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Identification of Progressive Cervical Epithelial Cell Abnormalities Using DNA Image Cytometry

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BACKGROUND. The objectives of the current study were to compare the capabilities of conventional cervical cytology and of DNA image cytometry (DNA-ICM) in the prediction of progressive or regressive behavior in atypical squamous cells (ASC), low-grade squamous intraepithelial lesions (LSIL), and atypical glandular cells (AGC).

METHODS. One hundred ninety-six women with Papanicolaou (Pap) smears that yielded diagnoses of ASC, LSIL, or AGC were included in a prospective cohort study. Slides were classified according to the Bethesda system. DNA-ICM was performed according to the consensus reports of the European Society of Analytical Cellular Pathology.

RESULTS. Reference standard verification was available in 108 patients. The rate of DNA aneuploidy in Pap smears increased significantly from cervical intraepithelial neoplasia 1 (CIN1) (54%) and CIN2 (64.3%) to CIN3 or greater (CIN3+) (83.3%) in subsequent biopsies ($P < 0.05$). Using ASC, LSIL, and AGC as input cytologic diagnoses and ≥ CIN2 as the output histologic diagnosis, the positive predictive values (PPVs) for conventional cytology and DNA-ICM were 35.2% and 65.9%, respectively ($P < 0.001$). The negative predictive value (NPV) of DNA-ICM was 85.0%. When ≥ CIN3 was used as the output histologic diagnosis, conventional cytology had a PPV of 22.2%. The PPV and NPV of DNA-ICM were 43.9% and 93.3%, respectively.

CONCLUSIONS. The results of the current study confirmed the prognostic validity of DNA image cytometry for differentiation between progressive and regressive lesions in patients with ASC, LSIL, and AGC diagnoses. ***Cancer (Cancer Cytopathol)* 2004;102:373–9.** © *2004 American Cancer Society.*

KEYWORDS: DNA image cytometry, atypical squamous cells, squamous intraepithelial lesion, atypical glandular cells, cervical cytology.

Cervical dysplasias are heterogeneous lesions, particularly with respect to their clinical behavior. Neither histologic nor cytologic evaluation can predict whether dysplastic cells will progress to carcinoma in an individual patient. Approximately 15–30% of all women who have low-grade squamous intraepithelial lesions (LSILs) on cervical cytology will have moderate or severe cervical intraepithelial neoplasia (CIN) identified on a subsequent cervical biopsy.[1,2] Consequently, in cervical cancer screening, large numbers of control procedures are performed, including conizations without evidence of CIN2, CIN3, or invasive carcinoma.

DNA aneuploidy, as measured by DNA image cytometry (DNA-ICM), represents the quantitative cytometric equivalent of chromosomal aneuploidy and has been accepted internationally as a well standardized marker of neoplastic cell transformation.[3–6] Various studies have demonstrated that it indicates either invasive carcinoma

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or prospectively neoplastic development in cervical dysplasia.[7–11] In addition, there is growing molecular biologic evidence that aneuploidy may play a causal role in carcinogenesis.[12] The International Consensus Conference on the Fight Against Cervical Cancer International Academy of Cytology (IAC) Task Force 8 recommended DNA-ICM as a useful adjunctive method for identifying cervical intraepithelial lesions, which require further clinical management.[13]

To the best of our knowledge, all previous studies on DNA-ICM of cervical dysplasias have been retrospective.[7–11] The objective of the current study was to investigate whether DNA-ICM significantly improved the diagnostic accuracy of Papanicolaou (Pap) testing in a prospective cohort of women with atypical squamous cells (ASC), LSIL, or atypical glandular cells (AGC).

MATERIALS AND METHODS

Patients

Between June 1996 and November 2003, Pap smears from 274 women yielded diagnoses of ASC, LSIL, or AGC at the Institute for Cytopathology, University of Düsseldorf (Düsseldorf, Germany). Cytologic samples were obtained consecutively from routine input at this institution and represent $< 3\%$ of the total workload, which is in line with diagnostic practice in Germany.[14] The diagnostic category ASC was used restrictively. In 78 patients, DNA-ICM was not performed because this procedure was not requested by the patients' gynecologists. The remaining 196 patients, who were referred from a total of 28 different institutions, were included in the current study (ASC, $n = 35$; LSIL, $n = 130$; AGC, $n = 31$). More than 1 diagnostic cytology/DNA-ICM study was performed in 24 of 196 patients. For these 24 patients, only the first DNA histogram was considered for the current study to avoid biases resulting from multiple testing of the same patient. The median patient age was 39 years (range, 16–78 years).

