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Malignant Potential of Dysplastic Endocervical Epithelium Assessed by Ploidy Status, S-Phase Fraction and C-myc Expression

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A B S T R A C T

The aim of this study was to determine if dysplastic endocervical cells (EC) posses a neoplastic potential as precursor lesions to adenocarcinoma in situ (AIS). The malignant potential was determined by assessing the ploidy status and proliferative activity by flow cytometry and c-myc expression by immunohistochemistry. The studied parameters were assessed separately in morphologically normal, dysplastic and malignant EC. The χ^2 test showed significant association of malignant EC with aneuploidy (p=0.008) and high proliferative activity (p=0.042). Since one third of the dysplastic EC are also aneuploid and show high mitotic activity, they probably have malignant potential as well. The dysplastic EC showed a significant association with c-myc oncogene expression (p=0.028). Our results indicate the existence of pre-malignant glandular lesions, while the immunohistochemical detection of c-myc protooncogene could be helpful in detection of EC with malignant potential, even without any dysplastic morphological changes.

Key words: *precursor lesions, cervical adenocarcinoma, ploidy, S-phase, c-myc oncogene*

Introduction

During the past three decades, the spectrum of endocervical glandular lesions has attracted increasing attention because of the absolute increase in the prevalence of invasive primary adenocarcinoma (ACA) of the uterine cervix all over the world[1–3].

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Unlike primary ACA of the cervix, the incidence of squamous cell carcinomas (SCC) has decreased[4–8]. A reduced morbidity and mortality of cervical SCC is due to early detection of its precursor lesions (cervical intraepithelial lesions – CIN grade II/III), for which there well-established morphological criteria exist[9,10].

The existence of precursor lesions of adenocarcinoma in situ (AIS) or invasive ACA is still a controversial topic[11–14]. Some authors believe that there are recognizable pre-neoplastic glandular lesions, while others think that morphological evidence is not sufficient to support the existence of pre-malignant lesions culminating in AIS[12,14]. Although the histological abnormalities of endocervical cells (EC) are frequent findings adjacent to AIS and invasive ACA, their potential pre-cancerous nature should be examined by objective parameters[11,14,15]. Aneuploidy, higher mitotic activity and over expression of the c-myc oncogene are nowadays well documented features of malignant cells[15–19]. The EC histomorphological abnormalities vary within a broad spectrum, with features intermediate between those of AIS and the normal endocervical epithelium[15]. In order to include all cytologically transformed EC the term dysplasia is used[15,20].

According to present insights into the carcinogenesis, neoplastic transformation is a long-term process from normal to malignant cell, during which many molecular mechanisms occur, leading after certain period of time to genomic instability and phenotypic variation[20].

The final step in the process of malignant transformation of the cell is the accumulation of many genetic events that lead to DNA damage manifested as quantitative changes in the DNA content and everlasting proliferation[16,20–22]. Aneuploidy represents an abnormal amount of DNA[23]. The period of the cell cycle during which cells are in preparation for cell division is called the S phase[23]. Ploidy status and S-phase fraction of certain cell populations are easily detected by flow cytometry. Consequently, in the malignant cell population in which the process of carcinogenesis has been completed, the DNA content is abnormal (aneuploidy and high S-phase fraction) displaying also visible malignant morphological changes that are easily recognized by a pathologist[17].

In attempt to detect carcinomas in the earliest phase of their development many new molecular studies simultaneously and continuously analyze the genetic events during the process of proliferation in both normal and malignant cells[18,19,24,25]. These studies discovered that the overexpression of the c-myc oncogene is the first critical genetic event in the process of carcinogenesis[18,19].

