DNA Ploidy Patterns in Cervical Intraepithelial Neoplasia Grade III, With and Without Synchronous Invasive Squamous Cell Carcinoma

Measurements in Nuclei Isolated From Paraffin-Embedded Tissue

A. G. J. M. HANSELAAR, MD, G. P. VOOIJS, MD, PhD, P. S. OUD, PhD, M. M. M. PAHLPLATZ, MS, AND J. L. M. BECK, CT

This study presents the results of cytophotometric (CPM) and flow cytometric (FCM) DNA ploidy measurements in cervical intraepithelial neoplasias grade III (CIN III) with and without synchronous invasive squamous cell carcinoma. Hysterectomy and biopsy material from 21 patients 35 years of age or younger and from 18 patients age 50 years or older was studied. The DNA analysis was performed in nuclei isolated from specific areas of paraffin-embedded tissue. There were significant differences in the distribution of DNA patterns between the two age groups. About 80% of CIN III lesions in women 50 years of age or older, with or without a coexisting invasive cancer were aneuploid. In the group of younger women a diploid DNA pattern was found in about 60% of CIN III with concomitant invasive cancer. In the absence of an invasive cancer, CIN III lesions were mostly polyploid. The DNA pattern of invasive cancers was generally identical with the adjacent CIN, thus suggesting that the two lesions were related. Although the prognostic value of DNA ploidy measurements in cervical intraepithelial lesions in women in these two age groups has to be further evaluated, these results are at considerable variance with previously published data on DNA values in CIN and invasive carcinoma. In four CIN III lesions without invasive cancer, in women of the group of 35 years of age or younger, human papilloma virus common antigen could be demonstrated by immunochemical procedure. In three of these cases a polyploid DNA pattern was present; the fourth case showed a bimodal aneuploid pattern.

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EPIDEMIOLOGIC STUDIES of cervical neoplasia have shown the incidence of carcinoma *in situ* and related lesions, grouped as cervical intraepithelial neoplasia grade III (CIN III) to be much higher than the incidence of invasive squamous cell carcinoma (ISC), indicating that only a relatively small number of CIN III lesions will progress to invasion. The majority of CIN III lesions, therefore, will either regress or persist.[1–3] Biopsies or treatment may account for some, but not all, of these cases.[4,5]

It is not possible to separate CIN III lesions into regressive, persistent, or progressive subtypes purely on the basis of histologic, cytologic, or clinical criteria. The use of objective parameters such as DNA measurements is being given increasing attention.[6–13] Aneuploidy has been a frequent finding in CIN III lesions using chromosome analysis,[6,7] cytophotometric (CPM) or flow cytometric (FCM) DNA measurements in cervical scrapings,[8,9] fresh biopsy material,[10] or paraffin sections.[11,12]

Cervical intraepithelial lesions with an aneuploid DNA pattern have been reported to have higher rates of persistence or recurrence than lesions with a diploid or polyploid pattern.[8,11,12] The results of these studies seem to be in conflict with DNA analyses which showed (peri-ploid) diploid or low ploidy DNA patterns in more than 50% of micro and macroinvasive squamous cell carcinomas.[13,14] Invasive carcinomas with low ploidy pattern were reported to have a higher frequency of lymph node metastases than occurred in high-ploidy carcinomas of comparable size and depth of invasion.[6,8,13,15] Furthermore, it was stressed by Atkin[15] that a diploid DNA pattern does not necessarily indicate a favorable outcome.

Recently the role of human papilloma virus (HPV) infection in the pathogenesis of cervical neoplasia has at-

From the Department of Pathology, University of Nijmegen, Nijmegen, The Netherlands.

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Address for reprints: A. G. J. M. Hanselaar, MD, Department of Pathology, University Hospital Nijmegen, Geert Grooteplein Zuid 24, NL-6525 GA Nijmegen, The Netherlands.

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TABLE 1. Cervical Epithelial Abnormalities, Subdivided for Type of Lesion and Age

	≤35 yr			≥50 yr	
	Patient group	Lesions (n)	Patients (n)	Patient group	Lesions (n)	Patients (n)
Cervical intraepithelial neoplasia grade III without adjacent invasive carcinoma (CIN)	I	11	11	III	8	8
Cervical intraepithelial neoplasia grade III with adjacent invasive carcinoma (CIN-INV)	IIA	10	10	IVA	10	10
Invasive squamous cell carcinoma adjacent to the CIN III-like lesions (INV)	IIB	10		IVB	10	
		31	21		28	18

tracted much attention. Along with koilocytotic atypia, which has been accepted as a pathognomonic cytologic marker for HPV infection,[16-19] HPV DNA can be demonstrated in most cases of cervical intraepithelial neoplasia and ISC using molecular hybridization techniques.[20-24] With immunohistochemical techniques, the presence of the common viral capsid antigen could be documented in some cases of cervical intraepithelial neoplasia, but not in invasive squamous carcinoma.[24-26] The DNA ploidy pattern of the lesions associated with HPV was studied by Fu and co-workers.[12,27] They stated that typical condylomas without a significant degree of nuclear pleomorphism have a polyploid DNA distribution, whereas "those changes having an aneuploid DNA pattern are true neoplastic processes."

