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Ploidy and chromatin pattern analysis as an aid for cervical smear diagnosis

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Summary. In the present study we used computer-assisted microscopy to analyze the morphology of Feulgen-stained cell nuclei in cell populations obtained at the same time as routinely performed cervical smears and in the same way. We investigated in a series of 110 cases whether the quantitative morphonuclear description of cytological cervical samples is able to aid pathologists to distinguish between benign and more suspect premalignant lesions. For this task nuclear DNA content, nuclear morphometry (size and anisonucleosis level) and chromatin pattern-related parameters were compiled for each specimen enrolled in the database. A set of 32 normal and 17 high-grade squamous intraepithelial lesion (HSIL) specimens (with diagnostic confirmations) were selected as references and used to establish a discriminant model on the basis of cytometry-generated variables. This model was then used to score the remaining 61 cases in our series (including cases exhibiting benign cellular changes, squamous cells of undetermined significance, low-grade SIL and cancers). The results show that a model discriminating efficiently between normal and HSIL groups can be obtained by combining 5 quantitative features (1 DNA ploidy-related, 2 morphometrical and 2 chromatin texture features). A 97% specificity and an 88% sensitivity characterized the boundary so established. When applied to new cases, the model was in fact able to correct diagnoses for cases which had been down- or up-graded on the basis of the Bethesda system, and provided scores in accordance with histological control.

Key words: Cervical cytology, Dysplasia, Diagnosis, Feulgen staining, Chromatin pattern, Computer-assisted microscopy

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Introduction

There is no doubt that the institution of widespread Papanicolaou smear screening has resulted in a marked reduction in mortality from cervical cancer (Koss, 1989; Raffle et al., 1995). Epithelial abnormalities, initially reversible but more apt to progress with increasing severity, have been classified by means of several different systems. The WHO system (Riotton and Christopherson, 1973) divides cervical dysplasia into mild, moderate and severe levels and defines a separate category for cervical cancer in situ (CIS). The Richart system (1980) classifies cervical lesions into three grades of cervical intraepithelial neoplasia, i.e. CIN1 and CIN2, which correspond to mild and moderate dysplasia respectively, and CIN3, which combines severe dysplasia and CIS. This system is still frequently used for histological classification. The more recent Bethesda system (Kurman and Solomon, 1994) has been designed to simplify cytological diagnosis and considers two categories of squamous intraepithelial lesions (SIL) only. Low-grade SIL (LSIL) corresponds to the CIN1 category (and also includes the cellular changes in HPV), and high-grade SIL (HSIL) combines CIN2 and CIN3. The correspondences between these 3 systems imply the same prognosis for the combined groups.

The accuracy of the cytological diagnoses in the case of Papanicolaou smears depends on the identification of morphological features characteristic of dysplastic or malignant cells. A wide range of reactive, inflammatory and even physiological conditions may give rise to cells whose cytomorphology closely mimics that of true precancerous or malignant changes. In these cases, colposcopy is required for differential diagnoses, creating anxiety among patients. Furthermore, numerous cases of spontaneous regression have been reported in the case of untreated patients affected by dysplasia (Bos et al., 1997; Lee et al., 1998; Holowaty et al., 1999). It follows that most women treated for dysplasia would never have developed cancer because their lesions could

have regressed spontaneously.

