Cyclins - a New Marker to Assess Severity of Cervical Lesions

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Abstract

Objective: *The purpose of this study is to highlight the presence of cyclins in cell cultures (CulCel) from cervical biopsies with varying degrees of lesions, using immunofluorescence with anticycline monoclonal antibodies (AcMcAC).* ***Material and methods:*** *The study included 68 samples from cases with cervical lesions (cervicitis, ASC-H, H-SIL, cervical cancer). Cultivation of explants and use of keratinocyte restraining media led to epithelial cell cultures free of contamination with fibroblasts. The obtained material was incubated with anticycline monoclonal antibodies (A, B, D1, D2, D3, E). The technique applied was indirect immunofluorescence using secondary antibodies. The interpretation of analyzed issues was based on observational criteria and was performed by three specialists in molecular and cellular biology. To provide a correlation between parameters, statistical analysis was performed using t-Student test.* ***Outcomes:*** *Cyclin A was identically expressed in the cytoplasm of the four categories of lesions, but presented significant differences in the nuclei: HPV negative cervicitis in 29% of cells, HPV positive HSIL in 42% of cells, HPV negative carcinomas in 62% of cells, HPV positive carcinomas in 71% of cells. Cyclin B in non-invasive lesions shows a moderate reaction in the dividing cells; in the presence of HPV, the response occurs in 6% of the cells in interphase. In HPV negative carcinoma, intense reaction occurs in the dividing cells and moderate reaction occurs in 21% of the interphase cells; in HPV positive carcinoma, the reaction is intense in the dividing cells and in 28% of the interphase cells. Cyclins D and E showed no significant differences between the categories of cases examined. Positive statistical correlations were observed between cyclin level in nuclei and cytoplasm, in cells with HPV negative ASC-H lesions ($r = 0.75$, $p = 0.05$), HPV positive HSIL ($r = 0.77$, $p = 0.05$), HPV negative CC - ($r = 0.78$, $p = 0.04$) and HPV positive CC ($r = 0.42$, $p = 0.01$).* ***Conclusions:*** *The presence of cyclin A in over 42% of CulCel and of cyclin B in over 21% of CulCel demonstrates their involvement as cell cycle regulatory markers in supporting the diagnosis of high grade cervical lesions/cancer.*

Keywords: *culture cells, cyclins, immunoflourescence, anticycline-antibodies*

Objective

The aim of this study is to draw up a model of fundamental research in cellular and molecular biology targeting the identification of progression markers of precancerous lesions of the cervix. To achieve this objective, the research consisted in obtaining stable epithelial cell cultures from biopsies performed in various degrees of cervical lesions. Subsequently, by initiating immunofluorescence techniques with anticycline monoclonal antibodies, different types of cyclins were identified in cultures from epithelial lesion. The research aims to discover to what extent some cyclins have the capacity of marker in aggravated and neoplasic cervical lesions. The paper addresses a new issue in the same line as the current research programs that seek to obtain new methods of diagnosis of precancerous and cancerous cervical lesions. Cyclins identification by immunofluorescence from epithelial cell culture is a laborious research activity carried out for the first time in Romania.

Material and Method

This study was conducted within the partnership between the Department of Obstetrics and Gynecology of "St. Pantelimon" Emergency Clinical Hospital and the Department of Molecular and Cellular Biology of Bucharest "Carol Davila" University of Medicine under PNII National Research Program (2007-2013) - Partnerships in priority fields, Research area: Biotechnology.

The methodology used in this study is based on the characteristics of basic research. Research was conducted on a group of 68 cases with abnormal cervical pathology, investigated in terms of detection and genotype of HPV, cases that required cervical biopsy and histopathology exam of the piece taken. Only 43 biological samples which corresponded to storage and processing standards for cell cultures could be obtained from these cases.

