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Short Communication

Adaptation of image cytometry methodology for DNA ploidy analysis of cervical epithelium samples: A pilot study



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ABSTRACT

Objective: To determine DNA ploidy in the cervical specimens of patients revealing a suspicion of cancer by image analysis performed by using a combination of commercial analysis software, conventional microscopy, and certified filters.

Materials and methods: This study followed a prospective design. Cervical samples were obtained from 20 patients undergoing routine screening in the Gynecologic-Oncology Unit of the University Hospital of the Federal University of Minas Gerais, Brazil. Three slides were prepared for each case and the DNA content was determined by image cytometry, post Feulgen staining. DNA ploidy, as well as events exceeding 5C and 9C, was assessed according to the guidelines and algorithms prescribed for diagnostic interpretation by the European Society for Analytical Cellular Pathology.

Results: By employing the adapted tool, identification of the lesions with euploid and aneuploid profiles was possible. Abnormal DNA content was found in 65% of the cases (13/20), with 45% (9/20) presenting nuclei with >5C content and 20% (4/20) with >9C content. In the analyses conducted in this study, the coefficient of variation with respect to DNA quantity was lower than the 5% threshold recommended by the European Society for Analytical Cellular Pathology.

Conclusion: Image cytometry of the cervical specimens revealed DNA aneuploidy, most probably resulting from chromosomal alterations and appearing as precancerous lesions in 65% of the cases. The adaptations implemented in this study, enabled the DNA-image cytometry to become more accessible, enhancing its extended use as an adjuvant strategy for the early screening of the cervical epithelium samples during routine analyses.

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Introduction

Cervical cancer has become one of the most common tumors detected in women worldwide, accruing approximately 530,000 cases and 275,000 deaths annually [1]. Tumors arise from pre-invasive precursor lesions with a slow progression rate, which enables detection and identification right at the initial phase [2]. The Papanicolaou smear is the universally used diagnostic method to detect cervical cancer [3]. Although this procedure has significantly reduced by as much as 75% of the number of deaths by

cervical cancer [4], considerable variation in its sensitivity and specificity has been reported [5–7]. Furthermore, this method is limited by the interpretation of the morphology, with little or no information regarding the risk of persistence, progression or regression and reveals large interobserver variability [8]. According to Böcking, neither histologic nor cytologic evaluation can predict whether the dysplastic cells will progress to carcinoma in an individual patient. Therefore, the early identification of precancerous changes remains a challenge [9].

Adjuvant diagnostic methods such as colposcopy, detection, and molecular typing of HPV and DNA image cytometry (ICM) have also been proposed. DNA-ICM is being used as a complementary detective method in clinical analyses in more than 40 laboratories across the United States, Europe, and Asia [10].

Combining microscopy and digital image analysis the DNA-ICM technique evaluates the stoichiometric nuclear DNA staining post

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Feulgen staining [11]. Diagnostic DNA cytometry aims at identifying the DNA stem lines outside the euploid regions as abnormal (or aneuploid) at a defined statistical level of significance [12].

This method enables the quantification of DNA ploidy by generating a quantitative cytometric equivalent of chromosomal ploidy, which can be conducted even in resting cells [12]. DNA aneuploidy is a proven good marker for prospective malignancy, as the chromosomal changes are related to malignant and premalignant lesions [13–15]. Thus, DNA image cytometry should provide information regarding the presence of rare cells with an abnormally high DNA content [12], attempting to distinguish the prospective malignant lesions from microscopically atypical or otherwise suspect cells. In various tissues, DNA-ICM has predicted malignant transformation, with high sensitivity and specificity [14,16,17]. Further, this technique has been recognized as a quantitative and reproducible method for the diagnosis of intraepithelial cervical lesions [18].

Image analysis using microscopic systems requires suitable and specific software tools for cell analysis, despite the high cost and limited availability [19]. In this work, a particular image analysis method was performed, using a combination of commercial analysis software (Image Pro-Plus 6.1 Media Cybernetics, Rockville, MD, USA), conventional microscopy, and certified filters. Furthermore, changes in the internal standard type and in the slide preparation method were adopted to yield more accurate DNA ploidy measurements.

