytopathologists (J.M.N., J.C.) with no knowledge of the results of HPV testing. The results were compared, and if the first 2 diagnoses disagreed, a third cytopathologist (P.B.) reviewed the case with no knowledge of preceding diagnoses. Consensus diagnoses were determined by a two-thirds majority when possible, and remaining discrepancies were resolved by conference review.

HPV Testing

For HPV testing, 4 mL of the sample was centrifuged, and the cell pellet was resuspended in 200 µL of phosphate-buffered saline for HPV testing. HPV DNA detection was performed by using the commercially available HC-II System. All cervical scrape specimens were analyzed for the presence of HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. The enzyme-linked immunosorbent assay is based on a sandwich hybridization followed by a nonradioactive alkaline phosphatase reaction with chemiluminescence in microplates. The positive threshold of this test was 1.0 pg/mL of HPV DNA. Samples were considered positive for HPV if the relative light unit reading obtained from the luminometer was equal to or higher than the mean positive control values.

DNA Image Cytometry

Every scrape showing discrepancies between cytology and HPV results, ie, cytology within normal limits associated with a positive HC-II test result (Cyto-/HPV+) or cytology with atypical cells (from ASCUS to smears suggestive of HSIL) with a negative HC-II test result (Cyto+/HPV−), was systematically submitted to a DNA ploidy profile determination. A new ThinPrep slide was prepared with the remainder of the cell sample. Slides were air dried, fixed for 30 minutes in buffered formalin, and stained with Feulgen (thionin) after a 1-hour acid hydrolysis (5N hydrochloric acid) at room temperature. In each staining procedure, calibration slides (fresh rat liver imprints) were added.

DNA image cytometry was performed with an image cytometer (CAS 200, Becton Dickinson, Leiden, the Netherlands) with ploidy measurement software. After calibration on rat hepatocytes (at least 20 nuclei with a coefficient of variation of <3%), the total ThinPrep slide was screened by the cytometrist. The selection of the nuclei that had to be measured was as follows: elimination of the nuclei of inflammatory cells; measurement of epithelial cell nuclei selected...
by their shape, size, and mode of grouping (cluster suggestive of exocervical cells) in normal smears; and measurement of nuclei of dyskaryotic cells and koilocytes in abnormal smears.

DNA cytometry histograms were classified into 2 groups: normal and suspect. The normal histograms corresponded to class I (diploid with low proliferation fraction) according to the classification of Auer et al\(^2\) and to the strictly polyploid (diploid + tetraploid) histograms without any cells exceeding 5c (a measurement on a DNA content scale). All other histogram types, ie, aneuploid, polyploid, or diploid with more than 2 cells exceeding 5c, and multiploid profiles (more than 1 aneuploid peak) were considered suspect.

**Follow-up**

All patients with cytologic abnormalities (from ASCUS to HSIL) were systematically recalled for colposcopy during subsequent weeks. Punch biopsy specimens were taken from the areas colposcopically suggestive of SIL. All patients with cytology within normal limits but with a positive HC-II test result were systematically recalled 6 to 12 months later for a new cytologic examination and new HPV testing followed by a colposcopy if a lesion was detected or if the HR-HPV infection was persistent. Punch biopsy specimens were taken from the areas colposcopically suggestive of SIL.

Patients with a second positive HR-HPV test result without a detectable lesion were recalled 6 to 12 months later for the third cytologic examination and HPV testing.

The primary end point of our study was the detection of histologically proven HSIL.

**Statistical Analysis**

Comparison of the percentages in the different groups was performed with the chi-square test. The efficiency of cytology, HPV testing, and DNA ploidy was evaluated by measuring sensitivities, specificities, positive predictive value (PPV), and negative predictive value (NPV).

**Results**

**General Results**

Of 7,949 ThinPrep cytology specimens associated with the HC-II test, 984 (12.4%) had conflicting results \*Table II, \*841 (85.5%) were Cyto–/HPV+, and 143 cases (14.5%) were Cyto+/HPV–.

In the Cyto–/HPV+ group, 258 DNA histograms (30.7%) were suspect, and 583 (69.3%) were normal. In the Cyto+/HPV– group, 78 DNA histograms (54.5%) were suspect, and 65 (45.5%) were normal.

