Interobserver reproducibility of DNA-image-cytometry in ASCUS or higher cervical cytology

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Received 17 August 2003
Accepted 19 April 2004

Abstract. In the present study, the aim has been to investigate the interobserver reproducibility of DNA-image-cytometry (DNA-ICM) applied to routine Pap smears classified as Atypical Squamous Cells of Undetermined Significance (ASCUS) or higher lesions (ASCUS+). 202 Pap smears diagnosed as ASCUS or higher were included in the study. After cytological assessment, smears underwent restaining according to Feulgen. First measurements were performed as routine workup. The second measurements were blinded to the result of the first and consecutively performed. DNA-ICM met the consensus statements of the European Society of Analytical Cellular Pathology (ESACP). Interobserver agreement was assessed by calculating Kappa statistics. The diagnosis of DNA-aneuploidy in the first measurements was confirmed in all cases. Second measurement detected 12 additional cases with aneuploidy. Nine out of these cases were classified as aneuploidy by detection of 9c Exceeding Events (9cEE). In three cases stemline-aneuploidy was disclosed. The overall proportion of observed agreement was 94.1%, $\kappa = 0.87$, 95% CI = 0.74–0.99. Our study shows a good inter observer reproducibility of DNA-ICM performed on cervical smears with ASCUS or higher lesions. DNA-ICM thus represents a highly reproducible diagnostic procedure.

Keywords: Interobserver reproducibility, DNA-image-cytometry, cervical cytology

1. Introduction

The reliability of a diagnostic method depends on different variables, like validity and reproducibility. The reproducibility of a method includes two aspects: intra- and interobserver agreement. Features, which have an impact on reproducibility, are objectivity of diagnostic criteria, number of diagnostic categories, study population and experience of the test performers. Interobserver variability has important implications for diagnostic error, thus patient care and also medical litigation.

In cancer screening and diagnosis, cytological and histological investigations so far played the crucial role and have greatly contributed to the fight against cancer worldwide. The most successful cancer screening program ever carried out is the early detection of cervical cancer and its precursors using exfoliative cytology, well-known as “Pap test”. Despite its great contribution to the increasing number of detected preinvasive cervical lesions and the decreasing number of invasive cancers, its reproducibility is still insufficient and causes clinical problems. Reproducibilities of cytological and histological diagnoses of precancerous lesions and cancers including typing and grading have been largely investigated.

Grading of dysplasia or intraepithelial lesions is a daily task in diagnostic pathology and cytopathology.
It is notoriously subjective and thus lacks sufficient intra- and interobserver reproducibility. This is partly due to the lack of validated morphological criteria, upon which pathologists and cytologists have reached consensus [4]. Variability among histopathologists was assessed in a study including 106 cervical biopsy specimens [7]. Four experienced histopathologists assigned them to one of five diagnostic categories: no dysplasia, mild, moderate, severe dysplasia and carcinoma in situ. Considerable disagreement among pathologists was observed: the unweighted kappa (a coefficient of correlation) was only 0.28.

Tezuka et al. (1992) reported a study on 70 cytological specimens containing endometrial cells. Nineteen pathologists assigned the smears to one of three diagnostic categories: negative for, suspicious of and positive for malignancy. The agreement was better on negative and positive categories (kappa = 0.46 and 0.47, respectively) and poor in grading suspicious cells. The overall kappa for all smears was only 0.36 [27].

Sherman and Paull (1993) investigated the reproducibility of cytological and histological diagnoses of vaginal intraepithelial neoplasia in a series of 124 smears and 70 corresponding biopsies [25]. Consensus in cytopathological diagnoses was reached in 46% and in histopathological diagnoses in 55% of cases. Using different morphological criteria, van Aspert van Erp et al. (1996) reported an interobserver agreement on endocervical columnar cell intraepithelial neoplasia of different grades from 74% to 94% [28]. The College of American Pathologists initiated an Interlaboratory Comparison Program in Cervicovaginal Cytology Study, which examined interobserver variability in the classification of SILs [29]. The concordance rates of LSIL and HSIL diagnoses were 77.4% and 65.9%, respectively. Using the modified Bethesda grading system for histological reporting on SILs, McCluggage and coworkers reported a weighted kappa of only 0.36 (95%CI 0.21–0.61) [22]. Thus the reproducibility of grading dysplasias is still insufficient and needs to be improved.

During the last decade, the major attempt to obtain a greater accuracy of diagnostic cytology with consequences in clinical management of CIN was the introduction of high-risk HPV DNA testing. The newest Consensus Guidelines of the American Society for Colposcopy and Cervical Pathology for the management of cervical cytological abnormalities recognized the improvement of sensitivity in the categories of atypical squamous cells by using HPV DNA testing and recommended it for further management of such lesions [30]. Recently, overexpression of p16INK4a induced by oncogenic high-risk HPV has been reported as a specific marker for CIN 2–3 or higher lesions [17, 23, 24]. Klaes et al. (2002) have demonstrated that immunostaining of p16INK4a may contribute to improve the interobserver agreement in the diagnosis of cervical intraepithelial neoplasia [18].