Sample Processing and Assessment

Samples from the uterine cervix were obtained using an Ayre spatula or a Cervex Brush (CooperSurgical, Trumbull, CT). Colposcopy generally was not performed. The specimens were fixed in alcohol, subjected to Pap staining, and screened by two of the authors (H.J.G. and H.V.Q.N.) or by medical technical assistants. Each abnormal smear also was examined by an experienced cytopathologist (A.B.) before a final diagnosis was rendered. The 1991 and 2001 Bethesda systems were used for cytologic classification.[15,16] The diagnoses of specimens that had been evaluated before publication of the 2001 Bethesda system were updated according to the revised nomenclature in the final compilation of the data.

Directly after morphologic investigation, the smears underwent destaining and restaining according to the method described by Feulgen.[17] Measurements of nuclear DNA content were performed as described previously using a computer-based image analysis system consisting of a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany) with a 40× objective (numeric aperture, 0.75; Köhler illumination) and a charge-coupled device black-and-white video camera with 572 lines of resolution (VariCam CCIR; PCO Computer Optics, Kehlheim, Germany).[18]

The software package used in the current study was the AutoCyte QUIC-DNA-Workstation (AutoCyte Inc., Burlington, NC), which provides shading and glare correction. The latter was performed at a rate of 2.2%. In each case, at least 30 intermediate squamous cells with normal appearance were measured as internal reference cells. Using squamous cells as an internal reference, latent human papillomavirus (HPV) infection must be considered as a potential cause of a slightly changed peridiploid DNA content.[6,19] Because a clonal change would be unlikely in cells with normal appearance, latent viral infection should increase the coefficient of variation of reference cells rather than shifting the respective DNA histogram peak. The former potential confounder was limited in the current study, because the coefficient of variation for reference cells was always $\leq 5\%$. At least 200 epithelial cells with abnormal (i.e., hyperchromatic), enlarged, or polymorphic nuclei were measured, starting with encircled areas. To increase the detection rate of 9*c*–exceeding events (9cEEs), all Feulgen-stained smears were checked during measurement. All technical instruments and all software used in the study met the standard requirements of the consensus reports of the European Society for Analytical Cellular Pathology (ESACP).[3–6]

A number of parameters were assessed for diagnostic interpretation.[3–6] *DNA stemline* is the G0/G1 cell phase fraction for a proliferating cell population (with a first peak and a second doubling peak or with nuclei in the doubling region). *DNA stemline ploidy* was defined as the modal value of a DNA stemline in *c* units (c = DNA content). DNA stemline aneuploidy was assumed if the modal value of a stemline was $< 1.80c$ or $> 2.20c$ and $< 3.60c$ or $> 4.40c$. *Rare DNA events* included the 9cEEs, which were defined as the number of cells with a DNA content $> 9c$. *Single-cell aneuploidy* was diagnosed when at least 1 cell per slide had DNA content $> 9c$ (9cEE > 1).[8] Single-cell aneuploidy was not applicable in patients who had a his-

tory of radiotherapy, because radiotherapy can induce polyploidization in excess of 9*c*.[20]

Follow-Up

Follow-up information was retrieved from a questionnaire, which was sent to patients' gynecologists. The reference standard was histologic examination. Histologic diagnoses were classified according to the CIN system.[21] In accordance with American Cancer Society guidelines, comparison with cytologic results was acceptable as a reference standard under defined circumstances.[22] We considered cytologic follow-up of at least 6 months to be equivalent to the reference standard if at least 2 consecutive Pap smears agreed with respect to the presence or absence of progressive disease. Final data retrieval of follow-up histology and cytology was carried out in December 2003.

Statistical Analysis

The diagnostic validity of cytologic or DNA-ICM categories within the study sample was evaluated by calculating prevalence rates and predictive values. Differences in proportions were evaluated using the chi-square test and the McNemar paired chi-square test. The level of significance was set at $P < 0.05$.