Irrespective of the HPV status, a suspect DNA profile was associated significantly with an abnormal cytologic result (chi square = 39.9; \(P < .001\)) with a sensitivity of 54.5%, a specificity of 69.3%, and a PPV and an NPV of 23.2% and 90.0%, respectively.

**Follow-up of the Cyto–/HPV+ Group**

In the group Cyto–/HPV+, which represented the large majority of the conflicting results between cytology and HPV testing (841 cases), follow-up information for 486 patients was available. In 292 (60.1%) of 486 cases, HPV testing was negative at the second smear, and the cytologic examination results remained normal except in 2 cases with ASCUS and 2 cases suggestive of LSIL. The colposcopic and histologic examinations of these cases did not reveal any HSIL. The DNA profile at the first smear for the patients in whom the viral infection cleared was suspect in 54 cases (18.5%) and normal in 238 (81.5%). On the other hand, in the 194 cases (39.9%) that were still HPV+ at the second examination, the DNA profile at the first smear was suspect in 88 cases (45.4%) and normal in 106 cases (54.6%). Thus, a normal DNA profile observed in HR-HPV+ smears within normal limits was associated significantly with the clearance of viral infection at the following smear (chi square = 40; \(P << .001\)). A normal DNA profile in an HR-HPV+ smear without a cytologic lesion could predict the clearance of the virus with a sensitivity of 81.5%, a specificity of 45.4%, a PPV of 69.0%, and an NPV of 62.4%.

In the group of 194 cases with a persistent HPV infection, 74 patients had a lesion at the cytologic examination: HSIL, 21, including 20 cases with a suspect DNA profile at the first examination; LSIL, 32, with 13 suspect DNA and 19 normal DNA profiles at the first smear; ASCUS, 21, with 8 suspect DNA and 13 normal DNA profiles at the first smear. In the group of 120 patients with persistent HPV infection without a lesion detected during the follow-up, 47 had a suspect DNA profile and 73 a normal profile at the first smear. Thus, a suspect DNA profile with a persistent HR-HPV infection could predict the appearance of a cytologic lesion with a sensitivity of 55.4%, a specificity of

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**Table II**

<table>
<thead>
<tr>
<th>DNA Profile for 984 Smears Showing Conflicting Results Between Cytology and Hybrid Capture II Test Results*</th>
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<tbody>
<tr>
<td>Hybrid Capture II</td>
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<td>Suspect DNA profile</td>
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* Data are given as number (percentage). Hybrid Capture II, Digene.
60.8%, a PPV of 46.6%, and an NPV of 68.9% Table 2. In the group of 194 patients with a persistent HPV infection, 21 had HSIL confirmed at the biopsy. The first smear was within normal limits associated with a suspect DNA profile in 20 cases. Thus, a suspect DNA profile at the first smear is a significant predictor of the detection of a histologically proven HSIL in cases of persistent HR-HPV infection (sensitivity, 95.2%; specificity, 60.7%; PPV, 22.7%; and NPV, 99.0%) Table 3.

Follow-up of the Cyto+/HPV– Group

Of 143 cases that were Cyto+/HPV– Table 4, follow-up information for at least 6 months was available for 50 patients. All had undergone colposcopic examination with a biopsy when a lesion was suspected. Patients with LSIL were not treated.

In the 105 cases of ASCUS, no HSIL was detected. For 32 patients, follow-up information was available (15 with a suspect DNA profile and 17 with a normal DNA profile at the initial smear). Among the 17 cases with ASCUS with a normal DNA profile, 13 cases were within normal limits at the second examination, 2 smears again revealed ASCUS, and 2 were suggestive of LSIL. Among the 15 initial cases of ASCUS with a suspect DNA profile, 12 were within the normal limits at the second examination, whereas 1 again had ASCUS and 2 were suggestive of LSIL. However, no HSIL was detected by follow-up histologic examination.

In the 35 smears suggestive of LSIL, no HSIL was detected. For 15 patients, follow-up information was available (3 with a normal DNA profile and 12 with a suspect DNA profile at the first smear). Among the 3 initial LSIL cases with a normal DNA profile, at the second examination 1 was within normal limits, 1 had ASCUS, and 1 remained suggestive of LSIL. Of the 12 initial smears suggestive of LSIL with a suspect DNA profile, at follow-up 10 were within normal limits, 1 had ASCUS, and 1 remained suggestive of LSIL with no HSIL at the biopsy.