DNA-image-cytometry (DNA-ICM) is a quantitative adjuvant method, which is more objective than traditional grading and typing methods, to establish the diagnosis of (prospective) malignancy in different preneoplastic lesions and for grading of tumor malignancy of manifest cancers. Four international consensus reports of the European Society of Analytical Cellular Pathology (ESACP) on standardized diagnostic DNA-ICM provided guidelines and performance standards for diagnostic DNA measurements, definitions of terms and algorithms for diagnostic data interpretation [1,10,14,15]. Increasing information on chromosomal aneuploidy not only as a highly specific marker of neoplastic cell transformation, but also on its role in tumor pathogenesis and progression supports the biological basis of diagnostic DNA-ICM [8,20]. International consensus has also been reached on the application of DNA-ICM for the identification of high-grade intraepithelial lesions in cervical cytology, which need further clinical management [13]. The finding of DNA-aneuploidy qualifies an Atypical Squamous Cell of Undetermined Significance (ASCUS) or Low-grade Squamous Intraepithelial Lesion (LSIL) as high-grade, obligatory precancerous or prospectively malignant, which should be removed. Grote et al. (2004) reported a positive predictive value (PPV) of 65.9% in ASCUS/LSIL lesions with a three-month follow-up [12], whereas after two years of follow-up, Böcking and Motherby observed a PPV of 92%. The negative predictive value of a DNA-euploid finding in ASCUS/LSIL lesions was 85.0% in the former study [12]. Grote et al. (2001) have also reported on the significant prognostic impact of DNA-ICM in invasive cervical cancer [11].

While data on diagnostic accuracy of DNA-ICM in cervical cytology are encouraging, no data have been published so far on the interobserver reproducibility of this adjuvant method with respect to the qualitative diagnosis of DNA-aneuploidy as a marker of progressive behavior in cervical intraepithelial lesions. Therefore the aims of this study were to investigate the interobserver reproducibility of DNA-ICM applied to routine Pap smears classified as ASCUS or higher lesions (ASCUS+).
2. Material and methods

2.1. Material

The material of this study consisted of 202 routine cervical cytology samples primarily classified as ASCUS+ lesions, including 25 cases of ASCUS, 72 cases of LSIL, 99 cases of High-grade Squamous Intraepithelial Lesion (HSIL) and 6 squamous cervical cancers. The specimens were collected at the Institute of Cytopathology, Heinrich-Heine University Düsseldorf, from 1996 to 2001. The mean age of patients was 34 years (range, 16–88 years).

2.2. Smear processing and measurements

After morphological investigation and classification using the Bethesda system [19], the smears underwent destaining and restaining according to Feulgen [9]. Feulgen staining was performed automatically using a modified staining machine, Varistain 24-4 (Shandon, Pittsburgh, Pennsylvania, USA), as described elsewhere [6]. Briefly, after rehydration in decreasing ethanol concentrations and re-fixation in buffered 10% formalin, 5 N HCl for hydrolysis was applied at 27° C for 60 minutes, followed by staining in Schiff’s reagent (Merck, Darmstadt, Germany, No. 1.09033.0500) for another 60 minutes in room temperature, rinsing in SO2-water and dehydration at increasing ethanol concentrations. The slides were then covered with Entellan (Merck, Darmstadt, Germany, No. 1.07961.0500).

Measurements of nuclear DNA contents were performed using a computer-based image analysis system consisting of a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany) with a 40× objective, NA 0.75; Köhler illumination was applied to reduce stray light. A CCD black and white video-camera with 572 lines resolution (VariCam, Modell CCIR, PCO Computer Optics, Kehlheim, FRG) was adapted to the microscope and connected to an IBM PC compatible computer through a frame-grabber board (Matrox Meteor Board/Matrox Electronic Systems, Unterhaching, FRG).

The software used in this study, co-developed by our group, was the AutoCyte QUIC-DNA-Workstation (TriPath Inc., Burlington, NC, USA), which provides shading- and glare correction. The latter was performed at a rate of 2.2%. In each case at least 300 nuclei of abnormal squamous cells were randomly measured. At least 30 normal intermediate squamous cells were measured as internal reference cells; a correction factor of 1.00 was used to obtain the normal 2c value. The coefficient of variation of reference cells was always below 5% [14]. All technical instruments and the software used in the study met the standard requirements of the European Society of Analytical Cellular Pathology (ESACP) consensus reports [1,10,14,15].