Harvesting was done with biopsy punch, from several colposcopically guided areas. Samples were carried in transport medium (Modified Eagle's Medium Dulbeco 2% antibiotic), with ice, in temperature isolated bags. Biological specimens processed to obtain cell cultures came from cases classified as follows: chronic cervicitis with HPV negative (7 cases), HSIL with positive HPV types 16 and 18 (22 cases), HPV negative carcinoma (three cases,) positive HPV carcinoma (types 16 and 18) (11 cases). The research was conducted during several successive steps. The first step was to obtain a primary cell culture by cultivating explants (small fragments of tissue) from biopsies.

The second phase of research consisted in applying a new protocol to obtain a stable cell culture, durable and made up of a greater number of cells. Keratinocytes restraining media were used as the method for cultivation. In the third stage, immunofluorescence techniques with monoclonal anticycline antibodies were carried out on all cell cultures, at passage 2. Step 4 of the study was performed in order to obtain the most accurate results possible in terms of cyclin immunofluorescence on keratinocytes. To achieve this goal, cytokeratin expression (marker of epithelial cells) was followed up to establish with certainty the epithelial origin of culture cells.

Immunofluorescence procedure used anti-pan Cytokeratin antibodies which recognize highly conserved sequences present in all types of cytokeratins to determine the epithelial nature of CulCel. The material obtained was incubated with anticyclin monoclonal antibodies (A, B, D1, D2, D3, E).

The technique used was indirect immunofluorescence (Dako Citomation, Glostrup, Denmark) with primary antibodies Santa Cruz Biotechnology California, USA on 1:50 dilution. Anti-rabbit secondary antibody, namely anti-mouse, goat produced, coupled with Alexa Fluor 488 or Fluor Alexa 555 (Invitrogen, USA) was used.

Nuclei highlighting was performed using DAPI dye (Sigma Chemical, St. Louis, MO). The examination of 43 cell cultures from cervical biopsies with varying degrees of lesions was performed with Nikon TE300 microscope equipped with Nikon DX1 image acquisition system, using 40x, 60x and 100x PlanApo Nikon lens. The interpretation was made by comparing image acquisition parameters of cultures incubated with fluorescently labeled primary antibodies to those without incubation with primary antibodies.

Aperture and sensitivity of the capture device remained fixed in all cases, only exposure times varied. The longest period of exposure was established as that when no residual fluorescence occurs in the negative control sections. The exposure time of preparations incubated with primary antibodies was not higher than the exposure time previously established for the experiment negative control.

There were 500 cells (from each epithelial culture obtained from various cervical lesions) fluorescently labeled for different types of cyclins. The counting was performed by three researchers specialized in cellular and molecular biology, and the final result was an average of the three counting procedures. To provide the correlation between parameters, statistical analysis was performed using Student's-t test, "paired samples for means" ("one-group two-Tails") variant. T-Student test is "robust" in terms of statistical analysis of a limited number of cases (n <30) and therefore the values obtained are good enough to draw a valid conclusion. Data were statistically analyzed using the Analysis Tool Pack for Microsoft Excel 2003 under Windows XP Professional. A predictive value $p < 0.05$ was considered statistically significant.

Results and discussions

Cell cultures derived from explants were unstable. These cultures were characterized by rapid decline over 7 to 10 days due to unidentified factors. Our research findings are in line with the data published in the international literature that indicate the same problem[1]. Initiating a new protocol for obtaining a time resisting cell culture and with a large number of cells using keratinocyte restraining media allowed us to obtain epithelial cell cultures not contaminated with fibroblasts[2]. Using this method allowed obtaining a high rate of reproducibility of epithelial cell cultures not contaminated with fibroblasts[3]. These cultures are an in vitro model for cyclins' expression. It was observed in this research that cultures infected with HPV have a difficult growth. It was also noted that these cultures are lost after 3-4 passages in comparison with HPV free cell cultures which persist over 6-7 passages[2]. The biotechnological innovation consisted in removal of this inconvenience by applying the immunofluorescence technique with monoclonal anticyclin antibodies on all cell cultures earlier, i.e. in passage 2. Using cells in a small passage prevents the possibility of radical change in culture cells when going through a number of passages[4] Lambert PF, et al 2005. It was noted that there are cells which no longer divide and remain isolated and cells that tend to rapidly divide, forming nests. The latter will be the niche for development of cell cultures throughout future passages. The passes are made when the cells reach a confluence of 80-100%. It was also found that in explant type cell cultures, in which small fragments of epithelium were dissected from biopsy specimens and grown with special keratinocyte restrai-