Materials and methods

Conducted on the lines of prospective design, this study involved the collection of cervical epithelium samples from 20 women undergoing routine screening at the Gynecologic-oncology ward of the University Hospital of the Federal University of Minas Gerais, Brazil, between December 2006 and February 2007. Patients were categorized by conventional cytology as having a low-grade lesion, high-grade lesion, or being negative for an intraepithelial lesion or malignancy (NILM). For analysis, the physician selected only samples suspicious for cancer, whereas DNA-ICM was performed independently, without knowledge of the clinical evaluation. Epithelium from the oral mucosa of clinically healthy women was used as control. The participants were informed regarding the purpose of the research and signed consent was taken from them. This study was approved by the Local Ethics Committee of the Federal University of Minas Gerais (coep ETIC 326/08).

Sample preparation and staining

The samples were collected in a universal cytological liquid (LucBASE, Florianópolis, Brazil) and fixed in 70% ethanol at -20°C (Merck KGaA, Darmstadt, Germany). Three slides were prepared for each patient by cytocentrifugation (Presvac, Balneário Camboriú, Brazil). A modified internal standard material was introduced, consisting of a cell suspension of the oral mucosa (reference cells). The reference cells and a sample of the cervical epithelium, were mounted on opposite ends of each slide [20]. Feulgen reaction was performed according to Gonçalves et al [18] and Carvalho et al [20]. The slides were postfixed in 4% paraformaldehyde (Merck) for 60 minutes and then washed in running water. Subsequently, the slides were hydrolyzed in 5M HCl (Merck) for 60 minutes at 24°C and stained with Schiff's reagent (Merck) for 12 hours at 4°C , in the dark. After washing the slides three times (for 2 minutes each time) in 0.5% SO_2 water, and again three times (for 1 minute each time) in distilled water, they were air-dried. The slides were then mounted in immersion oil and stored in the dark until image analysis.

Table 1

Summary of results from 20 cases analyzed by DNA-image cytometry showing total number of nuclei and nuclear DNA ploidy.

Case	Total nuclei	Nuclear DNA ploidy from three repetitions
01	327	Main peak at 2C and aneuploid nuclei 5cEE ^a = 63; 9cEE ^b = 5
02	363	Main peak at 2C and aneuploid nuclei 5cEE = 3; 9cEE = 0
03	293	Main peak at 2C and 5cEE = 0; 9cEE = 0
04	315	Main peak at 2C and aneuploid nuclei 5cEE = 3; 9cEE = 0
05	179	Main peak at 2C and aneuploid nuclei 5cEE = 1; 9cEE = 0
06	385	Main peak at 2C and 5cEE = 0; 9cEE = 0
07	303	Main peak at 2C and aneuploid nuclei 5cEE = 5; 9cEE = 0
08	625	Main peak at 2C and aneuploid nuclei 5cEE = 29; 9cEE = 4
09	232	Main peak at 2C and 5cEE = 0; 9cEE = 0
10	299	Main peak at 2C and 5cEE = 0; 9cEE = 0
11	149	Main peak at 2C and aneuploid nuclei 5cEE = 8; 9cEE = 0
12	644	Main peak at 2C and aneuploid nuclei 5cEE = 21; 9cEE = 1
13	183	Main peak at 2C and aneuploid nuclei 5cEE = 2; 9cEE = 0
14	408	Main peak at 2C and aneuploid nuclei 5cEE = 30; 9cEE = 4
15	341	Main peak at 2C and 5cEE = 0; 9cEE = 0
16	318	Main peak at 2C and aneuploid nuclei 5cEE = 25; 9cEE = 0
17	255	Main peak at 2C and 5cEE = 0; 9cEE = 0
18	311	Main peak at 2C and aneuploid nuclei 5cEE = 22; 9cEE = 0
19	259	Main peak at 2C and 5cEE = 0; 9cEE = 0
20	375	Main peak at 2C and aneuploid nuclei 5cEE = 5; 9cEE = 0

^a5cEE: 5C-exceeding events; ^b9cEE: 9C-exceeding events. Acronyms represent nonproliferating abnormal cells with different chromosomal aneuploidies and abnormally high number of chromosomes [12].

Image analysis system

The image analysis system for DNA measurements included a photomicroscope (BX-60; Olympus, Center Valley, PA, USA) equipped with a stabilized light source; UPlanFI objective 60/0.75; interference filter (green color 550–570 nm); and a monochromatic charge-coupled device digital video camera of 12-bit gray (Photometrics CoolSNAP Pro; Roper Scientific, Tucson, AZ, USA). The microscope was adjusted using the Köhler method, prior to the capture session of each slide.