This article reports the results of DNA measurements performed on whole nuclei derived from specific areas of the uterine cervix identified under the microscope. The method used in this study allowed us to compare the DNA content in intraepithelial lesions classified as CIN III without adjacent invasive cancer (CIN) with the DNA content in CIN III-like lesions adjacent to invasive cancer (CIN-INV). Furthermore, we compared the DNA content in intraepithelial CIN III-like lesions adjacent to areas of invasive carcinoma with the DNA content of the invasive component. The study was performed on two groups of women: age 35 years or younger and age 50 or older, corresponding to the biphasic distribution of invasive carcinoma.[2,3] In all patients representative histologic sections were stained for the presence of papilloma virus common antigen.

Patients and Methods

Patients

In this study cervical tissue derived from hysterectomy and biopsy material of 39 patients was examined. The CIN III was defined as an epithelial lesion in which undifferentiated basal or parabasal cells with nuclear enlargement, crowding, indistinct cell boundaries, and a high nucleo-cytoplasmic ratio occupied more than two thirds (or the full thickness) of the epithelium.[28] Superficial cytoplasmic keratinization could be present.

The lesions were divided into six groups, according to age of the patients and the presence or absence of invasive cancer adjacent to intraepithelial neoplasia (Table 1).

The first group contained 11 cases of CIN III in patients 35 years or younger, without concomitant microinvasive or macroinvasive squamous cell carcinoma (I:CIN). The second group (IIA and IIB) consisted of ten patients also 35 years of age or younger, but with CIN III lesions (IIA: CIN-INV) and an invasive carcinoma (IIB:INV) directly related to the CIN lesions in the same or in an adjacent tissue block. The CIN III lesions with or without concomitant invasive cancer were morphologically identical. However, the biological behavior is likely to be different. To differentiate between these two lesions we introduced for CIN III lesions with concomitant invasive cancer the term CIN III-like lesions (CIN-INV). Eight women 50 years or older with CIN III, without a concomitant invasive cancer constituted the third group (III:CIN).

The fourth group was composed of ten women, 50 years or older, with CIN III-like lesions (IVA:CIN-INV) and an invasive cancer in the same or in an adjacent tissue block (IVB:INV).

Using a recently described method for extracting intact nuclei from paraffin-embedded tissue,[29-31] it is possible to perform image and FCM DNA measurements of preselected areas in tissue sections.

Thus using selected areas, DNA content of pure intraepithelial lesions (CIN) can be compared with DNA content of intraepithelial lesions directly adjacent to invasive squamous cell carcinoma (CIN-INV) (Fig. 1). Also, the DNA content of invasive carcinomas (INV) can be compared with DNA content of both types of CIN III (CIN and CIN-INV).

Method of Extraction of Nuclei From Paraffin-Embedded Tissue

Two consecutive 50-μm sections were cut from paraffin-embedded formalin-fixed tissue containing CIN III and/or invasive cancer. Fifty micrometers' thickness was

proven to cause the least number of artifacts in DNA histograms.[32] Sections were deparaffinized and rehydrated in the following sequence: xylene (twice), 100% ethanol (twice), 80% ethanol, 70% ethanol, 50% ethanol and distilled water (at least 10 minutes in each solution). Thereafter tissue sections were placed on microscopic slides. For orientation and localization of relevant areas 5-μm tissue sections were made before and after the two "thick" sections were cut. These were stained with hematoxylin and eosin (H & E). An example of a CIN III-like lesion with an adjacent invasive carcinoma is shown in Figure 1.

Under a dissecting microscope the CIN III and/or invasive carcinoma regions were localized and prepared for DNA analysis by scraping off nonrelevant areas with a scalpel. The target tissue was then taken from the glass slides, put in a centrifuge tube, and incubated in phosphate-buffered saline (PBS), with 0.1% protease (type VII from Bacillus amylolique faciens, Sigma Chemical Co, MI) at 37°C, for 60 minutes. During incubation the specimens were gently syringed using needles ranging in diameter from 0.4 to 1.1 mm (gauge, 23–19), at 15, 30, 45, and 60 minutes. Incubation was terminated by adding 4 to 5 ml of cold (4°C) PBS, after which the tubes were put on ice. The nuclei were washed twice with PBS with intermediate centrifugation steps, and counted with a Coulter Counter Model ZB1 (Coulter Electronics, Hialeah, FL). About 10,000 nuclei were centrifuged, and 150 μl fetal calf serum (normal pooled Gibco, Paisly, UK) was added. This cell suspension then was centrifuged (5 minutes at 500 rpm), placed on slides using a cytocentrifuge (Shandon, UK) for 10 to 20 seconds, air dried, and fixed in a mixture of methanol, 37% formaldehyde, and acetic acid (85-10-5 by volume) for 1 hour.