The c-myc gene controls cell growth and proliferation of each type of human cell[26]. During the process of proliferation, the normal, non-neoplastic cells showed the c-myc gene product localized in the nucleus. Due to its short half-life of 15–20 minutes, it desintegrated very soon[27,28]. In cells, which suffered malignant transformation triggered by overexpression of c-myc oncogen, the enormous production of oncoproteins accumulated in the cytoplasm of the transformed cells[27,28]. Therefore, the earliest manifestation of malignant transformation of the cell without visible morphological changes could be an increased cytoplasmatic expression of the c-myc oncoprotein.

The aim of this study was to determine the DNA content (ploidy and proliferative status) and the expression of the c-myc oncogene in the dysplastic EC, and to compare them results with the DNA content and the c-myc expression in the morphologically normal or malignant EC of AIS or ACA. The idea was to determine whether dysplastic EC have features different from normal cells, which would indicate their malignant potential.

Samples and Methods

Sample selection

Serial sections of the cervices stained with hematoxylin and eosin (H&E) were obtained from 49 patients who underwent cone biopsy or hysterectomy because of a previously diagnosed AIS or primary invasive ACA of the cervix by punch biopsy. Only one representative slide was chosen from each patient, showing simultaneously the presence of the morphologically normal endocervical glands and the areas of endocervical glandular dysplasia adjacent to the area of malignant EC in AIS or invasive ACA (Figure 1).

Morphological criteria

In order to be able to study the biological behavior of the endocervical dysplastic cells, the morphological criteria had first to be established. The minimum histological criteria for dysplasia were applied, knowing the definite morphological definitions of the normal and malignant EC[15,17].

According to many different histological descriptions of the dysplastic EC found in literature[12–14] and in our personal experience based on observation, we established well-defined criteria for dysplasia. The most obvious abnormal changes are nuclear. The nuclei are slightly enlarged, elongated and often cigar -shaped, with some variations in size and shape. Hyperchromasia is usually a prominent feature. Mitotic figures are absent or very rare. The epithelium may also show the loss of polarity, so that the nuclei are haphazardly arranged in relation to the basement membrane and the luminal surface. These changes are clearly different from the single, basal, small, uniform nucleus of normal EC or from the enlarged and elongated nuclei with frequent mitosis and prominent pseudostratification.

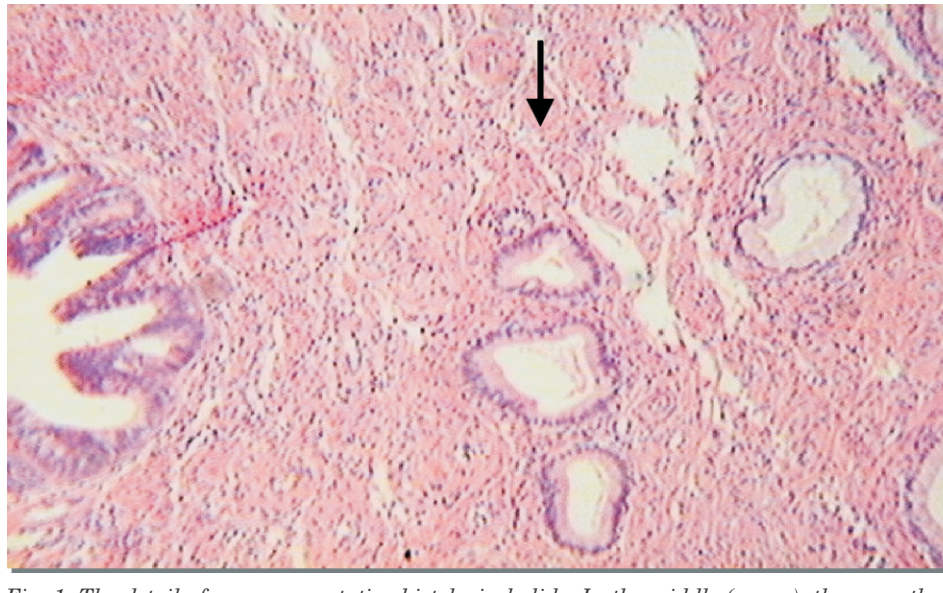

Fig. 1. The detail of one representative histological slide. In the middle (arrow), there are three glands with the dysplastic EC between the normal endocervical glands on the right and the malignant endocervical epithelium of AIS on the left.