Three patients had an initial smear suggestive of HSIL and all had a suspect DNA profile. The biopsy was within normal limits in 2 cases, and the other case had only LSIL.

Thus, in the absence of HR-HPV infection, no HSIL was detected, but if a suspect DNA profile was associated frequently with the presence of a cytologic lesion, it could not predict the outcome of the cytologic lesions during follow-up (chi square = 0.14; $P > .05$).

Discussion

One of the potential benefits of liquid-based cytology, in addition to its higher sensitivity for the detection of cytologic abnormalities, is that the remainder of the cell sample can be used for adjunctive testing (HPV detection and DNA measurement). To our knowledge, the present study is the first to apply these 3 approaches to select patients at high risk for HSIL on a unique initial sample. We confirmed previous observations showing that a suspect DNA profile is associated with the presence of cytologic abnormalities. We also clearly demonstrated that in cases of normal smears with HR-HPV infection, a suspect DNA profile can predict the appearance of a cytologic lesion, specifically a histologically proven HSIL, with a sensitivity of 95.2%, a specificity of 60.7%, and a PPV of 22.7%, whereas persistent HR-HPV infection alone has a PPV of 10.8% in these cases. In contrast, a normal DNA profile is associated strongly with the clearance of HPV infection. Thus, the use of a complementary DNA measurement in smears with apparent discrepancies between cytology and HPV testing brings powerful and useful prognostic information.

The presence of cytologic lesions without detectable HR-HPV infection may be because of the presence of an
HR-HPV type undetected by the HC-II assay, to a low-risk HPV infection, or to a regressive HPV infection. In our series, we found no histologically proven HSIL in cases of HR-HPV– abnormal cervical smears. These results need to be related to similar observations on ASCUS and LSIL in which a positive HPV test result could help identify patients who have underlying HSIL with an NPV near 100%. However, in these conditions, the DNA profile could not significantly predict the regression of cytologic lesions in the cases for which follow-up was available. As a consequence, the absence of HR-HPV infection in smears suggestive of cervical lesions remains the principal predictive variable, with an NPV of 100% for detecting HSIL.

All of these findings can be interpreted through the natural history of HPV infection. The persistence of HR-HPV infection is associated significantly with progressive disease. HR-HPV persistence causes constitutive expression of the viral oncogene proteins in host cells, which leads to chromosomal changes and finally to polyploidy, followed by aneuploidy. Indeed, we noted a strong association between the persistence of HR-HPV infection and the presence of a suspect DNA profile on smears within normal limits, whereas the great majority (81.5%) of the patients in whom the viral infection was cleared had a normal DNA profile at the first smear. Consequently, we can hypothesize that a normal DNA profile in Cyto–/HPV+ smears likely signals a transient and regressive HR-HPV infection. The heterogeneous spectrum of DNA profiles associated with cytologic abnormalities without detectable HR-HPV may be because of persistent but regressive lesions after the clearance of HR-HPV infection. Most HPV infections are known to regress spontaneously, especially in young women, and the mean HPV infection duration is between 8 and 14 months. In parallel, most LSILs, but also a significant percentage of HSILs (10%-30%) also regress. Interestingly, longitudinal studies have found that the regression of abnormal cytology in women with a positive HR-HPV result at baseline was associated strongly with viral clearance but occurred 0.3 year later than HPV clearance. In such cases, the presence of a normal DNA profile could argue in favor of a regressive diploid LSIL. In the same way, the determination of a suspect DNA profile may be related to a more advanced but also regressive LSIL or HSIL. Thus, the clearance of HR-HPV infection remains the principal prognostic factor in Cyto+/HPV– cases, preceding the regression of the lesion.

The analysis of the DNA profile on the same liquid-based cytology sample with results that are within normal limits but with a positive HR-HPV test results provides a powerful complement of tests to select women with a high risk for developing a histologic lesion. A suspect DNA profile seems to be the clue of a persistent HR-HPV infection and dramatically increases the PPV of the HC-II test alone. It is possible that the automation of DNA measurement with image analysis software could be used to discriminate among patients with normal smears with HR-HPV infection, the patients at high risk of developing HSIL who will benefit from more intensive follow-up.