The following parameters were assessed for diagnostic interpretation:

- DNA stemline: the G0/G1 cell-phase fraction of a proliferating cell population (with a first peak and a second doubling one, or nuclei in the doubling region) [1,15].
- DNA stemline ploidy: the modal value of a DNA stemline in the unit c (c = content of DNA) [1,15].
- DNA-euploidy: the types of DNA distributions which cannot be differentiated from those of normal cell populations (resting, proliferating, or polyploidization) [15].
- Diploid euploidy: DNA-stemlines with a modal value between 1.8c and 2.2c [15].
- Tetraploid euploidy: DNA-stemlines with a modal value between 3.6c and 4.4c [15].
- DNA stemline aneuploidy: this was assumed, if the modal value of a stemline was < 1.80c or > 2.20c and < 3.60c or > 4.40c [1,15].
- Single cell aneuploidy: occurrence of at least one cell with a DNA content > 9c (9cEE ⩾ 1) per slide [5].

Two measurements were performed on each sample. The first measurements were done as routine workup of ASCUS or higher cases by an experienced cytotechnologist (K.K.), diagnostic interpretations were performed by an experienced cytopathologist (A.B.). The second measurements were blinded to the result of the first measurement and consecutively performed and interpreted by a gynecologist with experience in gynecological cytology (V.Q.H.N). Interobserver agreement was assessed by Kappa statistics.

3. Results

The prevalence of DNA-aneuploidy in two measurements is shown in Table 1. The group of HSIL and invasive cancer revealed an increased proportion of DNA-aneuploidy, reaching 100% in the invasive cancer cases. Parallel to this observation, the rates of DNA-aneuploid lesions also increased from the group
of histologically confirmed CIN I to invasive cancer as shown in Table 2.

Table 3 demonstrates the results from detailed DNA-histogram interpretation of the two independent measurements. DNA-euploidy was divided into two categories (diploid and polyploid); DNA-aneuploidy was split into three categories (9cEE only, aneuploid stemline only or both 9cEE and aneuploid stemline). None of the cases with DNA-aneuploidy in the first measurements were interpreted as DNA-euploidy in the second measurements. Yet, there were 12 cases with a discrepancy between the first and second measurements. Nine out of these cases were classified as aneuploidy by detection of only one or a few cells with 9cEE. In three cases stemline-aneuploidy was disclosed. Figure 1 illustrates some concordant and discrepant results from two measurements. The correlation of the two diagnostic categories is shown in Table 4. The overall proportion of observed agreement was 94.1%. Kappa statistics was computed from the results shown in Table 4 and yielded a weighted value of $\kappa = 0.87$, 95% CI = 0.74–0.99.

### 4. Discussion

DNA-ICM is an indirect quantitative method for evaluation of nuclear DNA content of cells or tissues. Since diagnosis based on DNA-ICM has an impact on diagnosis and prospective behavior of precancerous lesions and invasive cervical cancers, its application can help to improve the reproducibility of grading cervical dysplasias and invasive cancers. DNA-ICM is somewhat time-consuming and needs cytological expertise. One of its advantages is that it may be performed retrospectively, irrespective of the type of preceding fixation and staining and even on archived slides. Up to 1995 DNA-ICM lacked international standardization of measurement performance and diagnostic interpretation of data. This has changed since the ESACP has published its "Consensus Reports for Standardized Diagnostic DNA-image-cytometry" [1,10,14,15].

All smears included in our study, even within the routine framework, were processed and measured in strict accordance with these standards: e.g., more than 30

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| Prevalence of DNA aneuploidy in different cytodiagnostic categories |
|---------------------------------------------------------------|
| Cytological diagnosis | N | 1. Measurement | 2. Measurement |
|-----------------------|---|----------------|----------------|
| ASCUS                 | 25| 9 (36)         | 11 (44)        |
| LSIL                  | 72| 27 (37.5)      | 29 (40.3)      |
| HSIL                  | 99| 86 (86.9)      | 94 (94.9)      |
| Invasive carcinoma    | 6 | 6 (100)        | 6 (100)        |

ASCUS: atypical squamous cells of undetermined significance; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion.

| Prevalence of DNA-aneuploidy in correlation to histological follow-up |
|-------------------------------------------------------------------|
| Histological diagnosis  | N | 1. Measurement | 2. Measurement |
|-------------------------|---|----------------|----------------|
| WNL                     | 10| 6 (60)         | 6 (60)         |
| CIN I                   | 18| 11 (61.1)      | 12 (66.7)      |
| CIN II                  | 31| 24 (77.4)      | 26 (83)        |
| CIN III                 | 72| 65 (90.3)      | 67 (93.1)      |
| Invasive carcinoma      | 4 | 4 (100)        | 4 (100)        |