Collecting samples for flow cytometry analysis

In order to determine the ploidy status and proliferative activity of the normal, dysplastic and malignant EC from the chosen samples with diagnosed AIS or invasive ACA, a different marker encircled the representative areas on each chosen slide. Another section from paraffin block was cut in order to get similar slide. From that unstained slide, the foci at the matching location with marked areas on H&E stained slide were microdissected in three pieces to get normal, dysplastic and malignant cell populations separately and then prepared for the flow cytometry analysis.

For the purpose of identifying the ploidy status and S-phase standard of the normal EC, a control group of 20 tissue samples with morphologically normal EC from the patients undergoing hysterectomy for uterine leiomyoma was analyzed.

The control tissues with the normal EC, as well as the microdissected tissues with the normal, dysplastic, and malignant endocervical epithelium from the representative samples, were separately processed for flow cytometry performed by FACSCalibur (Becton Dickinson, San Jose, CA, SAD) using the computer programs –Cellquest™ and ModFit LT™ and the method described by Leers et al[29].

The flow cytometer measured the DNA content (ploidy and S-phase) of each disaggregated nucleus in the cell suspension and plotted results as a histogram showing relation between DNA content (x-axis) and total cell count (y-axis). All histograms that have only one peak represent the DNA diploid population.

The definition of the DNA aneuploid cell populations requires the presence of two distinct peaks in the histogram[23]. The results of the ploidy status of the normal EC from the control group are shown in Table 1.

The assessment of the S-phase fraction of the dysplastic and malignant EC was possible after we determined the standard S-phase of the normal endocervical epithelium in the control cervical tissues. The percentage of the normal EC ranged from 0.48% to 3.42% (Table 1). According to this S-phase standard of normal cervical tissue, all studied dysplastic and malignant EC were divided into two groups: the first group of cells which S-phase fraction was lower than 3.42% (S_1) and considered normal, and the second group of cells, which S-phase fraction was higher than 3.42% and considered to have higher mitotic activity (S_2).

Immunohistochemical staining

From the same paraffin blocks with chosen samples, several more 3 μm sections were cut for immunohistochemical staining with the anti-c-myc monoclonal antibodies (Serotec, Oxford, UK). To detect the reaction product the immunohistochemical staining used the avidin-biotin-peroxidase complex technique with 3.3'-diaminobenzidene as the chromogene. Anti-c-myc antibody was used at 1:50 dilution after antigen retrieval in EDTA at pH 8.0, and microwaved for 15 minutes at room temperature.

Because the different cellular localization of c-myc product was first noticed and well documented in normal colon epithelium, adenomas and adenocarcinomas of the colon, the samples of the colon carcinoma associated with an accumulation of cytoplasmic c-myc oncoprotein were used as positive control[27,28]. The normal mucosal cells adjacent to the colon carcinoma showed no or only nuclear staining (data not shown).

For the negative control the same procedure was used, but for the omission of the primary antibody.

The positive c-myc oncoprotein overexpression in studied EC was considered when diffuse brown cytoplasmic staining

TABLE 1
RESULTS OF FLOW CYTOMETRY AND IMMUNOHISTOCHEMISTRY ANALYSIS ON 20 CONTROL CERVIX TISSUE SAMPLES CONTAINING NORMAL ENDOCERVICAL EPITHELIUM