The aim of the present paper is to propose a tool to assist in the cytological diagnosis of cervical smears. For this, we used computer-assisted microscopy to analyze the morphology of Feulgen-stained cell nuclei in a cell population obtained at the same time as routinely performed cervical smears and in the same way. As already demonstrated in various pathological fields (Decaestecker et al., 1998; Yeaton et al., 1998, François et al., 1999; van Velthoven et al., 2000) including the analysis of pancreaticobiliary brush cytology specimens (Sears et al., 1998) image cytometry of Feulgen-stained nuclei is able to quantitatively measure the subtle changes arising in the chromatin texture of premalignant and malignant cells. This is why, in the present study, we investigated in a series of 110 cases whether the quantitative morphonuclear description of cytological cervical samples is able to aid pathologists to distinguish between benign and more suspect premalignant lesions. For this task nuclear DNA content, nuclear morphometry (size and anisonucleosis level) and chromatin pattern-related parameters were compiled for each specimen enrolled in the database. A set of 32 normal and 17 HSIL specimens (with diagnostic confirmations) were selected as references and used to establish a discriminant model on the basis of cytometry-generated variables. This model was then used to score the remaining 61 cases in our series (including those exhibiting benign cellular changes, atypical squamous cells of undetermined significance or ASCUS, LSIL and cancers). The results show that this kind of model can aid the evaluation of new cases by distinguishing between benign and suspect cervical lesions.

Materials and methods

Clinical data

The complete series consisted of 110 cytological samples from women of between 18 and 74 years of age (mean = 37). The cytological samples were collected using a Cervex-brush (Yvsolab, Antwerp, Belgium) at the same time and location as conventional Papanicolaou smears were performed (at the cervical or at transformation zone level). It can be thus considered that cytological diagnosis and image cytometry have been carried out on very similar cytological samples which thus include the same potential information. The cytological samples so collected were immediately placed in a tube containing 1-2 ml of EFA fixative [i.e. by volume containing 95% ethanol (75%), 40% formalin (20%), and glacial acetic acid (5%)] and stored until analysis. The corresponding Papanicolaou smears were diagnosed in accordance with the Bethesda system (Kurman and Solomon, 1994) and included 32 normal, 14 LSIL, 18 HSIL and 4 cancers. In addition, 14 cases exhibited benign cellular changes; of these, 10 were associated with inflammation, 3 with atrophy and 1 with mycosis. The remaining 4 cases were atypical squamous cells of undetermined significance (ASCUS). The criteria used for ASCUS identification are those described in the Bethesda System, i.e. cellular abnormalities that are more marked than those attributable to reactive changes but fall short of a diagnosis of SIL (Kurman and Solomon, 1994). More often, ASCUS involves nuclear enlargement in squamous cells with mature, superficial/intermediate-type cytoplasm.

From this series we selected 32 normal and 17 HSIL cases for which we had diagnostic confirmations (normal smears from women regularly examined and HSIL cases with histological confirmation). These cases were used as references, while the remaining ones were taken as tests. The test series also included 6 cases for which histological diagnoses made on biopsy controls disconfirmed the cytological diagnoses. These cases included 4 LSILs which were upgraded to CIN2s or CIN3s (i.e. moderate to severe dysplasia associated with the HSIL class), 1 LSIL reclassed as benign and, finally, one HSIL reclassed as HPV (associated with the LSIL class in the Bethesda system). These "disconfirmed" cases were used to test if the methodology proposed in the present study (based on image cytometry, see below) was able to rectify the cytological diagnoses of these cases in order to be more in accordance with the histological ones.

Cytological sample preparation and Image cytometry

The samples were subjected to a technique described elsewhere (Kiss et al., 1993) which makes it possible to obtain single-cell nuclei suspensions (after pronase digestion) that are centrifuged onto glass slides (cytospins) and stained by the Feulgen reaction.

Image analysis was performed using a SAMBA 2005 image processor (Alcatel-TITN, France). The way in which the cell nuclei were selected for analysis and digitized is detailed elsewhere (Budel et al., 1997; Sears et al., 1998; Yeaton et al., 1998). Briefly, the Feulgen-stained nuclei were digitized and accepted by the observer on the basis of conventional criteria required for a correct analysis (i.e. an intact nuclear membrane, satisfactory segmentation, and an absence of staining artifacts or overlapping nuclei). No other selection criterion was used (such as nucleus shape or texture) to avoid selection bias. (This nuclei selection can be made easily and very quickly by any observer at the time of image analysis and does not must be considered as a limitation). Between 200 and 300 cell nuclei per case were analyzed for statistical requirements (Bartels et al., 1985). For each nucleus registered, 15 numeric attributes were calculated based on algorithms reflecting the nucleus morphometric, densitometric and textural features, providing a quantitative description of the chromatin pattern, i.e. its distribution and level of organization. Which precise morphonuclear features describe each of the 15 nuclear attributes is detailed elsewhere (Budel et al., 1997; Sears et al., 1998; Yeaton