The system was calibrated based on the recommendations of Hardie et al [11], Puech and Giroud [21], Vilhar and Dermastia [22], Mendonça et al [23], and basically involved three tests performed in our laboratory: (1) stability—the system acquired stability after 12 minutes, from the time it was connected [23]; (2) linearity—linear correlation between the optical density (OD) calculated by the software and certified OD values was $R^2 = 0.9978$ [23]; and (3) uniformity—the OD determination of a nucleus was found to be independent of its position in the visual field [23].

UPlanFI objective magnification $\times 60$ and tools from the Image ProPlus 6.1 analysis system (Media Cybernetics, Rockville, MD, USA) were used to capture the nuclei images. The macro function programming was used to automate the capture of the individual nuclei. Approximately, 200–350 nuclei of the cervical epithelium and at least 30 cell nuclei of the reference material were scanned and segregated into groups for comparative analysis. Nuclei sampling was performed in a semi-interactive manner, discarding overlapping or poorly preserved nuclei and leukocytes.

Using the image analysis program Image Pro-Plus 6.1 the integrated OD (IOD) was automatically calculated by multiplying the nuclear area (μm^2) by the OD of the object. IOD values were rescaled in terms of the relative DNA content, based on the reference cells with a known DNA content [24]. SPSS 13.0 software (Macintosh package version; SPSS, Chicago, IL, USA) was used to calculate the modal IOD values in each repetition. The data generated were then plotted as nuclear DNA ploidy histograms for statistical assessment of the G_0/G_1 and G_2/M peaks, according to prescribed criteria of the European Society for Analytical Cellular Pathology (ESACP) [24].