ning media, their efficiency in generating epithelial cells used in the following passages was lower than in cell cultures made by enzyme dissociation.

Immunofluorescence technique with anti-pan Cytokeratin antibodies allowed to establish the epithelial nature of CulCel. Differentiation of fibroblast-like cells and epithelial cells by testing the cytokeratin expression on allowed the obtaining, within the second passage cells, of epithelial origin cells in a percentage of over 95%. Percentage of poor cell cultures has been minimized by obtaining high quality cell cultures, further to the use of keratinocyte restraining media, a method which led to the non-occurrence of unstable cell cultures characterized by rapid decline after 7-10 days[2]. The analysis of the cell cultures obtained, incubated with fluorescently labeled anticyclin antibodies, showed the following aspects:

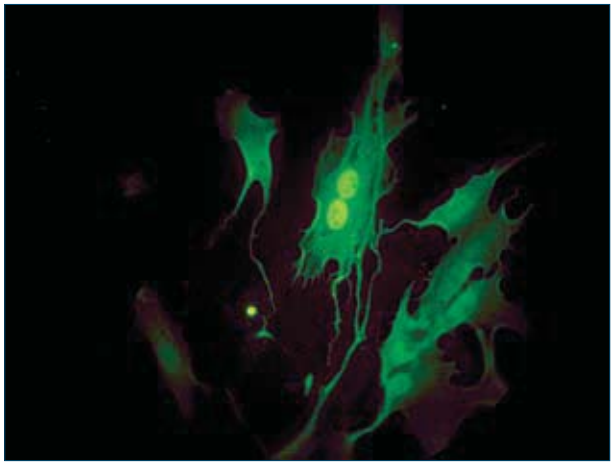


Figure 1. Expression of cyclin D1 in epithelial cell culture from bioptic fragments with HPV infected cervical neoplastic lesion

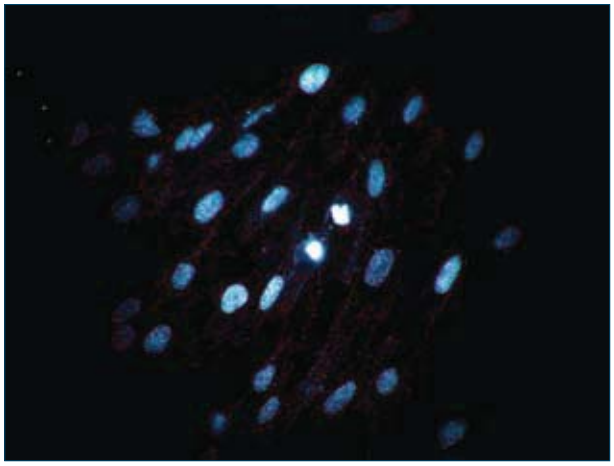


Figure 2. Immunofluorescence: expression of cyclin D3 in epithelial cell culture from bioptic fragments with cervical neoplastic lesion with no demonstrated HPV infection; ob. 60X nuclei (DAPI)