et al., 1998). Each cytological cervical sample was then characterized by 30 morphonuclear variables describing the mean and the standard deviations (SD) of each of the 15 nuclear attributes in the cell population analyzed (listed in Table 1). The SD variables were considered to take into account cell heterogeneity. In addition, the distribution of the DNA content per sample was described by means of 8 variables (listed in Table 1) including the DNA index, and seven determining the proportions of the different ploidy-related cell subpopulations in each histogram (Decaestecker et al., 1996). (The variables particularly involved in the present study are described in the Results).

Statistical analysis

The statistical comparisons of the data groups were performed by means of parametric t-tests (if the conditions of applicability were satisfied) or their non-parametric equivalent Mann-Whitney U-tests (if they were not). Furthermore, the association between 2 binary variables was tested by means of the exact Fisher test. Multivariate analysis was carried out by means of a conventional stepwise linear discriminant analysis. All the statistical analyses were effected with Statistica software (StatSoft, Tulsa, OK, USA).

Results

Distinguishing between the normal and the HSIL cases by means of cytometry-generated variables

The percentage of tetraploid cells (%4C), i.e. a ploidy-related variable, constituted the most discriminant variable (Mann-Whitney test, exact p-value = 0.0001) taken for the purpose of distinguishing between the reference groups (normal and HSIL). The value distribution of this variable is illustrated in Figure 1A. The presence/absence of 4C cells in a smear provided a significant cut-off value separating the above-mentioned risk groups. Indeed, 27 out of 32 normal cases had no 4C cells, against 4 out of 17 HSILs. The significance of the difference between the corresponding proportions (84% normal versus 24% HSIL) was confirmed by an exact Fisher test ($p < 0.00008$).

Stepwise discriminant analysis selected 4 complementary variables for distinguishing between the reference groups. These variables consisted of two morphometrical and two textural features. They were the mean nuclear area (NA, estimating the mean nuclear size in pixels), the anisonucleosis level (SDNA, i.e. NA standard deviation in the cell population), the Local Mean (LM), which estimates overall chromatin condensation, and the standard deviation of a nuclear attribute, which measures the co-occurrence matrix coefficient variance (SDCV) and characterizes the heterogeneity of the different levels of chromatin condensation. Fig. 1B-D illustrate the value distributions of 3 of these variables by means of the inter-quartiles ranges (i.e. the ranges of the 50% values which were the most central). Two of these variables exhibited significant differences across the two reference groups. The LM variable (Fig. 1B) showed significantly lower values in the HSIL group ($p = 0.02$, Mann-Whitney test) and, in contrast, the SDNA variable (Fig. 1D) exhibited significantly higher values in this group ($p = 0.006$, t-test). The HSIL group also showed a slight (not significant) increase in nuclear size (Fig. 1C).

The combination of the %4C variable with the 4 others listed above enabled an efficient distinction to be made between the normal and the HSIL groups. To illustrate this, the top of Figure 2 shows the value distribution of the discriminant factor resulting from the linear combination (performed by linear discriminant analysis) of the 5 variables selected, and the boundary between the two groups established by the model. This factor is a new variable (i.e. a new route into the data space) which contributed best to the separation of the two groups under consideration (as confirmed by a Mann-Whitney test, $p < 10^{-6}$). Negative values for the

Table 1. Computer-assisted microscope analysis of Feulgen-stained nuclei: variables computed