Cytophotometric Analysis

For CPM isolated nuclei from tissue blocks of all 39 patients were stained with Thionin-Feulgen.[33] DNA content of 200 stained intact CIN III (CIN and CIN-INV) or invasive cancer (INV) nuclei was measured using the Nijmegen Image Analysis System (NIAS). The NIAS system is composed of an Orthoplan Scanning microscope (Leitz Wetzlar, West Germany), and provided with a linear diode array (Fairchild). The microscope is interfaced to a MINC minicomputer with a LSI 11/23 microprocessor (Digital Equipment Corp., Maynard, MA). Nuclei were measured with monochromatic light with a wavelength of 585 nm (band width, 20 nm). Sampling density was four pixels per micrometer. As an internal standard for DNA content 20 lymphocytes per slide were measured.

Flow Cytometric Analysis

The suspension containing residual nuclei was centrifuged briefly and fixed in cold (−20°C) 70% ethanol.

The nuclei were then stained for DNA with propidium iodide[34] and measured in a Cytofluorograph 50H flow cytometer (Ortho-Instruments, Westwood, MA).

In seven patients the number of cells was sufficiently high for both CPM and FCM analysis.

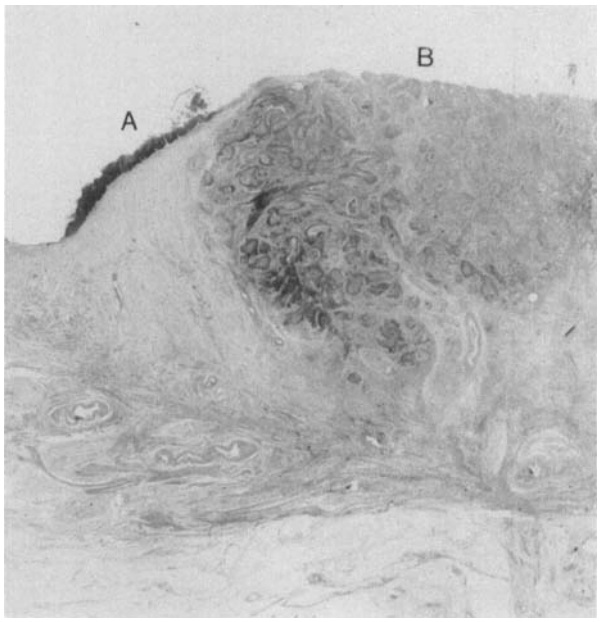


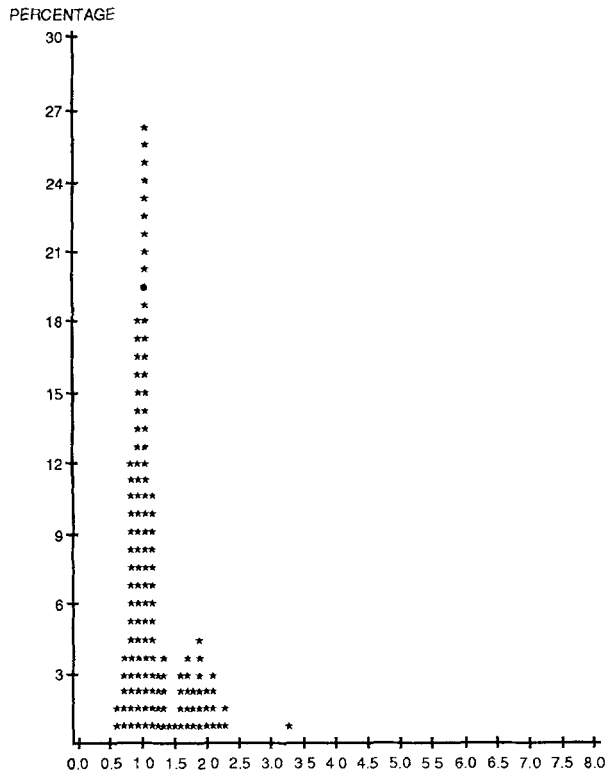
FIG. 1. Invasive squamous carcinoma (B) and an adjacent CIN III (A) (H & E, ×4).