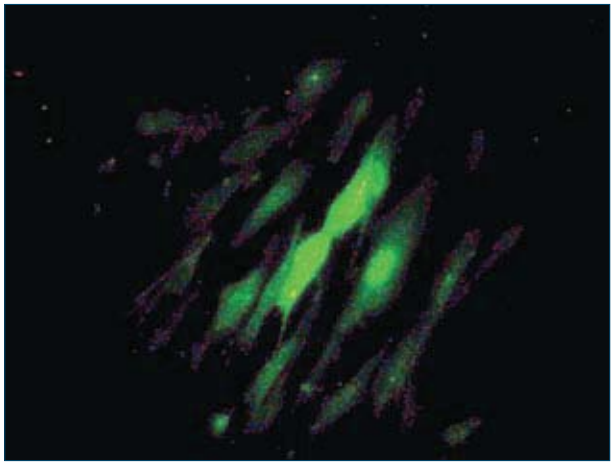


Figure 3. Immunofluorescence: expression of cyclin D3 in epithelial cell culture from bioptic fragments with cervical neoplastic lesion with no demonstrated HPV infection; ob. 60X

Cyclins D are the first to enter the cell cycle, namely G1 phase[10,11,12,14].

Cyclin D1 in cervical lesions with ASC-H cytology shows a weak positive reaction in the cytoplasm of 475 cells of 500 counted, which accounts for 95% of cell culture. The reaction in the nucleus was weakly positive in 105 cells and intense in 75 cells, which accounts for 36% of cell culture. The counting in HSIL lesions showed identical results as in ASC-H. The same result was revealed in HPV negative cervical carcinoma. Instead, in HPV positive cervical carcinoma, if the cytoplasm weak positive reaction has been maintained in 95% of cells, the nucleus has revealed a weak positive reaction in 90 of 500 counted cells and an intense reaction in 110 cells, which means the presence of the D1 cyclin in 40% of cell culture (figure 1).

Cyclin D3 in ASC-H and HSIL cervical lesions presented a weak reaction in the dividing cells. In HPV positive and negative cervical carcinoma, the reaction was revealed as weak positive in the cytoplasm of 500 counted cells and as moderate in the nucleus of 30 cells, which accounts for 6% of the cell culture (figures 2 and 3).

Cyclin E, which regulates transition from G1 phase to S phase presented significant changes in relation to severity of injury. In ASC-H lesions, 460 cells which account for 92% of cell culture showed a diffuse positive reaction with perinuclear emphasis. Reaction is intense in the dividing cells. In HSIL lesions, 445 cells which account for 89% of cell cultures have a diffuse positive reaction with perinuclear emphasis; the reaction is intense in the dividing cells. In HPV negative carcinoma, 475 cell, i.e. 95% of the cell culture, had a positive reaction with perinuclear emphasis; the reaction is intense in the dividing cells. The same results were found in the epithelial cultures from HPV positive cervical carcinoma (figures 4 and 5).

Cyclin A starts its activity in G1 phase of the cell cycle but reaches its activity peak in G2 phase. In ASC-H lesions, cyclin A has a weak positive reaction in the cytoplasm of the 435 cells, which accounts for 87% of cell culture, and the reaction in the nucleus is moderate in 145 cells, i.e. 29% of cell culture. In HSIL lesions, the reaction is weakly positive in the cytoplasm of 460 cells, i.e. 92% of cell culture. The reaction in the nucleus is moderate in 210 cells, which accounts for 42% of cell culture. In HPV negative carcinoma, the cytoplasmic reaction remains weak in 460 cells,

i.e. in 92% of cells, but in the nucleus, the reaction has a moderate intensity in 360 cells, i.e. in 64% of cell culture. In HPV positive carcinoma, the weak cytoplasmic reaction stands out in 480 cells, i.e. in 96% of cell culture. Moderate reaction of the nucleus occurs in 355 cells, i.e. in 71% of cell culture (figure 6).

Cyclin B is essential for the transition from G2 phase into M phase[16].This cyclin gradually accumulates in G2 phase and is abruptly destroyed as the cell exits from mitosis. In ASC-H lesions, cells have a moderate positive reaction in the dividing cells. In HSIL lesions, the moderate positive reaction in the dividing cells maintains, but also appears in interphase cells (about 6% of cell culture). In HPV negative carcinoma, an intense reaction was noted in the dividing cells and in 105 of the 500 counted cells, i.e. 21% of cell culture, whereas a moderate reaction was noted in the cytoplasm of interphase cells. In HPV positive carcinoma, the intense reaction in the cytoplasm of dividing cells maintains and a moderate reaction occurs in the cytoplasm of interphase cells (in 140 cells, i.e. 28% of the cell culture)(figures 7 and 8).