NUCLEAR DNA CONTENT	MORPHOMETRIC AND CHROMATIN TEXTURE PARAMETERS	
	(Sample Mean)	(Sample Standard Deviation)
DNA index (DI)	Nuclear Area (NA)	Standard Deviation NA (SDNA)
% Diploid Cell Nuclei (%2C)	Integrated Optical Density (IOD)	SDIOD
% Hyperdiploid (%H2C)	Mean Optical Density (MOD)	SDMOD
% Triploid (%3C)	Skewness (SK)	SDSK
% Hypertriploid (%H3C)	Variance of Optical Density (VOD)	SDVOD
% Tetraploid (%4C)	Kurtosis (K)	SDK
% Hypertetraploid (%H4C)	Short Run Length (SRL)	SDSRL
% Pentaploid (%5C)	Long Run Length (LRL)	SDLRL
	Grey Level Distribution (GLD)	SDGLD
	Relative Distribution Frequencies (RLD)	SDRLD
	Relative Distribution Percentage (RLP)	SDRLP
	Local Mean (LM)	SDLM
	Energy (E)	SDE
	Matrix Coefficient Variance (CV)	SDCV
	Contrast (C)	SDC

discriminant factor were thus considered to characterize a normal cell population while positive ones were seen as characterizing an HSIL one. Only one normal and 2 HSIL cases were misclassified by the model established in this way (i.e. a 6% error rate against 18% if %4C had been used alone, as reported above).

Application of the model on new cases

We then applied the model to the rest of our series, i.e. to the test cases which were not used to construct the discriminant model (see Materials and methods). The discriminant scores were computed for all these cases, as indicated at the bottom of Figure 2 (where we report the reference boundary established at the top). The 4 cancer cases each obtained a positive score and were thus correctly associated with severe epithelial abnormalities. In contrast, the benign cellular changes (INFL, mycosis and ATROPH) were all (except one atrophy case) on the right side of the line (i.e., the scores associated with normal cells). The ASCUS cases in our series also seemed associated with normal scores. The scores for the LSIL (mild dysplasia) cases appeared to be more heterogeneous, including 2 cases with high scores for which neither cytological follow-up nor histological control were available. With respect to the cases with conflicting cytological and histological diagnoses the model provided scores in accordance with the histological diagnoses, (whether the case was under- or up-graded on the basis of the Bethesda system), i.e. positive scores for cases associated with severe epithelial abnormalities (CIN2 and CIN3 combined in the HSIL category) and negative scores for cases associated with benign or mild epithelial abnormalities (HPV belonging to the LSIL category).

Discussion

Palcic et al. (1995) reported that about 8% of the 60 million Pap smears performed in the USA in 1995 exhibit "not negative" cytology (ASCUS, LSIL, HSIL, etc). These authors report that possibly 15% of these