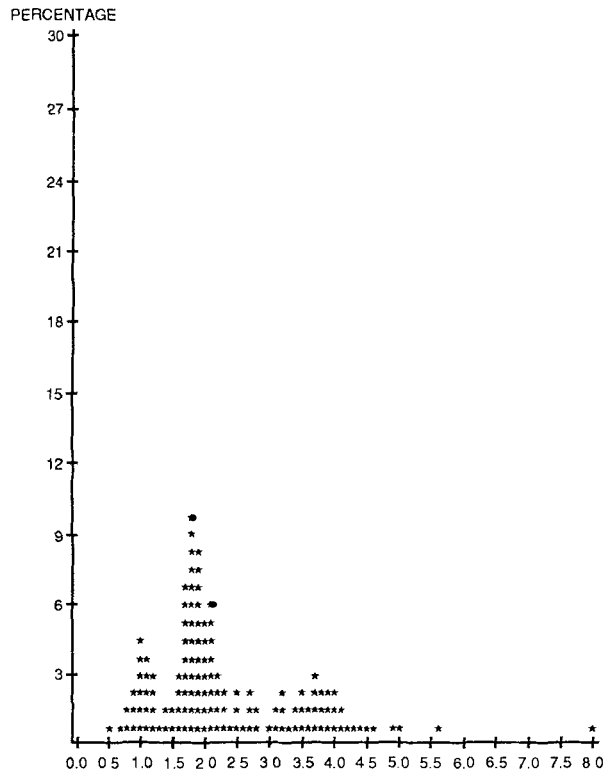
Histogram Interpretation

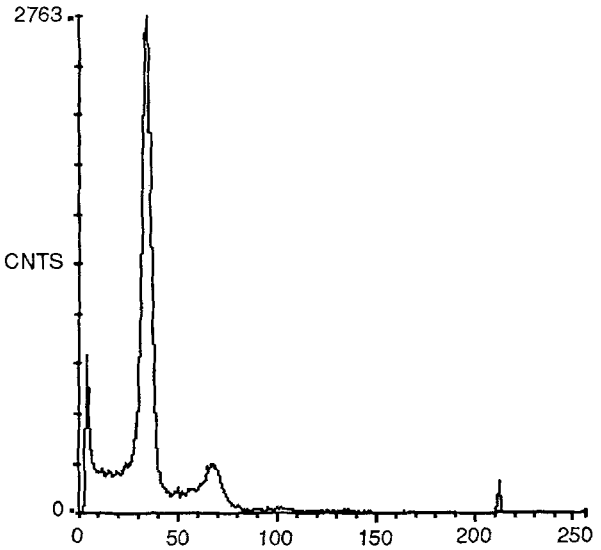
The DNA content was expressed as DNA index (DI). DNA index was defined as the nuclear integrated optical density of the dominant peak or peaks of the histogram divided by the median integrated optical density of the "internal" control sample of lymphocytes.[35,36] In cytophotometry the DI distributions were defined from histograms using a bin size of 0.1. Histograms were classified as diploid, polyploid, or aneuploid.[11,31] DNA ploidy patterns were designated diploid (2C; DI = 1.0) when a distinct peak was found in the diploid or near diploid (DI = 0.9–1.1) region, polyploid when distinct peaks were present either in the diploid (2C; DI = 1.0), and tetraploid (4C; DI = 2.0) or in the diploid, tetraploid and octaploid (8C; DI = 4.0) regions. Ploidy patterns were considered to be aneuploid in all cases with scattered unimodal, bimodal, or multimodal distribution of DNA peaks in the histograms.

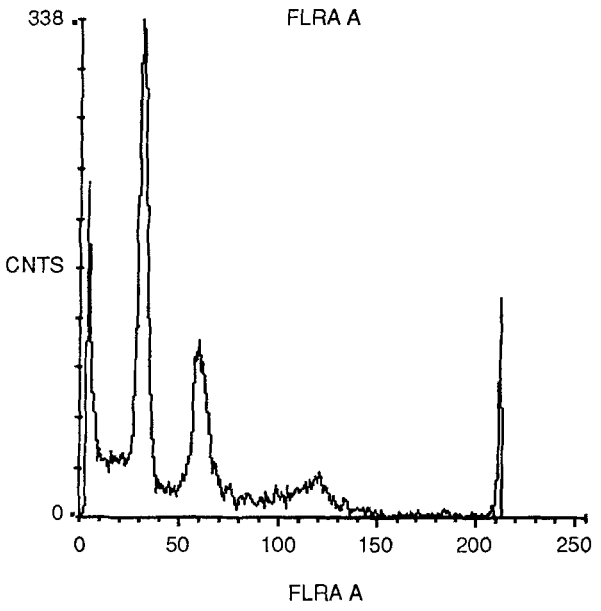
Identification of Papilloma Virus Infection

In all cases of CIN III and invasive cancer the presence of papilloma virus infection was studied. Paraffin sections (5–6 μm) were cut from the same tissue blocks and submitted for immunohistochemical staining with rabbit antisera, prepared against disrupted bovine papilloma virus