The distribution of cyclin immunofluorescence reaction in cytoplasm and nuclei of cells from different cervical lesions is illustrated in graphs 1-3.

Positive statistical correlations were observed between the cyclin level of nuclei and the cytoplasm of cells with ASC-H HPV negative lesions ($r = 0.75$, $p = 0.05$), HSIL - HPV positive ($r = 0.77$, $p = 0.05$), CC - HPV negative ($r = 0.78$, $p = 0.04$) and CC - HPV positive ($r = 0.42$, $p = 0.01$) significant.

Discussion

The results of our research reveal that cell cultures obtained by growing explants of cervical biopsies are characterized by instability (decline in 6-7 days). These results are identical to those reported in the literature [1]. As regards cell cultures, two major lines of action were identified. The growing can be achieved with regular medium (DMEM - SFB10%), but it is necessary to use a "feeder layer" system of fibroblastic nature. This methodology is not applicable in this research that aims to investigate cyclins and other parameters in the cultures' development, without detecting the degree of participation of the "feeder layer" substrate in the expression/alteration of a particular parameter.

The current research shows that the optimal cell cultivation method is the method using keratinocyte restraining media.

The cells obtained from the culture have a different morphology also depending on the type of lesion. If cells cultured from the cervix free of lesions and from the cervix with minimal lesions show a classic and somewhat uniform epithelial morphology, those cultured from invasive cancers may have a distinct morphology. Although some morphological characteristics of these cells are similar to fibroblasts, the probability to select cells, other than the epithelial ones, is minimal when using restraining media[2].

The role of cyclins in cell cycle can be summarized as follows[5,6,13]:

- G1-phase cyclins: D and E;
- S-phase cyclins: A;
- M-phase cyclins: B.

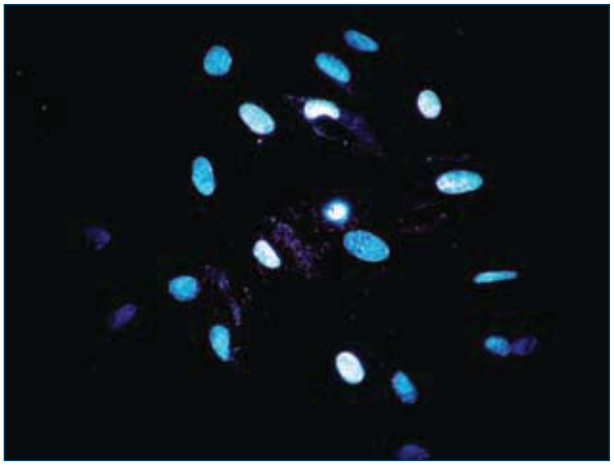


Figure 4. Immunofluorescence: expression of cyclin E in epithelial cell culture from bioptic fragments with cervical neoplastic lesion with no demonstrated HPV infection; ob. 60X nuclei (DAPI)

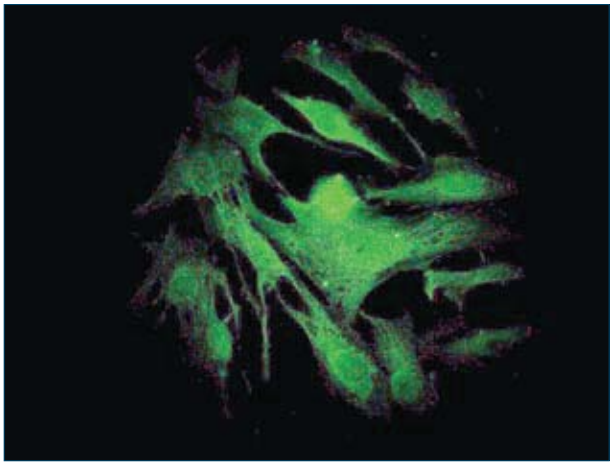


Figure 5. Immunofluorescence: expression of cyclin E in epithelial cell culture from bioptic fragments with cervical neoplastic lesion with no demonstrated HPV infection; ob. 60X