No. of patients	Flow cytometry		Immunohistochemistry: c-myc oncoprotein
	Ploidy	S-phase (%)	
1.	Diploid	2.87	–
2.	Diploid	0.48	–
3.	Diploid	1.96	–
4.	Diploid	1.63	–
5.	Diploid	3.42	+
6.	Diploid	1.28	–
7.	Diploid	1.33	–
8.	Diploid	2.27	–
9.	Diploid	1.58	–
10.	Diploid	2.05	–
11.	Diploid	1.87	–
12.	Diploid	1.07	–
13.	Diploid	2.64	–
14.	Diploid	2.15	–
15.	Diploid	1.97	–
16.	Diploid	2.58	–
17.	Diploid	1.94	–
18.	Diploid	1.05	–
19.	Diploid	0.91	–
20.	Diploid	2.53	–

was seen (Figure 2). Table 1 also shows the results of the anti-c-myc antibody immunohistochemical staining in the control group with the morphologically and clinically normal cervical tissues.

Statistical analysis

χ^2 test was performed using a computerized statistical package (SPSS 7.0 for Windows) to determine whether an association between the dysplastic and malignant EC exists. The level of significance was defined as $p<0.05$.

Results

Table 1 shows the flow cytometry and the immunohistochemistry data for the control group, enabling us to compare the results of the flow cytometry analysis of normal, dysplastic and malignant EC from studied tissues (Table 2).

Ploidy status

All 20-control samples with normal EC were diploid.

In the experimental samples (N=49), almost all normal EC were diploid (98%), whereas one third of dysplastic EC were aneuploid.

In the majority of cases, the malignant EC were characterized by aneuploidy (59.2%), but the rest were diploid despite malignant morphological features.

χ^2-test showed a significant association between the malignant EC and aneuploidy (χ^2=6.944; p=0.008; Table 3).

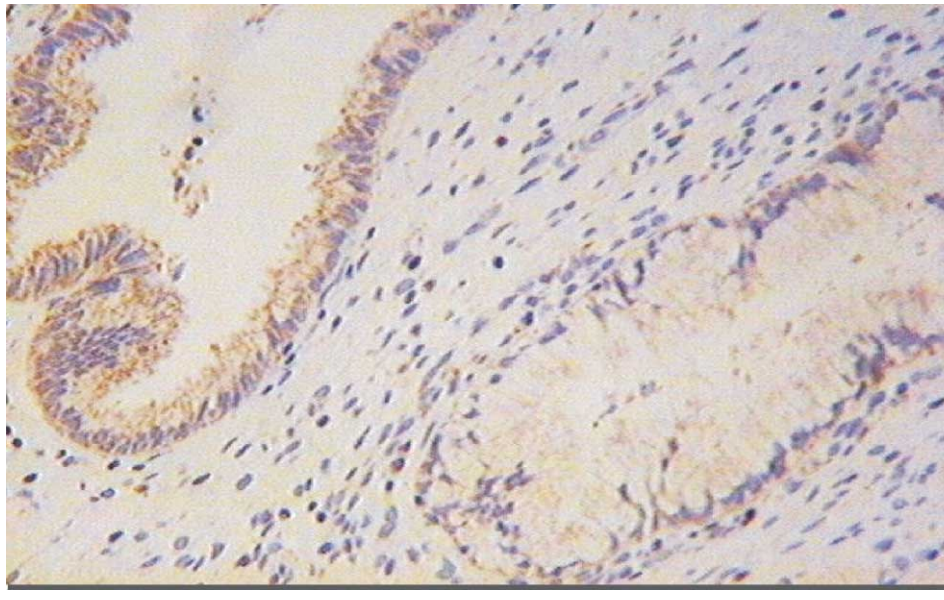

Fig. 2. Immunohistochemical demonstration of c-myc oncogene product in morphologically different EC. C-myc oncoprotein positivity is obvious in the cytoplasm of the endocervical gland with morphologically clear dysplastic EC (upper left corner). In morphologically normal EC of the gland located in the down right corner of the pictur c-myc oncoprotein expression is lacking.