FIGS. 2A–2D. Diploid DNA pattern of an invasive squamous cell carcinoma is shown in both cytophotometry (CPM) (A, top left) and flow cytometry (FCM) (B, top right). The DNA index is shown on the X axis in cytophotometry histogram (woman 31 years of age). (C, bottom left) Cytophotometric (left) and (D, bottom right) flow cytometric histograms of an invasive cancer showing a polyploid DNA pattern. The first (diploid) peak is higher in the FCM histogram, because lymphocytes and stromal cells are measured as well (patient 31 years of age).

type 1 virions (Dako Corporation, Santa Barbara, CA). These antisera react with the capsid antigens of both animal and HPV.[25–27] Immunohistochemical identification of papilloma virus common antigen was performed using the peroxidase–antiperoxidase (PAP) technique. Paraffin sections were deparaffinized, hydrated, treated with hy-

TABLE 2. DNA Ploidy Patterns in Cervical Intraepithelial Neoplasia Grade III With and Without Coexisting Invasive Squamous Cell Carcinoma*

DNA ploidy pattern	CIN III											
	CIN						CIN-INV					
	Total		≤35 yr		≥50 yr		Total		≤35 yr		≥50 yr	
	N	Percent	N	Percent	N	Percent	N	Percent	N	Percent	N	Percent
DNA diploid	2	11	2	18	0	—	8	40	6	60	2	20
DNA polyploid	7	37	6	55	1	13	2	10	1	10	1	10
DNA aneuploid	10	53	3	27	7	88	10	50	3	30	7	70
Total N	19		11		8		20		10		10	

CIN: cervical intraepithelial neoplasia grade III without adjacent invasive carcinoma; CIN-INV: CIN with adjacent invasive carcinoma.

* Total and subdivided for two age categories.

drogen peroxide in methanol, rinsed in PBS, preincubated for 10 to 30 minutes in normal swine serum, then incubated overnight with the antiserum, diluted 1:3000. The next day the specimens were treated with swine anti-rabbit (1:30) and PAP (1:100), and visualized with diamine benzidine (DAB). Slides with deep brown nuclei in two successive stainings were scored as positive for papilloma virus common antigen.[25]

Results

Comparison of Flow Cytometric With Cytophotometric Findings

In seven patients with invasive squamous carcinoma but in none of the CIN III cases, sufficient material was available for FCM DNA analysis. In all seven cases, results of FCM and CPM analyses were similar. Two examples are shown in Figures 2A through 2D.

Cytophotometric Results

Cytophotometric DNA analysis was done in all 39 CIN III lesions (CIN and CIN-INV) and in 20 invasive cancers (INV).

The results of cytophotometric analysis of CIN III without adjacent invasive carcinoma (CIN), compared with CIN III with adjacent invasive carcinoma (CIN-INV) are shown in Table 2. In two cases of CIN the DNA histogram showed a diploid pattern, seven patients had a polyploid pattern, and ten an aneuploid pattern. DNA analysis in 20 CIN III-like lesions with adjacent invasive carcinoma (CIN-INV) disclosed a diploid DNA-pattern in eight cases, a polyploid pattern in two, and aneuploidy in ten cases.

When age of the patients was taken into consideration (Table 2), in CIN III lesions (CIN and CIN-INV) an aneuploid DNA pattern was most frequently found in patients 50 years of age and older, both in the absence (seven of eight patients) and in the presence (seven of ten patients) of an adjacent invasive squamous cell carcinoma.