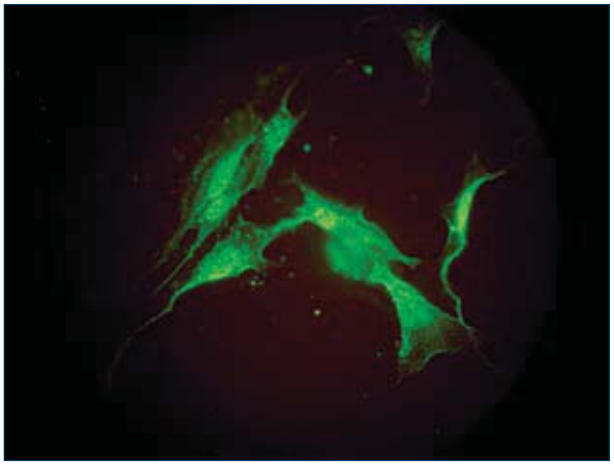


Figure 6. Immunofluorescence: expression of cyclin A in epithelial cell culture from bioptic fragments with cervical neoplastic lesion with no demonstrated HPV infection; ob. 60X

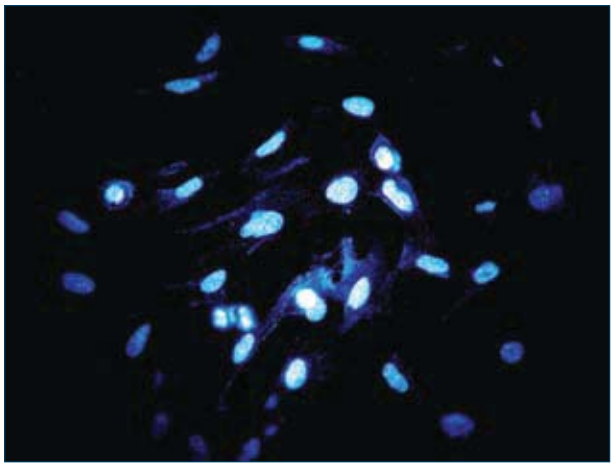


Figure 7. Immunofluorescence: expression of cyclin B1 in epithelial cell culture from bioptic fragments with cervical neoplastic lesion with no demonstrated HPV infection; ob. 60X nuclei (DAPI)