TABLE 2
RESULTS OF FLOW CYTOMETRY AND IMMUNOHISTOCHEMISTRY ANALYSIS OF 49 EXPERIMENTAL CERVICAL SAMPLES CONTAINING NORMAL, DYSPLASTIC AND MALIGNANT ENDOCERVICAL EPITHELIUM

No. of patients	Ploidity*		S-phase*		C-myc***		
	Dysplastic	Malignant	Dysplastic	Malignant	Normal	Dysplastic	Malignant
1.	diploid	diploid	normal	normal	negative	positive	negative
2.	aneuploid	aneuploid	high	high	negative	positive	negative
3.	diploid	diploid	normal	high	negative	positive	positive
4.	diploid	diploid	normal	normal	negative	negative	positive
5.	aneuploid	aneuploid	high	high	positive	positive	positive
6.	aneuploid	aneuploid	normal	high	negative	negative	negative
7.	aneuploid	aneuploid	high	high	negative	negative	negative
8.	aneuploid	aneuploid	high	high	negative	positive	negative
9.	aneuploid	aneuploid	normal	high	negative	negative	negative
10.	aneuploid	aneuploid	normal	high	negative	negative	negative
11.	diploid	aneuploid	high	high	positive	positive	positive
12.	aneuploid	aneuploid	high	high	negative	positive	positive
13.	aneuploid	aneuploid	high	normal	negative	positive	positive
14.	diploid	diploid	normal	normal	negative	positive	positive

TABLE 2 Continued

No. of patients	Ploidity*		S-phase*		C-myc***		
	Dysplastic	Malignant	Dysplastic	Malignant	Normal	Dysplastic	Malignant
15.	aneuploid	aneuploid	high	high	positive	positive	negative
16.	aneuploid	aneuploid	normal	normal	negative	positive	positive
17.	diploid	diploid	normal	normal	negative	positive	negative
18.	diploid	aneuploid	high	high	negative	positive	positive
19.	diploid	diploid	normal	normal	negative	positive	positive
20.	diploid	diploid	normal	normal	negative	negative	positive
21.	diploid	diploid	normal	normal	negative	negative	positive
22.	diploid	aneuploid	normal	high	negative	positive	positive
23.	diploid	aneuploid	normal	high	negative	negative	negative
24.	diploid	diploid	normal	normal	negative	positive	positive
25.	diploid	aneuploid	normal	normal	negative	positive	positive
26.	aneuploid	aneuploid	high	normal	negative	positive	negative
27.	diploid	diploid	normal	normal	negative	positive	positive
28.	diploid	diploid	normal	high	negative	positive	negative
29.	aneuploid	aneuploid	normal	high	negative	positive	positive
30.	diploid	aneuploid	normal	high	negative	negative	positive
31.	diploid	diploid	normal	normal	negative	positive	positive
32.	diploid	diploid	normal	normal	negative	positive	negative
33.	diploid	diploid	normal	normal	negative	positive	positive
34.	diploid	aneuploid	high	high	negative	positive	positive
35.	aneuploid	aneuploid	high	high	negative	positive	negative
36.	diploid	diploid	normal	normal	negative	positive	positive
37.	diploid	aneuploid	normal	high	negative	positive	negative
38.	aneuploid	aneuploid	high	high	positive	positive	positive
39.	diploid	diploid	normal	high	negative	positive	positive
40.	diploid	aneuploid	normal	high	negative	positive	positive
41.	diploid	aneuploid	normal	normal	negative	positive	positive
42.	diploid	diploid	normal	normal	negative	negative	negative
43.	diploid	aneuploid	high	high	positive	positive	negative
44.	diploid	aneuploid	high	high	negative	positive	negative
45.	diploid	diploid	normal	normal	negative	positive	negative
46.	aneuploid	aneuploid	high	high	negative	positive	positive
47.	diploid	diploid	normal	normal	negative	positive	positive
48.	diploid	aneuploid	high	high	negative	positive	negative
49.	diploid	diploid	normal	normal	negative	positive	positive