RESULTS

Descriptive Statistics

Among the 196 patients examined, 89 had a history of abnormal pathologic findings with respect to the genital tract preceding the Pap smear investigated in the current study. These findings included uterine malignancy (cervical carcinoma, $n = 8$; endometrioid carcinoma, $n = 1$; leiomyosarcoma of the uterus, $n = 1$; non-Hodgkin lymphoma, $n = 1$), hysterectomy for other reasons ($n = 3$), conization ($n = 8$), and abnormal Pap smear findings made elsewhere ($n = 67$). Altogether, hysterectomy and/or irradiation had previously been performed in 14 patients (hysterectomy, $n = 10$; irradiation, $n = 7$). Reference standards were available for 108 patients (biopsy, $n = 2$; conization, $n = 49$; curettage, $n = 2$; hysterectomy, $n = 6$; cytologic follow-up of at least 6 months with at least 2 concurring Pap smears, $n = 50$). With two exceptions, all patients who were without progressive disease on cytologic follow-up presented with two normal Pap smears as controls; the two exceptions were patients who had irradiation-induced dysplasia, which remained stable. In 34 patients, cytologic follow-up did not meet the criteria for equivalence to the reference standard (i.e., duration > 6 months, with 2 concurring Pap smears). Fifty-four patients were lost to follow-up because they did not revisit their gynecologist for further diagnostic procedures.

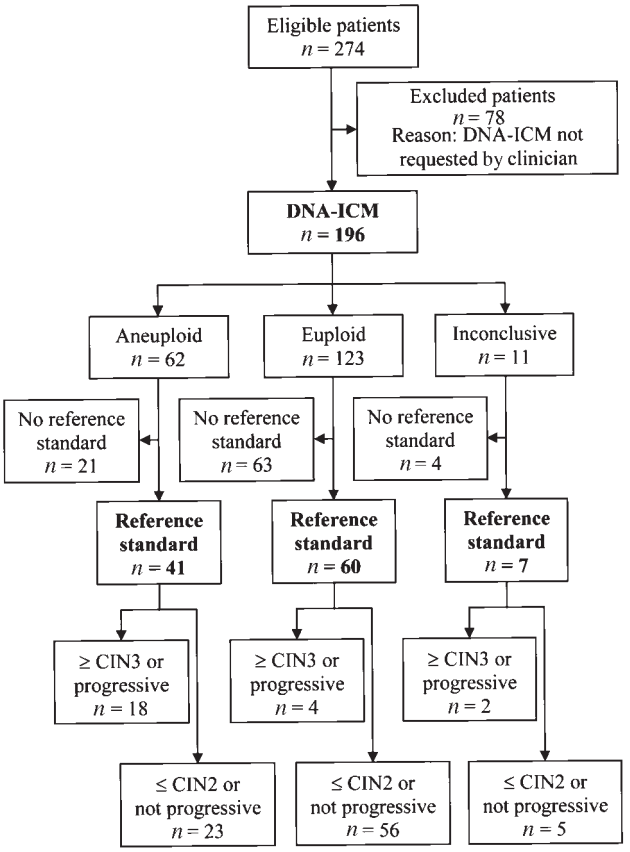

FIGURE 1. Flow chart illustrating the flow of participants within the study. ICM: image cytometry; CIN: cervical intraepithelial neoplasia; *c*: DNA content.

The median interval between the initial cytologic/DNA-ICM diagnosis and its histologic verification was 3 months (range, 1–30 months). In patients who had cytologic findings that were equivalent to the reference standard, the median follow-up was 25 months (range, 6–66 months). Figure 1 depicts the design of the study and the flow of participants using the standardized flowchart suggested by the Standards for Reporting of Diagnostic Accuracy initiative.[23]

Prevalence of DNA Aneuploidy

The overall prevalence of DNA aneuploidy in the current prospective cohort of 196 patients with ASC, LSIL, or AGC was 31.6% (Table 1). The prevalence of DNA aneuploidy differed with respect to cytologic subgroup and was correlated with the grade of histologically proven CIN, although this trend failed to reach statistical significance (chi-square ≤ 1.55; $P \geq 0.11$). The highest rate of stemline aneuploidy was observed in patients with AGC (chi-square ≥ 3.73; $P < 0.05$). Altogether, single-cell aneuploidy was the most frequent type of aneuploidy detected in 85.5% of all patients who had abnormal DNA distributions (Table 2).