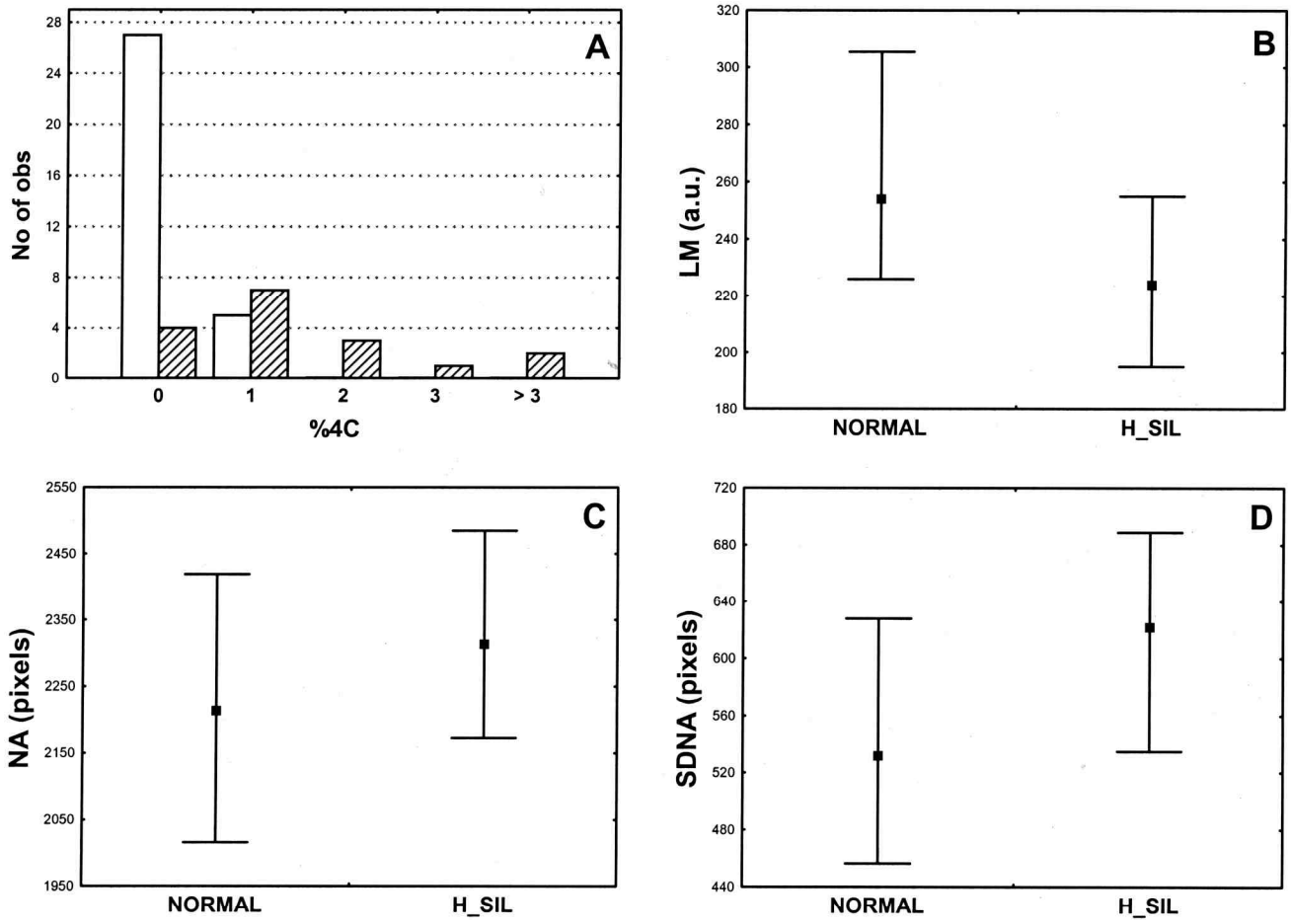

Fig. 1. A. Distribution histogram of the percentage of tetraploid cells (%4C) in the cell populations analyzed for the normal (white columns) and the HSIL (hatched columns) cases. **B-D.** Value distributions of the local mean (LM), the nuclear area (NA) and the anisonucleosis level (SDNA) features across the normal and HSIL groups. The results are given as median values (small squares) with the inter-quartiles ranges (bars).

cases will have positive follow-ups (repeated Pap smears, colposcopy or biopsy). This means that there are about 85% of cytologically false-positive cases (which represent more than 4 million cases in the data of Palcic et al., 1995). Furthermore, of the cases with positive follow-ups, about 3% only are seen as possibly developing into invasive cancers. These data seem to result from the considerable attention paid to avoiding false-negative cases. However, these data (among others) indicate that cytological (and also histological) diagnostic criteria might benefit from some refinement. Furthermore, the diagnosis of cervical smears remains a subjective procedure with consequent problems of reproducibility. In addition to these problems arising from morphological interpretation, pathologists are faced with interpretative dilemmas posed by the patients' condition or clinicians' techniques in obtaining the cervical smears. New techniques such as the "monolayer" screening devices have recently been introduced in order to attempt to improve slide interpretation (Vassilakos et al., 1996, 1998; Takahashi et al., 1998; Johnson et al., 2000).