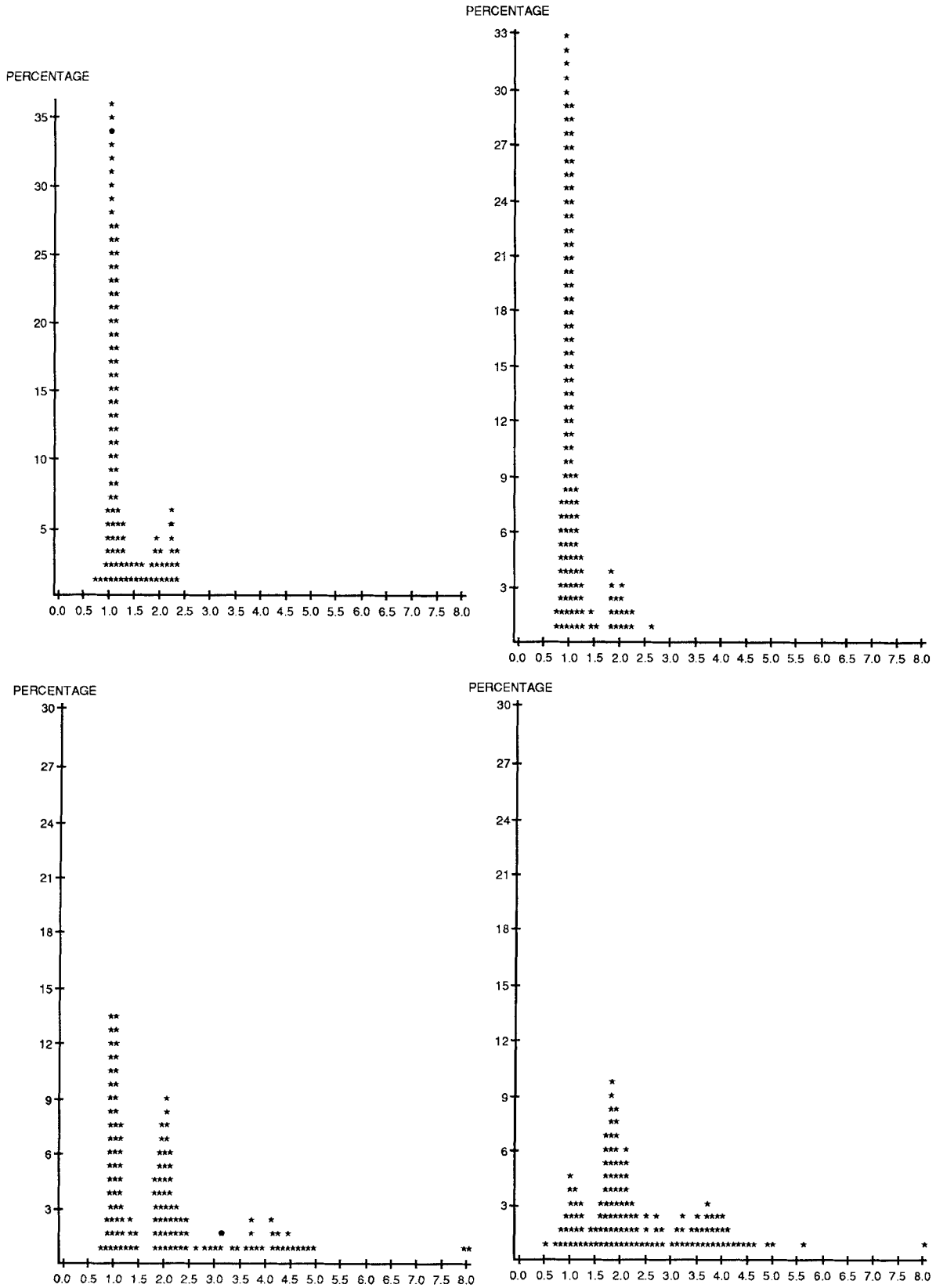
In the younger age group (≤35 yr) aneuploidy was present in only three of 11 (27%) patients with CIN III and in three of ten (30%) patients with a CIN-INV lesion. A diploid pattern was observed in two (two of 11, 20%) cases of (CIN) lesions in younger patients. In patients with CIN-INV lesions, diploidy was found in six (60%) younger women and in two (20%) women older than 50 years. A polyploid DNA pattern was identified in six (six of 11, 55%) patients from the younger age group with a CIN lesion, only in one older patient (one of eight, 13%) with a CIN lesions and in patients with CIN-INV lesions in one patient (10%) of each age group.

In Table 3 distribution of DNA patterns in 20 invasive squamous cell carcinomas (INV) and adjacent CIN lesions (CIN-INV) is compared. In 18 of 20 cases (90%) DNA patterns in invasive carcinomas were similar to those of adjacent (CIN-INV) lesions (eight diploid, one polyploid, nine aneuploid). In Figures 3A through 3D two characteristic examples are given. In these two cases with DNA aneuploidy in both the intraepithelial lesion (CIN-INV)