TABLE 1
DNA Aneuploidy in the Various Cytologic Subgroups

		DNA-ICM (%)				Outcome (PPV [%])		
Cytology	No. of cases	Single-cell aneuploidy	STL aneuploidy	Single-cell and STL aneuploidy	Total	N_{RS}	≥ CIN2	≥ CIN3
ASC	35	7 (20.0)	1 (2.9)	1 (2.9)	7 (20.0)	7	1 (14.3)	1 (14.3)
LSIL	130	39 (30.0)	11 (8.5)	7 (5.4)	43 (33.1)	84	29 (34.5)	17 (20.2)
AGC	31	7 (22.6)	7 (22.6)	2 (6.5)	12 (38.7)	17	8 (47.1)	6 (35.3)
Total	196	53 (27.0)	19 (9.7)	10 (5.1)	62 (31.6)	108	38 (35.2)	24 (22.2)

DNA-ICM: DNA image cytometry; PPV: positive predictive value; STL: stemline; N_{RS}: number of samples with reference standard available; CIN: cervical intraepithelial neoplasia; ASC: atypical squamous cells; LSIL: low-grade squamous intraepithelial lesion; AGC: atypical glandular cells.

TABLE 2
Positive and Negative Predictive Values for DNA Image Cytometry

				Outcome (%)	
DNA-ICM finding	No. of cases	No. of aneuploid samples (%)	N_{RS}	≥ CIN2	≥ CIN3
Aneuploid	62	62/62 (100.0)	41	65.9[a]	43.9[a]
Single-cell aneuploidy	53	53/62 (85.5)	34	61.8[a]	41.2[a]
STL aneuploidy	19	19/62 (30.7)	13	92.3[a]	76.3[a]
Single-cell and STL aneuploidy	10	10/62 (16.1)	6	100.0[a]	100.0[a]
Euploid	123	—	60	85.0[b]	93.3[b]

DNA-ICM: DNA image cytometry; N_{RS}: number of samples with reference standard available; CIN: cervical intraepithelial neoplasia; STL: stemline.
[a] Positive predictive value.
[b] Negative predictive value.

TABLE 3
Prevalence of DNA Aneuploidy in Association with Histologic Follow-Up

Histology	No. of cases	Single-cell aneuploidy (%)	STL aneuploidy (%)	Single-cell and STL aneuploidy (%)	All aneuploidy (%)
WNL	8	2 (25.0)	—	—	2 (25.0)
CIN1	13	6 (46.2)	1 (7.7)	—	7 (53.9)
CIN2	14	7 (50.0)	2 (14.3)	—	9 (64.3)
CIN3	23	13 (56.5)	10 (54.2)	6 (26.9)	19 (82.6)
Invasive carcinoma	1	1 (100.0)	—	—	1 (100.0)

STL: stemline; WNL: within normal limits; CIN: cervical intraepithelial neoplasia.

Cytologic/Histologic Follow-Up and DNA Aneuploidy

ASC/LSIL and AGC eventually were found to be CIN3 or invasive carcinoma in 18 of 91 patients (19.8%) and 6 of 17 patients (35.3%), respectively (Table 1). Histologic examination was the reference standard in all cases of progressive disease—i.e., no patient with cytologic follow-up of at least 6 months and at least 2 concurring Pap smears had a progressive lesion. The proof of DNA aneuploidy prompted some of the clinicians to seek histologic verification, even in patients with ASC or LSIL. Table 3 describes the correlation between histologic diagnoses and DNA-ICM results on preceding Pap smears. The prevalence of DNA aneuploidy increased from 54% in patients with CIN1 to 64.3% in patients with CIN2 and to 83.3% in patients with ≥ CIN3 (CIN1 and CIN2 vs. ≥ CIN3: chi-square = 3.71; $P < 0.05$). The combination of single-cell aneuploidy and stemline aneuploidy was observed exclusively in patients with CIN3. No aneuploidy was found in 4 patients who had histologically proven CIN3. In