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

| Comparison of detailed results from repeated diagnostic DNA-image-cytometry of cervical smears |
|---------------------------------------------------------------------------------------------|
| 2. Measurement | 1. Measurement | Euploidy | Aneuploidy |
|----------------|----------------|----------|------------|
|               |                | Diploid  | Polyploid  |
| Euploidy      | 5              | 2        | 0          | 0          |
| Polyploid     | 4              | 51       | 0          | 0          |
| Aneuploidy    |                |          | 0          |            |
| 9cEE only     | 1              | 4        | 29         | 2          |
| Stemline only | 0              | 3        | 0          | 9          |
| 9cEE + stemline| 0             | 4        | 19         | 14         |

9cEE: 9c exceeding events.
Fig. 1. DNA histograms of concordant and discrepant measurements. (A) Minor differences of DNA histograms with identical diagnostic interpretation: LSIL; DNA-aneuploidy, peridiploid, peritetraploid and perioktoploid stemlines, 9cEE = 7 (left) and = 4 (right). (B) Minor differences of DNA histograms with identical diagnostic interpretation: ASCUS; DNA-aneuploidy, peridiploid and peritetraploid stemlines, 9cEE = 1 (left) and = 2 (right). (C) Minor differences of DNA histograms resulting in different diagnostic interpretations: HSIL; DNA-euploidy, peridiploid and peritetraploid stemlines, 9cEE = 0 (left), -aneuploidy, 9cEE = 2 (right).
reference cells were measured, coefficients of variation always were below 5%, coefficients of correlations between nuclear areas and integrated optical densities (IODs) of reference cells below $r = 0.4$. This was mainly achieved applying a software correction of glare- (at 2.2%) and diffraction errors. Internal reference cells were only measured within the same slides and nearby the abnormal epithelial cells under analysis. In order to provide most reliable results, diagnostic interpretation of DNA-histograms has been carried out based on unified criteria from these Consensus Reports.

During the last five years, the role of DNA-ICM in identification of progressive CINs has been well studied and proven by various authors. The positive and negative predictive values of DNA-ICM for detection of progressive CIN 1–2 reported by Hering et al. were 85.2% and 77%, respectively [16]. Bollmann and coworkers found that DNA-ICM supported the binary classification of the Bethesda System [2]. In another study, DNA-ICM has been able to differentiate CIN 3 from CIN 1–2 lesions [Shirata et al., 2001]. Two recently published studies confirmed the usefulness of combination HPV DNA testing and DNA-ICM to identify cases, which are at elevated risk to develop HSIL and cancer, thus need further appropriate clinical management [3,21].

The high value of interobserver agreement of 94.1% achieved in this study is at least 20% superior to the rates reported in the literature for subjective histological or cytological diagnoses of cervical dysplasias. Explanations for this high value are, on the one hand, the high standardization of DNA measurements and diagnostic data interpretation and, on the other hand, the objectivity of the method. In contrast to the subjective assessment of conventional diagnostic morphology-based methods, DNA-histogram interpretation is based on well-defined algorithms, which already proved their diagnostic validity in cervical pathology.

The diagnosis of DNA-aneuploidy in the first measurement was confirmed by second measurement in all cases. No false positive findings of DNA-aneuploidy were observed. The second measurement revealed missed 9cEE in 9 cases. As these rare events can sometimes only be detected by thorough screening of the slides, they may be missed if the screening is not done carefully enough in a routine setting. Every slide should be firstly randomly screened. To avoid seldom-false negative results, if aneuploidy could still not be detected, slides should be then carefully screened for dark and large nuclei.

In few cases, sampling of 300 abnormal cells may not be representative enough to detect aneuploid DNA-

Table 4
Comparison of two diagnostic categories of DNA-image-cytometry applied to cervical smears

| 2. Measurement | 1. Measurement |
|----------------|----------------|
| Euploidy       | 62             | 0              | 62             |
| Aneuploidy     | 12             | 128            | 140            |
| Σ              | 74             | 128            | 202            |
stemplines. In our series, repeated DNA-ICM disclosed additional 3 cases with stemline aneuploidy. Measuring more than 300 cells may increase the ability of the method to detect diagnostically relevant DNA-aneuploidy. Reference cells should be measured nearby the respective analysis cells to avoid scaling errors due to staining inhomogeneity.

In accordance with previous studies, Grote et al. recently demonstrated again the diagnostic validity of DNA-ICM to identify progressive cervical intraepithelial lesions [2,3,12,16,21,26]. The present study now could prove the reliability of this method. We therefore propose DNA-ICM as a valid and reliable tool to identify progressive cervical intraepithelial lesions.

Our study yielded a good interobserver reproducibility of DNA-ICM performed on cervical smears with ASCUS + lesions. DNA-ICM thus represents a highly reproducible diagnostic procedure.

Acknowledgement

Dr. V.Q.H. Nguyen was supported by a grant from the Düsseldorf Entrepreneurs Foundation, Düsseldorf, Germany.

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