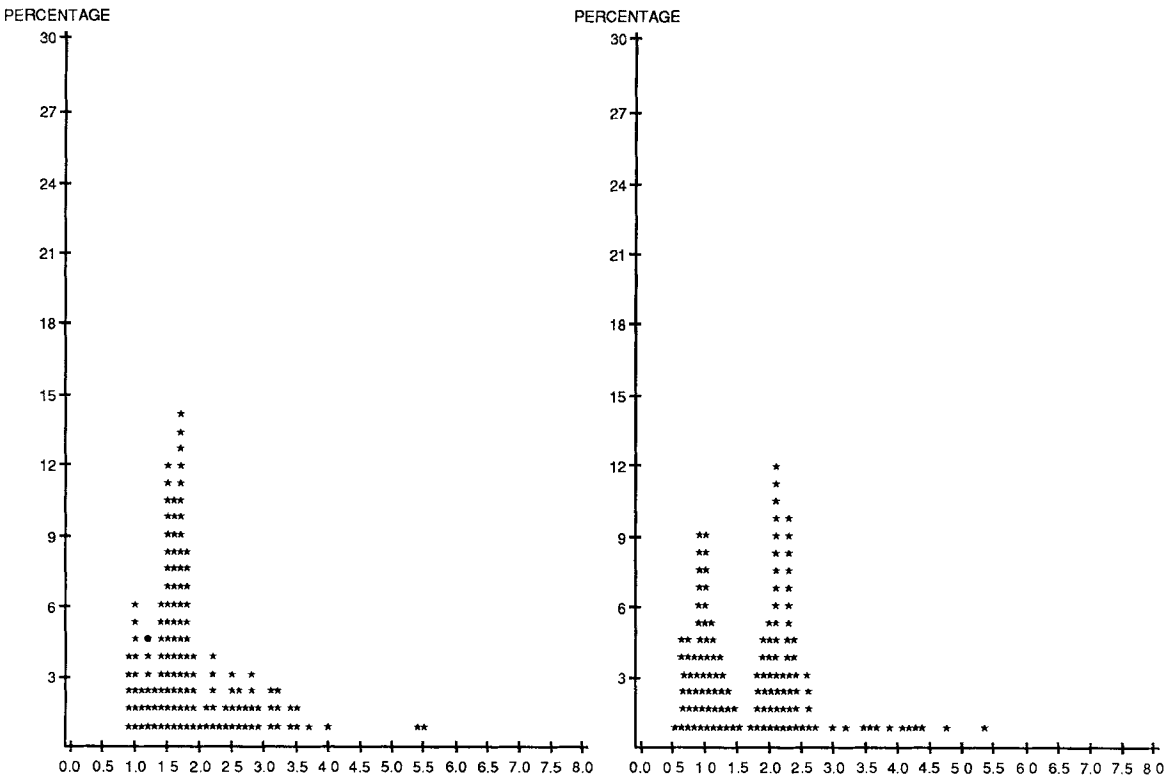
TABLE 3. Distribution of DNA Patterns in 20 Cases of Invasive Cancer and Adjacent Cervical Intraepithelial Neoplasia Grade III-Like Lesions

DNA ploidy pattern	CIN III-like lesion (CIN-INV)			
	Diploid	Polyploid	Aneuploid	N
Invasive cervical cancer (INV)				
Diploid	8	—	—	8
Polyploid	—	1	1	2
Aneuploid	—	1	9	10
N	8	2	10	20

CIN: cervical intraepithelial neoplasia grade III without adjacent invasive carcinoma; CIN-INV: CIN with adjacent invasive carcinoma.



FIGS. 3A–3D. Diploid DNA patterns present in both (A, top left) the CIN III-like lesion (CIN-INV) and (B, top right) the adjacent invasive cancer (INV) (patient 33 years of age). (C, bottom left) Polyploid CIN III-like lesion (CIN-INV) and (D, bottom right) polyploid invasive cancer (INV) (same patient as Figs. 2C and 2D).



FIGS. 4A AND 4B. (A, left) Aneuploid DNA pattern of CIN III-like lesion (CIN-INV) and (B, right) polyploid DNA pattern in invasive cancer (INV) in the same patient (patient 62 years of age).

and the adjacent invasive carcinoma, DNA distribution in invasive cancers usually was more scattered. In two cases (10%) DNA patterns of the intraepithelial lesion and the invasive cancer were different. In one case the (CIN-INV) lesion was polyploid whereas the invasive cancer had an aneuploid pattern. In the second case (Figs. 4A and 4B) the opposite was noted.

Identification of Papilloma Virus Infection

Papillomavirus common antigen could be detected in four women younger than 35 years of age with a (CIN) lesion. Papillomavirus was not detected in any of the other lesions.

The DNA pattern was polyploid in three of these four younger patients. In the fourth patient a bimodal aneuploid DNA pattern was seen.

Discussion

Cytophotometric and flow cytometric DNA analysis of selected areas of cervical intraepithelial neoplasia grade III and invasive squamous cell carcinoma was performed using whole nuclei extracted from paraffin-embedded tissue.[29-31] With this method selected areas of archival paraffin-embedded tissues can be analyzed retrospectively. Stromal and normal epithelium cells can be removed from the specimen under a dissecting microscope, thus reducing the mostly diploid component of benign cell populations,[29] which may obscure small aneuploid fractions. The selection of areas of CIN III or invasive cancer by elimination of nonrelevant tissue segments before nuclear isolation makes DNA histograms truly representative of the epithelial lesion under study.[29,30] Furthermore, DNA measurements of intact nuclei are more reliable than measurements in nuclei in tissue sections.[8,11,12,29]

In this study sufficient material was available in seven cases of invasive cancers for both cytophotometric and flow cytometric DNA analysis. The results of both measurements were comparable: a high first (diploid) peak in the FCM histograms was the result of residual lymphocytes and stromal cells still present in the sample.

In this study cytophotometric DNA analysis was possible in all cases of CIN III and invasive cancer. In 18 of the 20 cases (90%) DNA patterns of CIN III-like lesions and adjacent invasive squamous cancers were similar, implying that these CIN lesions are true precursors of invasive cancer.