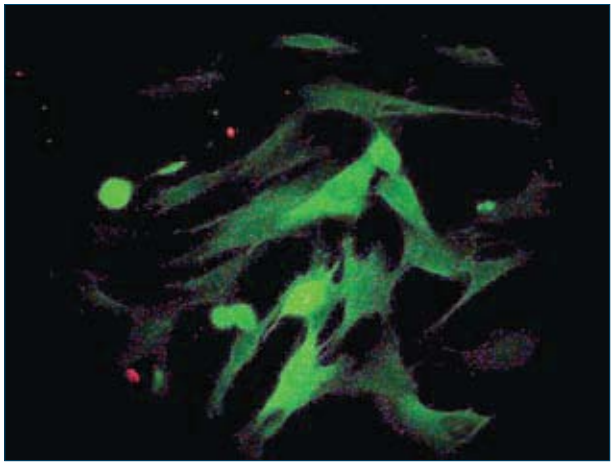
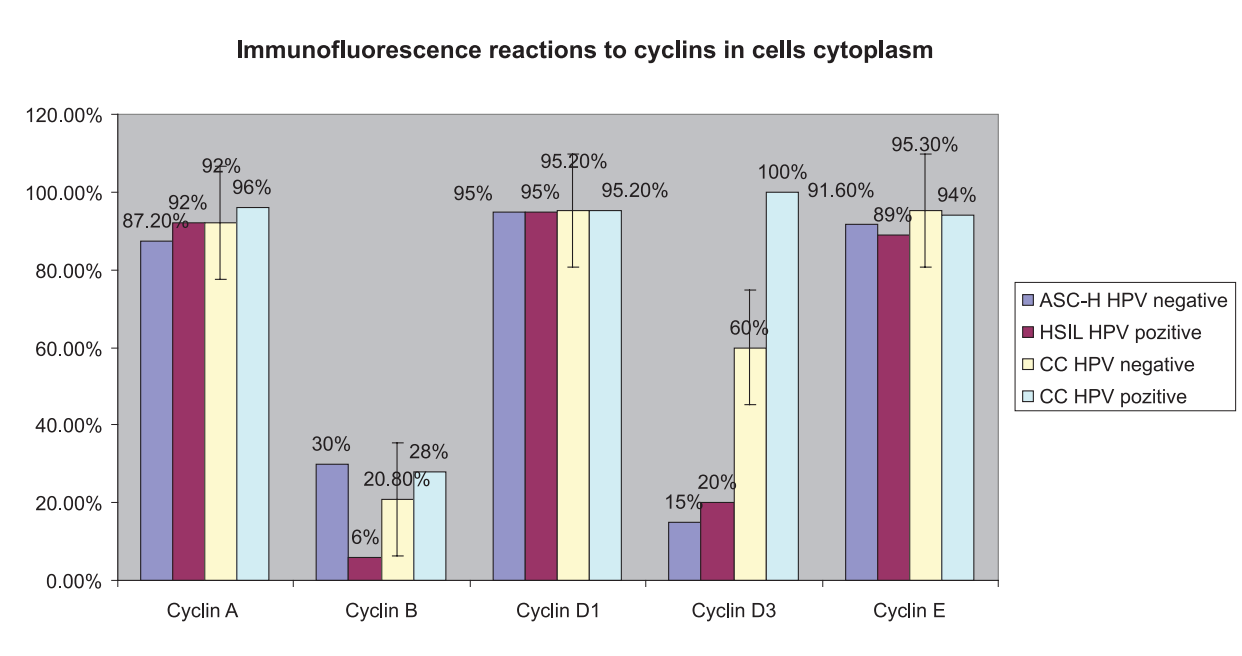


Figure 8. Immunofluorescence: expression of cyclin B1 in epithelial cell culture from bioptic fragments with cervical neoplastic lesion with no demonstrated HPV infection; ob. 60X

Cyclins are named this way because their concentration varies periodically during the cell cycle. Cyclins are produced or degraded as needed so as to drive the cell through various stages of the cell cycle. They were discovered in 1982 by R. Timothy Hunt while studying the cell cycle. Cyclin forms Cyclin/Cdk complexes[9,10]. The formed complex leads to activation of Cdk active site. Tim Hunt is a Nobel Laureate in Physiology or Medicine (2001), a distinction awarded for highlighting cyclins in the cell cycle. When cyclin cell concentration is low, the cyclin dissociates from Cdk, due to a Cdk protein chain that blocks the active site leading to the dissociation of Cyclin / Cdk complexes. The independent cyclins do not have enzymatic activity. The binding of cyclins by protein-dependent kinase as p34 (cdc2) leads to the formation of maturation promoter factor (MPFs) which is involved in the phosphorylation of other proteins responsible for formation of microtubules and chromatin remodeling during division. Cyclins contain two domains, i.e. N-terminus and C-terminus. There are several types of cyclins that work in different parts of the cell cycle and cause Cdk to phosphorylate several substrates[8]. Cyclins D act in early G1 phase cell and facilitate the cell transition to S phase[11,12,14]. Cyclins D/CDK6 and D/CDK4 lead to activation of cyclin E and then cyclin A. Cyclins D and E push the cells from G0 phase into G1 phase of cell cycle. Cyclins E and A affect the S phase and cyclins A and B control the mitosis. Cell cycle progression from G1 phase to S phase depends on pRb phosphorylation by the complexes formed by cyclins D and E along with cyclin-dependent kinases[13]. The role of cyclin D in the determinism of aggravated dysplasia and cervical cancer is not clear