* Ploidy status was assessed by flow cytometry of dysplastic and malignant EC epithelium;
** S-phase of dysplastic and malignant EC epithelium was normalizedto control group and considered normal ($1 \leq 3.42\%$) or high ($> 3.42\%$);
*** c-myc oncoprotein expression in normal, dysplastic and malignant EC cells

TABLE 3
PLOIDY OBTAINED BY FLOW CYTOMETRY ANALYSIS IN THE DYSPLASTIC AND MALIGNANT ENDOCERVICAL EPITHELIUM FROM 49 PATIENTS WITH AIS OR INVASIVE ACA OF THE CERVIX

EC cells	Diploid	Aneuploid	Total
Dysplastic	33	16	49
Malignant	20	29	49
Total	53	45	98

$\chi^2 = 6.944$; p=0.0084

S-phase fraction

Among the dysplastic EC, 65.3% displayed normal and 34.7% high proliferative activity. The malignant EC of AIS or ACA had 44.9% samples with normal and 55.1% with high mitotic activity.

χ^2-test showed a significant association between the malignant EC and higher proliferative activity (χ^2=4.125; p=0.042; Table 4).

TABLE 4
THE S-PHASE DATA OBTAINED BY THE FLOW CYTOMETRY ANALYSIS IN THE DYSPLASTIC AND MALIGNANT ENDOCERVICAL EPITHELIUM FROM 49 PATIENTS WITH AIS OR INVASIVE ACA OF THE CERVIX

EC cells	S^1	S^2	Total
Dysplastic	32	17	49
Malignant	22	27	49
Total	54	44	98

$\chi^2 = 4.124$; p=0.0423

As one third of dysplastic cells were aneuploid and had a high mitotic activity it may be expected they have a malignant potential.

Expression of the c-myc oncogene

Only one sample of the normal endocervical epithelium from the control group showed a mild positive cytoplasmic c-myc oncoprotein expression.

In the glands with the normal endocervical epithelium from the experimental cervical tissue, the c-myc oncoprotein was expressed in only 10.2% samples. The proportion of the c-myc positive cells was increased in the morphologically changed EC. In the endocervical glands with the morphologically obvious dysplastic changes c-myc oncoprotein expression was positive in 79.6% samples whereas only 59.2% of malignant EC samples were positive.

χ^2-test showed a significant association between the dysplastic endocervical cells and the expression of the c-myc oncoprotein (χ^2=4.804; p=0.028; Table 5).

TABLE 5
CYTOPLASMIC C-MYC EXPRESSION DETECTED BY IMMUNOHISTOCHEMISTRY IN DYSPLASTIC AND MALIGNANT ENDOCERVICAL EPITHELIUM FROM 49 PATIENTS WITH AIS OR INVASIVE ACA OF THE CERVIX

EC cells	C-myc negative	C-myc positive	Total
Dysplastic	10	39	49
Malignant	20	29	49
Total	30	68	98

$\chi^2 = 4.803$; p=0.0284

As the c-myc oncoprotein is the marker of neoplastic transformation of the cell, its cytoplasmic expression confirms the malignant potential of dysplastic EC.

Discussion

Frequent histological findings of dysplastic endocervical glands in the border areas between the normal and malignant glands awakened our suspicions as to their pre-malignant potential. The main problem was that the dysplastic endocervical epithelium forms a wide histological spectrum between the AIS and the normal endocervical epithelium and we, on the other hand, had no standard morphological definitions applicable to such a histological spectrum.

According to present knowledge, the biological behavior of the dysplastic cells is unpredictable, because some of them may be included in potentially reversible processes, while the others may progress to AIS[15].