Cytophotometric DNA analysis of both pure CIN III and CIN III-like lesions in the presence of invasive cancer showed DNA aneuploidy in 51%, polyploidy in 23%, and diploidy in 26%. These figures differ considerably from data published by others[9-11] even when taking into ac-

TABLE 4. Comparison of the Current Results With Data From Previous Studies by Jakobsen *et al.*[10] and Fu *et al.*[11]

Study by	Fu *et al.*[11]	Jakobsen *et al.*[10]	Current study
Yr	1981	1983	1987
Material	Paraffin-embedded tissue, 14-μm section	Fresh biopsy specimen	Paraffin-embedded tissue: nuclei isolated from 50-μm sections
Technique of DNA analysis	Microspectrophotometry, single wavelength, plug method	Flow cytometric analysis	Cytophotometric analysis
No. of patients	100	121	39
No. of CIN III	10	47	39
DNA pattern			
Diploid	0	10	10
Polyploid	0	—	9
Aneuploid	10	37	20
Age of patients	Not investigated	Not investigated	21 patients ≤35 yr 18 patients ≥50 yr

CIN III: cervical intraepithelial neoplasia grade III without adjacent invasive carcinoma.

count that these authors were using different methods or somewhat different definitions of aneuploidy (Table 4).

Jakobsen *et al.* described FCM analysis of fresh cervical biopsy material of 121 patients. From 47 cases identified as severe dysplasia or carcinoma *in situ* in 37 (79%) DNA histograms were aneuploid.[10]

Fu *et al.* described the results of microspectrophotometric DNA measurements using a single wavelength plug method in 14-μm paraffin sections. In their study of 100 patients all ten cases diagnosed as CIN III were aneuploid.[11] Nasiell *et al.*[9] used scanning microspectrophotometry for DNA measurements in cytologic preparations, defining aneuploidy by the "5-c exceeding rate." In all six cases of carcinoma *in situ* in their study they found a significant aneuploid cell fraction.

In none of these studies age distribution of patients was taken into account. In the current study 78% (N:14) of CIN III lesions in women 50 years of age or older, with as well as without coexisting invasive cancer, were aneuploid. In contrast the frequency of aneuploidy in CIN III and CIN III-like lesions in younger women was remarkably low (29%, N:6).

These differences in frequency of aneuploidy in the two age groups may indicate two biologically different neoplastic processes. In the group of younger women a diploid DNA pattern was found in about 60% (N:6) of intraepithelial lesions with adjacent invasive cancer (CIN-INV). This high percentage is at variance with data from others.

In the study of Fu *et al.* none of the 10 CIN III lesions was diploid.[11] A diploid pattern was recorded in CIN I and II lesions only. Diploid and polyploid DNA patterns were previously thought to be found almost exclusively in reactive processes and in regressive CIN lesions.[11,13,36] When Jakobsen *et al.* noted about 20% diploidy in CIN III lesions,[10] these authors attributed this observation to the small size of the intraepithelial lesions in their study. However, Spriggs *et al.* using cytogenetic techniques, in specimens of carcinoma *in situ,* already demonstrated the modal chromosome number in 29% of cases to be in the diploid range.[37] Atkin also stressed that diploid DNA patterns of CIN III lesions did not necessarily indicate good prognosis.[15]

Finally, diploid DNA patterns also have been found in cervical invasive cancer and in other malignant tumors in a variety of organs such as breast, esophagus, stomach, colon, endometrium, head and neck squamous carcinomas, and ovaries.[13,36] Our data strongly support the fact that a diploid DNA pattern in a CIN III lesion does not exclude progression to invasive cancer and is not necessarily indicative of a reactive or regressive change.

The significance of polyploidy in CIN III lesions is uncertain. The assumption that polyploidy indicates a reactive change is supported by the relatively high percentage (55%) of polyploid CIN III lesions in the absence of an invasive carcinoma (CIN) in younger women and the low percentage (10%) in older women (13%), in (CIN-INV) lesions (10%), and in invasive cancer (10%).

In three of the polyploid CIN III lesions without invasive cancer (CIN), HPV common antigen was present. This supports an infectious nature of these lesions. Further behavior of the polyploid lesions is as yet unknown.

Based on our analysis of DNA ploidy in cervical intraepithelial neoplasia grade III, using nuclei isolated from paraffin-embedded tissue, the conclusion is warranted that a significant proportion of diploid CIN III lesions in younger women have invasive potential and thus a diploid DNA pattern in CIN III lesions should not automatically be considered to be an indicator of good prognosis. Our findings also suggest that processes leading to invasive cancer of the cervix may be biologically different in younger and older women. However, the current group of 39 patients is too small to draw definitive conclusions.

Further analysis using a larger group of patients should provide more detailed information on the relation between DNA ploidy patterns of CIN III lesions of the uterine cervix and their progressive or regressive behavior.

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