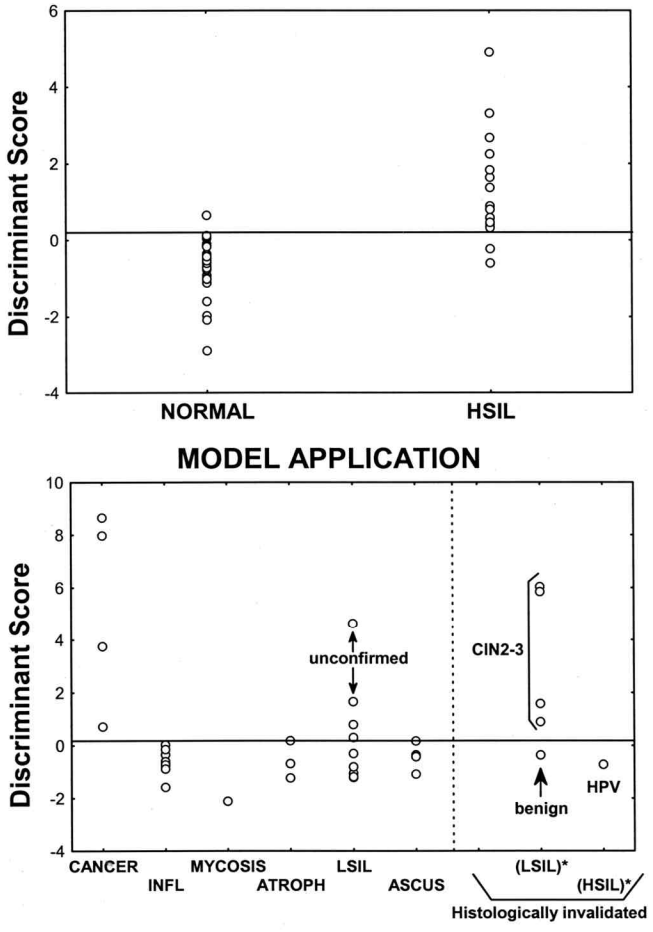

Fig. 2. Top: Value distribution of the discriminant factor (combining 5 cytometry-generated features) across the normal and HSIL reference groups, and the boundary (horizontal line) established by the model between these two groups. **Bottom:** Discriminant scores obtained for new cases reported in function of their diagnostic groups. (INFL = inflammatory, ATROPH = atrophy). Two cases with unconfirmed diagnoses (follow-up or histological control unavailable) are indicated in the LSIL group. The right-hand side reveals the scores established for 6 cases first diagnosed as LSIL (5 cases) and HSIL (1 case), and histologically invalidated (the respective histological diagnoses are indicated).

Many attempts have been made to refine and objectify some of the criteria used for cervical smear diagnosis. With respect to cytological features, high-resolution image cytometry applied to Feulgen-stained nuclei enables three distinct sets of morphological characteristics to be objectified, i.e. the morphometrical, the densitometrical and the textural variables. Morphometrical variables are any variable quantitatively describing the shape and the size of a nucleus. Densitometrical features globally describe the DNA distribution in cell nuclei and thus include measurements related to DNA ploidy level, thus enabling a DNA histogram to be drawn up for each cell sample. Finally, textural features characterize the chromatin patterns in cell nuclei more subtly and, particularly, the aspect and distribution of chromatin clumps.