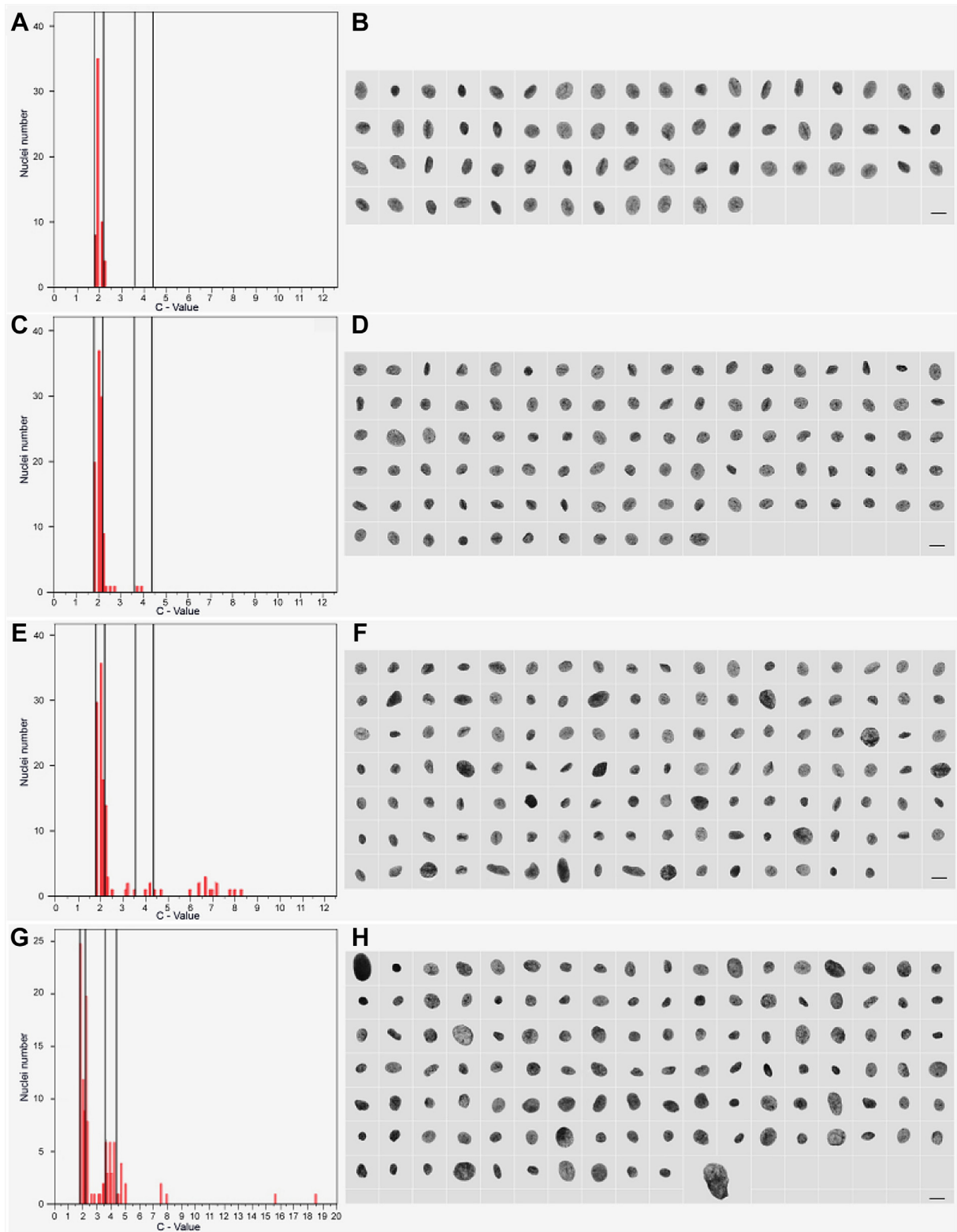


Fig. 1. Representative histograms and nuclei collections analyzed by image cytometry. (A) Histogram of oral epithelium nuclei used as control (internal standard). Main peak at 2C; 5C-exceeding events (5cEE) = 0; 9C-exceeding events (9cEE) = 0. (B) Corresponding nuclei collections. (C) Histogram from a Papanicolaou (Pap) smear diagnosed as negative for intraepithelial lesion or malignancy showing normal DNA ploidy (Case 17). Main peak at 2C; 5cEE = 0; 9cEE = 0. (E) Histogram from a Pap smear diagnosed as high-grade lesion, with DNA ploidy showing 5C-exceeding events (Case 16). Main peak at 2C; 5cEE = 13; 9cEE = 0. (G) Histogram from a Pap smear diagnosed as high-grade lesion, with DNA ploidy showing 9C-exceeding events (Case 14). Main peak at 2C; 5cEE = 5; 9cEE = 2. (D, F, H) Cervical epithelium nuclei collection used for integrated optical density measurements and elaboration of the respective histograms. Bar = 10 μ m.

Criteria for DNA ploidy classification

Based on the algorithms from the ESACP guidelines, a sample was classified as diploid when only one peak was found in G_0/G_1 phase (2C), with <1% of the cells exceeding 5C and less than 10% in the G_2/M phase (4C). Aneuploidy was identified when the number of nuclei exceeding 5C (5cEE) or 9C (9cEE) was >1% of the total number of the nuclei analyzed [24]. DNA-ICM was conducted without identifying the nature of the lesion.

Results

According to the classification criteria, 35% of the cases sampled (7/20) were considered normal, presenting only histograms with the G_0/G_1 peak of diploid nuclei. Abnormal DNA content was observed in 65% of the cases (13/20), with 45% (9/20) presenting nuclei with content greater than 5C, and 20% (4/20) revealing content more than 9C. The total number of nuclei analyzed and the ratio of the aneuploid nuclei detected in each case are summarized in Table 1.

In Fig. 1, four of the 120 nuclei collections used for the IOD measurements and elaboration of the respective histograms are represented. The histogram of the material used as control showed the main peak at 2C, (diploid nuclei in G_0/G_1 phase; Fig. 1A). Fig. 1C shows a normal DNA ploidy case histogram (main peak at 2C; 5cEE = 0; 9cEE = 0), while Fig. 1E and 1G represent histograms of cases showing aneuploid nuclei (main peak at 2C; 5cEE > 0; 9cEE > 0). In such examples, the occurrence of nuclei with altered morphology could be observed (Fig. 1F–H).

In all the samples analyzed, the coefficient of variation (CV) corresponding to the ratios between the modal IOD values of the reference cells and nonpathologic G_0/G_1 cells ranged from 0.8% to 4.49%.

The DNA-ICM measurements in the different cytological subgroups are listed in Table 2. The high-grade lesion group revealed increased DNA aneuploidy, while 40% (2/5) of the NILM smears showed DNA aneuploidy.

Discussion

In this study, the steps in the image analysis system calibration and the algorithms accomplished to identify precancerous and cancerous lesions enabled the execution of quantitative analysis. System calibration included stability, linearity, and uniformity tests, and stability was achieved after 12 minutes. According to Vilhar and Dermastia [22], the stabilized light source prevents variations in the OD values measured. In the linearity test, the calculated coefficient of determination ($R^2 = 0.9978$) fulfills the requirements ($R^2 > 0.9$) for performing densitometric measurements [22]. The CV obtained in the uniformity test was <1%. Values below 3% are acceptable according to the international standards prescribed in

the medical arena for DNA-ICM analysis [11]. Quality control of the instrumentation used for analysis is the first step required to standardize the quantification of nuclear DNA ploidy [21]. Our laboratory fulfilled the requirements for application of the technique.