The existence of the aneuploid nuclear content, a higher mitotic activity, and the overexpression of cytoplasmatic c-myc oncoprotein among the spectrum of the endocervical dysplastic cells, helped us to discover the biological nature of some of them. The results of our study show that one third of the studied dysplastic population has a DNA aneuploidy and a higher S-phase, the well-documented and statistically significant markers of malignant cells[15–19]. These data, obtained by the flow cytometric analysis, support the hypothesis about the existence of the precancerous lesions to AIS. Although we used standardized procedures to prepare and analyze the control and representative samples, as well as to analyze and interpret the cytometry data, we must stress that the flow cytometry technique has an important limitation. It consists in the inability to detect loss of 3–5% of the DNA content[23]. That is the reason that certain number of aneuploid cells is counted automatically as diploid. Thus, even among our malignant cell populations with a definite malignant transformation, almost over 40% of them were diploid and have a normal mitotic activity. Therefore, the measurement of the DNA content by means of flow cytometry provides support for the hypothesis regarding the existence of the precursor lesions to AIS, but the clinical utility for their recognition is not satisfactory.

Many studies showed that the overexpression of the c-myc oncoprotein was involved in the initial phase of the malignant transformation, when the cell has no chromosomal aberrations yet, but shows very subtle or none morphological changes[18,19]. Immunohistochemical methods on the histological samples that simultaneously showed the spectrum of the endocervical changes ranging from the normal to dysplastic and malignant cells were used in order to determine whether the differences in expression of that oncoprotein exist in cells of different morphology. Our results show that almost 80% of the dysplastic EC had the cytoplasmic c-myc oncoprotein expression and definitively confirm the hypothesis of their malignant significance. It is possible that, the rest of the morphologically dysplastic cells without the c-myc expression have reactive changes without the neoplastic potential. Our results also support the hypothesis about importance of the c-myc oncogene activation in carcinogenesis. Ten percent of the morphologically normal EC and almost 60% of the morphologically malignant cells (with lower expression than in the dysplastic cells) support the hypothesis that the c-myc expression is important as one of the switches that turn the normal cell into the pre-malignant one, while afterwards, its importance diminishes[19].

Our data showed that some endocervical dysplastic cells have an aneuploid DNA content and a high S-phase, as well as cytoplasmatic expression of the c-myc oncoprotein, supporting the role of the EC dysplasia in the evolution of AIS.

But since the flow cytometry and immunohistochemical methods have some limitations in detection the malignant potential of the cell, additional, more accurate molecular techniques should be used.

Our findings that a pathologist can not morphologically distinguish the dysplastic endocervical epithelium with malignant potential from the reactive, reversible dysplastic epithelium, emphasize the clinical importance of the detection of c-myc oncoprotein in the cell cytoplasm using the immunohistochemical method.

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MALIGNI POTENCIJAL DISPLASTIČNOG ENDOCERVIKALNOG EPITELA U ODNOSU NA STANJE PLOIDIJE, FRAKCIJOM S-FAZE I EKSPRESIJOM C-MYC

SAŽETAK

Cilj ove studije bio je odrediti imaju li displastične endocervikalne stanice (ES) neoplastični potencijal kao prekursorne lezije za adenokarcinoma in situ (AIS). Maligni potencijal određen je procjenom stanja ploidije i proliferativne aktivnost putem citometrije te c-myc ekspresije imunohistokemijski. Ispitivani parametri procijenjeni su

zasebno u morfološki normalnim, displastičnim te malignim endocervikalnim stanicama. χ^2 test je pokazao značajnu povezanost malignih EC s aneuploidijom (p=0,008) i visokom proliferativnom aktivnosti (p=0,042). Kako je jedna trećina displastičnih EC također aneuploidna i pokazuje visoku mitotsku aktivnost, one vjerojatno također imaju maligni potencijal. Displastične EC su pokazale značaju povezanost s c-myc onkogenom ekspresijom (p=0,028). Prikazani rezultati upućuju na postojanje pre-malignih glandularnih lezija, dok imunohistokemijska detekcija c-myc protoonkogena može biti od pomoći u detekciji EC s malignim potencijalom i to onda kada displastične morfološke promjene nisu prisutne.