As stated above, image cytometry enables both DNA ploidy level and morphonuclear features to be measured, and is able to identify morphological changes in samples with relatively few cells (some hundreds). As emphasized by O'Leary (1998), this is a significant advantage over flow cytometry, which is limited to ploidy-related measurements, requires samples with a large number of cells, and is thus less adapted to smear analysis. Furthermore, image cytometry also allows a reasonable assessment of the G1-, the S-, the G2- and the M-fractions to be made (Pauwels et al., 1995). Several groups of authors have already pointed out the significant diagnostic information offered by image cytometry. In this context, the cellular and nuclear features of intermediate cervical cells were examined by Wied et al. (1984), who found significant differences between dysplastic lesions and cervical carcinomas in situ. Similarly, Guillaud et al. (1995) and, more recently, Kasper et al. (1997) studied the prognostic relevance of malignancy-associated changes (MACs). These studies (and others such as the one by Palcic et al., 1995) are based on morphonuclear features. In contrast, information on DNA ploidy level (also obtained by means of image cytometry) have been used by others (van Leeuwen et al., 1996; Monsonego et al., 1997; Davey et al., 1998), showing an association between aneuploidy and lesion severity. However, very few (if any) studies fully use the complete potential of image cytometry enabling a combination of DNA ploidy levels and morphonuclear measurements to be set up, as in the present study. The morphonuclear features were calculated by the computer at the same time (without any additional cost) as the integrated optical density, which led to the determination of the DNA ploidy level. Hence, any laboratory having a computer-assisted microscope equipped with a black & white CCD camera

for DNA measurement purposes can also use the morphonuclear features described in this paper.

We have based the present study on the fact that smear samples from extreme groups (such as normal versus HSIL) have a major chance of exhibiting marked differences with respect to their nuclear-related features. To combine the diagnostic information generated by quantitative variables we used stepwise discriminant analysis. This method selected only one ploidy-related variable, i.e. the percentage of tetraploid cells (but in first position, i.e. as the most discriminant feature as confirmed by conventional univariate analysis). This means that in the presence of quantitative morphonuclear information the determination of DNA ploidy level also offers useful diagnostic information for distinguishing between HSIL and normal cells. Two morphometrical and two textural features (but no densitometrical ones) effectively completed the model. It will be noted that of these four additional features two are standard deviation measurements (SDNA and SDCV). This type of feature is able to take into account cell heterogeneity into the cell population analyzed (such as the anisonucleosis level) and is sensitive to any deviation around the mean value. It can be hypothesized that these SD-related features increase the model sensitivity to the presence of a reduced number of atypical cells, as also made by considering the different proportions of the different ploidy-related cell subpopulations (see Decaestecker et al., 1996 for an extended discussion about this latter point). The discriminant factor, resulting from the linear combination of the five variables selected, effectively contributed to separating the normal and the HSIL groups. Negative values for the discriminant factor were thus seen as characterizing a normal cell population, while positive ones were seen as characterizing an HSIL one. A 97% specificity and an 88% sensitivity characterized the boundary established in this way as compared with an 84% specificity and a 76% sensitivity if the ploidy-related variable (%4C) was used alone. When the model was applied to new cases, an interesting result was that it corrected diagnoses for cases which had been down- or up-graded on the basis of the Bethesda system, and provided scores in accordance with the histological diagnoses. Furthermore, except for one case, the benign lesions (including the inflammatory and atrophy cases) all received classification scores associated with normal cases. While encouraging results have been obtained, the present series has to be extended in order to refine the characterization and classification of LSIL and ASCUS cases.

A number of studies have emphasized the usefulness of combining DNA ploidy measurement and oncogenic Human Papillomavirus detection for prognosis of cervical lesions. However efficient HPV testing involves DNA-tests which can be heavy routinely. As the presence of aneuploidy was shown as strongly suggestive of the presence of oncogenic HPV types (Multhaupt et al., 1993; Rihet et al., 1996; Monsonego et al., 1997), it will be interesting to investigate whether the approach proposed in the present study (combining DNA ploidy and morphonuclear features) is able to provide information strongly correlated with oncogenic HPV, and whether it enables HPV testing to be eventually avoided.

In conclusion, high-resolution image cytometry applied to cervical smear analysis enables DNA ploidy-related variables to be used at the same time as morphometrical and chromatin pattern-related ones. The data obtained in the present pilot study strongly suggest that this kind of measurement can be usefully combined in order to aid pathologists in cervical smear diagnosis.

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