these patients, the median interval between DNA-ICM and conization was 15 months (range, 8–23 months), considerably longer than the corresponding interval in the remaining patients for whom histology was the reference standard. Two of four patients exhibited aneuploidy on repeat DNA-ICM before conization. In two patients who had single-cell aneuploidy, histologic examination did not reveal dysplasia. One of these patients, who had ASC, was diagnosed with follicular cervicitis at biopsy; further cytologic follow-up revealed ASC once again but was negative for the presence of an intraepithelial lesion 14 months after biopsy. In the other patient, single-cell aneuploidy was diagnosed, as DNA-ICM detected 1 cell with a DNA content of 9.2*c*; subsequent investigation disclosed that this patient's specimen was sent to our laboratory for a second opinion due to the discrepancy between abnormal cytology (AGC) and negative histology after the patient underwent dilatation and curettage. No further follow-up data were available. Four of the 7 patients who had previously received irradiation had polyploid histograms with 9cEEs (maximum, 28*c*). All seven histograms were evaluated as being indicative of DNA euploidy, because no stemline aneuploidy was present. Five of these seven patients had lesions that were not progressive; for the remaining two, no reference standard was available.

Diagnostic Accuracy of Cytology and DNA-ICM

The diagnostic accuracy of cytologic and DNA-ICM diagnoses was calculated by comparing initial cytologic/DNA-ICM findings with follow-up findings as evaluated by cytology and/or histology (Tables 1, 2). When CIN2+ was used as the output histologic diagnosis, the positive predictive values (PPVs) for conventional cytology and DNA-ICM were 35.2% and 65.9%, respectively. When CIN3+ was selected as the output histologic diagnosis, the PPVs were 22.2% for cytology and 43.9% for DNA-ICM. The improvement in PPV yielded by DNA-ICM was highly statistically significant for each patient (paired chi-square $\geq$ 29.4; $P < 0.001$). Like PPV, the negative predictive value (NPV) of DNA-ICM was dependent on output criteria (Table 2). Because the initial cytologic diagnosis of all study smears was at least ASC, it was not possible to calculate NPVs for cytologic diagnoses, nor was it possible to calculate sensitivity and specificity for cytology and DNA-ICM.

DISCUSSION

In the current study, we demonstrated that the use of DNA-ICM on Pap smears that yielded diagnoses of ASC, LSIL, or AGC significantly improved the diagnostic accuracy of conventional cytology. When CIN2+ was used as the output histologic diagnosis, the PPVs of conventional cytology and DNA-ICM were 35% and 66%, respectively ($P < 0.001$). In addition, DNA-ICM yielded a relatively high NPV (85%). To our knowledge, the current investigation is the first prospective cohort study of the diagnostic accuracy of DNA-ICM in cervical cytology. Previous studies applied a retrospective design and reported high PPVs (84–100%) for the development of in situ carcinoma or invasive carcinoma from mild/moderate cervical dysplasias with proven DNA aneuploidy.[7–11] However, several factors, such as differences in definitions of input and output criteria, latency periods between Pap smear and histologic diagnosis, sampling error, subjectivity of morphologic diagnoses, acquisition and work-up of biopsy tissues, definitions of reference standards, and DNA-ICM algorithms and procedures, limit our ability to compare these studies with the current one.

Predictive values largely depend on the definition of diagnostic input and output criteria. Cytologic diagnoses of $\geq$ ASC or $\geq$ LSIL represent commonly used input criteria, and histologic diagnoses of $\geq$ CIN2, $\geq$ CIN3, or high-grade squamous intraepithelial lesion (HSIL) commonly serve as output criteria.[1,2,24] In the current study, the use of ASC and LSIL yielded PPVs ranging from 14.3% to 34.5%, values that are in accordance with previous studies on the diagnostic accuracy of conventional cytology that used the same input and output criteria.[1,2,24] Another important confounding variable is the latency period between Pap smear and histologic verification. Because the interval required for a mild or moderate squamous dysplasia cytologically diagnosed on a Pap smear to develop into histologically proven carcinoma may range from months to as long as 10 years,[25] a simultaneously obtained histologic diagnosis may not be adequate for an accurate assessment of the predictive value of a cytologic diagnosis or an adjuvant method. In the current study, the median interval between the initial cytologic/DNA-ICM diagnosis and subsequent biopsy was only 3 months (range, 1–23 months). In contrast, the interval between detection of DNA aneuploidy and histologic follow-up in previous retrospective studies has been reported to be 1–3 years.[7–11] Sudbø et al. demonstrated that although only 10% of all DNA-aneuploid oral squamous dysplasias progressed to invasive carcinoma after 1 year, the progression rate increased to 90% after 5 years.[26]