Use of the cytocentrifuge for slide preparation is another modification introduced in this study. This procedure was used to supply the flattened cells uniformly scattered in a delimited circular area, with slight overlapping and cell density per field suitable for DNA-ICM analysis.

Oral epithelial cells were used as the internal standard [18] as their nuclear morphology has characteristics similar to the cervical epithelial cells [25]. This raises the reliability of the rescaling step. Two types of reference systems, namely external and internal, are present, based on the ESACP guidelines. Böcking and Nguyen [12] recommend the use of the internal reference cells, which are prepared in parallel with the clinical sample, because they have the advantage of being subject to all the preparatory steps as the clinical specimen cells being analyzed. Different cell types have been used for the quantification of nuclear DNA ploidy in tumors, such as lymphocytes [14,16] and normal cells of the tissue being analyzed [12,17,26]. However, the cytomorphologic differences between the standard and analyzed samples, particularly with respect to the nuclear area and the degree of chromatin condensation are relevant factors for selection of the reference standard [27]. These authors found that the degree of tumor ploidy could be modified by the type of tissue used as the diploid reference standard. Furthermore, Grote et al [26] reported that the virus incubation period should be considered when using normal cervix cells as the reference, as they can generate histograms having a peridiploid DNA profile.

In this study, the diploid populations and nuclei having DNA content >5C and 9C were identified. Among the cases evaluated, 35% (7/20) were classified as diploid. According to Böcking et al [28], patients with a normal DNA histogram (main peak at 2C; 5cEE = 0; 9cEE = 0) may return to routine screening intervals. Nuclei with DNA content >5C (main peak at 2C; 5cEE > 0; 9cEE = 0) were identified in 45% of the samples (9/20), even among those with a histopathologic diagnosis of NILM (2/5). Sun et al [29] suggested that these patients be referred for additional tests, such as colposcopy and biopsy. Nuclei with a DNA content >9C (main peak at 2C; 5cEE > 0; 9cEE > 0) were detected in 20% (4/20) of the cases evaluated and were classified as aneuploid lesions with poor prognosis. Various authors report that the presence of DNA aneuploidy defines a lesion of undetermined significance as high-grade, obligatory precancerous, or prospectively malignant [12,30]. Böcking and Nguyen [12] recommend surgical intervention when such aneuploid lesions with poor prognosis are identified.

On nuclei IOD analysis, the maximum CV measured was 4.49%. This result is consistent with the medical DNA-ICM parameters, which accept CV values below 5% [24]. The coefficient value obtained confirms the adequacy of the procedures adopted.

Diagnostic DNA-ICM is an accurate and quantitative method that has been internationally standardized. The DNA-ICM may contribute to cut down the costs that result from repeated and unnecessary cytologic, histologic and clinical controls [12]. In fact, the expenses for such analyses are paid, for instance, by the German health insurance in the cases of cytologically doubtful diagnoses [31], and cost approximately 50 euros per measurement [12]. Furthermore, surgical intervention in DNA-ICM detected aneuploid lesions also avoids the high costs that are normally incurred when advanced carcinomas require treatment. This study adapted a procedure for quantitative analysis of DNA ploidy by combining commercial analysis software, conventional microscopy and certified filters. Such an adaptation makes DNA-ICM more accessible,

Table 2
DNA-image cytometry measurements in the cytologic subgroups.

No. of cases	Cytological diagnosis*	DNA-image cytometry (%)**		
		5cEE	9cEE	Diploid
14	HSIL	7(50)	3(21)	4(29)
1	LSIL	1(100)		
5	NILM	1(20)	1(20)	3(60)

*According to the Bethesda nomenclature system [2]; **According to the European Society of Analytical Cellular Pathology [24].

5cEE = 5C-exceeding events; 9cEE = 9C-exceeding events; HSIL = high-grade lesion; LSIL = low-grade lesion; NILM = negative for intraepithelial lesion or malignancy.

enabling the extended use of this tool as an adjuvant strategy for early screening of the cervical epithelium samples during routine analyses. Our results confirm the immense benefits of the DNA-ICM methodology and validate its application as an effective auxiliary diagnostic tool.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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