Sampling errors remain the principal source of discrepancy between cytologic and histologic findings.[27] Biopsies of small, discrete lesions can be performed correctly only under colposcopic visualization. This procedure generally was not performed in the patients investigated in the current study. Koss

pointed out that low-grade intraepithelial lesions may develop peripherally with respect to high-grade lesions or carcinoma in situ or at sites adjacent to invasive malignancies.[28] However, the high-grade lesion, carcinoma in situ, or invasive malignancy in question may not be represented in a given smear, which may only contains cells from the accompanying low-grade intraepithelial lesion; this type of error is known as a *geographic error*. Geographic errors in cell sampling may also explain the observed discrepancies between cytologic/DNA-ICM findings and histologic findings in the current series.

Morphologic criteria for cervical dysplasias in cytology and histology are used inconsistently. Many authors reported poor intraobserver and interobserver reproducibility of histologic and cytologic diagnoses in pathology specimens of the uterine cervix.[2,29,30] A correct cytologic diagnosis may be followed by an incorrect histologic diagnosis, and vice versa. The subjectivity of morphologic diagnoses, therefore, may influence the results of studies on the diagnostic accuracy of cervical cytology and DNA-ICM. In our experience, thorough examination of the entire Feulgen-stained slide (i.e., not only encircled areas) often leads to a dramatic increase in the number of abnormal cells detected. Most of these cells show only minor abnormalities, but cells with major abnormalities also are detected in this way, thereby increasing the detection rate of 9cEEs. A major drawback of the current study was that Pap smears were recruited from 28 different institutions. Consequently, subsequent pathologic work-ups and evaluations of histologic material were not standardized. We attempted to organize such a standardized review but were unable to do so. However, the results of the current study reflect the predictive value of DNA-ICM in a routine setting, rather than its potential validity under experimental conditions.

Before 1995, DNA-ICM results were largely dependent on test procedures and diagnostic algorithms. This made older DNA-ICM studies of cervical dysplasia difficult to interpret. Since 1995, the standardization of DNA-ICM has been promoted by 4 consecutive consensus reports of the ESACP.[3–6] Recently, Nguyen et al. reported a 94% interobserver correlation with respect to the detection of DNA aneuploidy in Pap smears.[31] This correlation is at least 20% greater than the rates reported in the literature for histologic or cytologic diagnoses of cervical dysplasias based on morphology alone.[29,30] Thus, it appears that standardized DNA-ICM represents an objective and a highly reproducible diagnostic procedure.

DNA stemline aneuploidy reflects the clonal expansion of cells with distinct chromosomal aneuploidy. Abnormal stemlines have been reported in 41–85% of invasive cervical carcinomas and have exhibited some degree of correlation with tumor grade and histologic subtype.[32–34] In addition to being a sign of stemline abnormality, *rare events* may indicate DNA aneuploidy. These rare events are likely to be attributable to nonproliferating abnormal cells with different chromosomal aneuploidies and abnormally high numbers of chromosomes.[19] Therefore, rare events may serve as markers of malignant cell transformation, even if they are not relevant to tumor growth. This may explain the considerably higher PPV associated with stemline aneuploidy compared with single-cell aneuploidy (92% vs. 62% for $\geq$ CIN2). It is noteworthy that the highest PPV observed was associated with combined stemline and single-cell aneuploidy (100% for CIN2+).

HPV DNA testing is another adjuvant method that can be used to increase diagnostic accuracy in cervical cancer screening. High-risk HPV detection has a very high NPV, ranging from 98.9% to 99.9% when CIN2+ is used as an output diagnosis.[24,35,36] However, the PPV associated with positive findings of high-risk HPV is low (19.6–29.1%).[24,35,36] Combining high-risk HPV testing, with its high NPV, and DNA-ICM, with its high PPV, may be a major step toward improved diagnostic accuracy in cervical cancer screening.[37] In a related development, the complementary value of direct sequencing of high-risk HPV and laser-scanning cytometry was recently demonstrated.[38,39]

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