Chart 1. Immunofluorescence reaction to cyclins in cell cytoplasm



yet. Some studies show low levels of cyclin D in H-SIL lesions and ISCC compared with normal cervical epithelium and HPV negative lesions. The expression of cyclins depends on HPV subtype[7,8,15]. Some authors show cyclin D overexpression in invasive cancer while other authors showed a low importance of cyclins in CIN lesions and squamous cell carcinoma[5]. Cyclin A controls S phase of cell cycle [Masson M, et al., -2003] and therefore the synthesis of nuclear DNA.

Cyclins B are synthesized in the early S1 phase and induce the formation of MRF promoter factor. Cyclin B is associated with p34cd complex in G2 phase for-

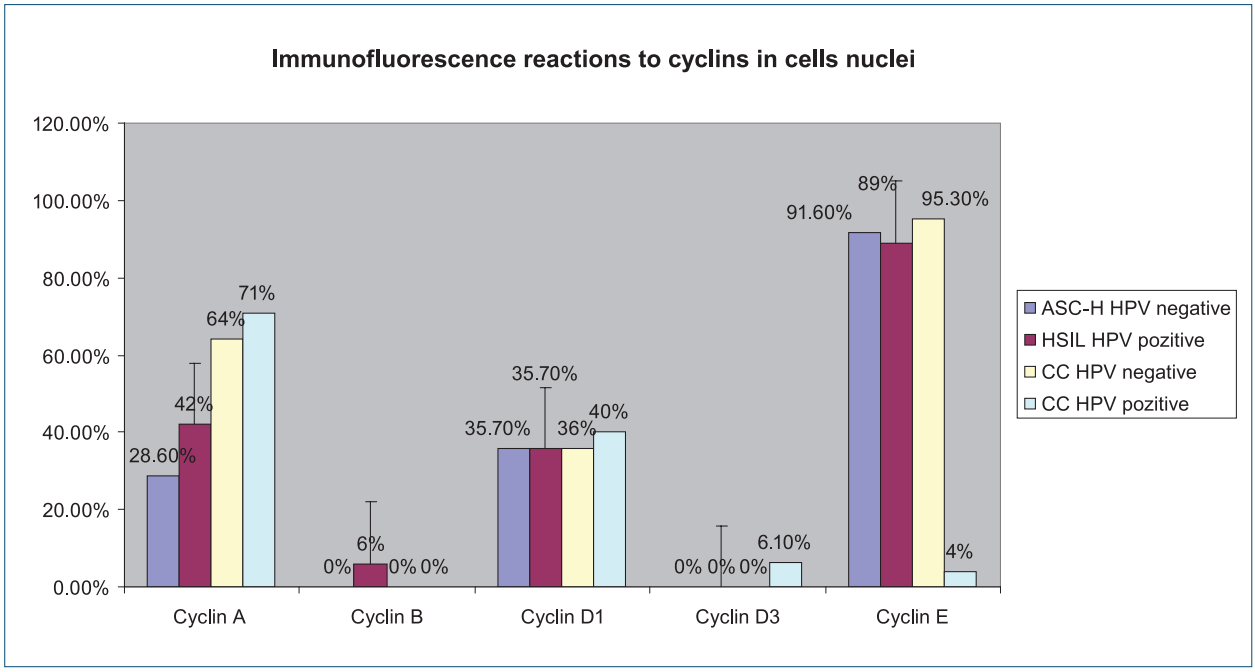


Chart 2. Immunofluorescence reaction to cyclins in cell nuclei

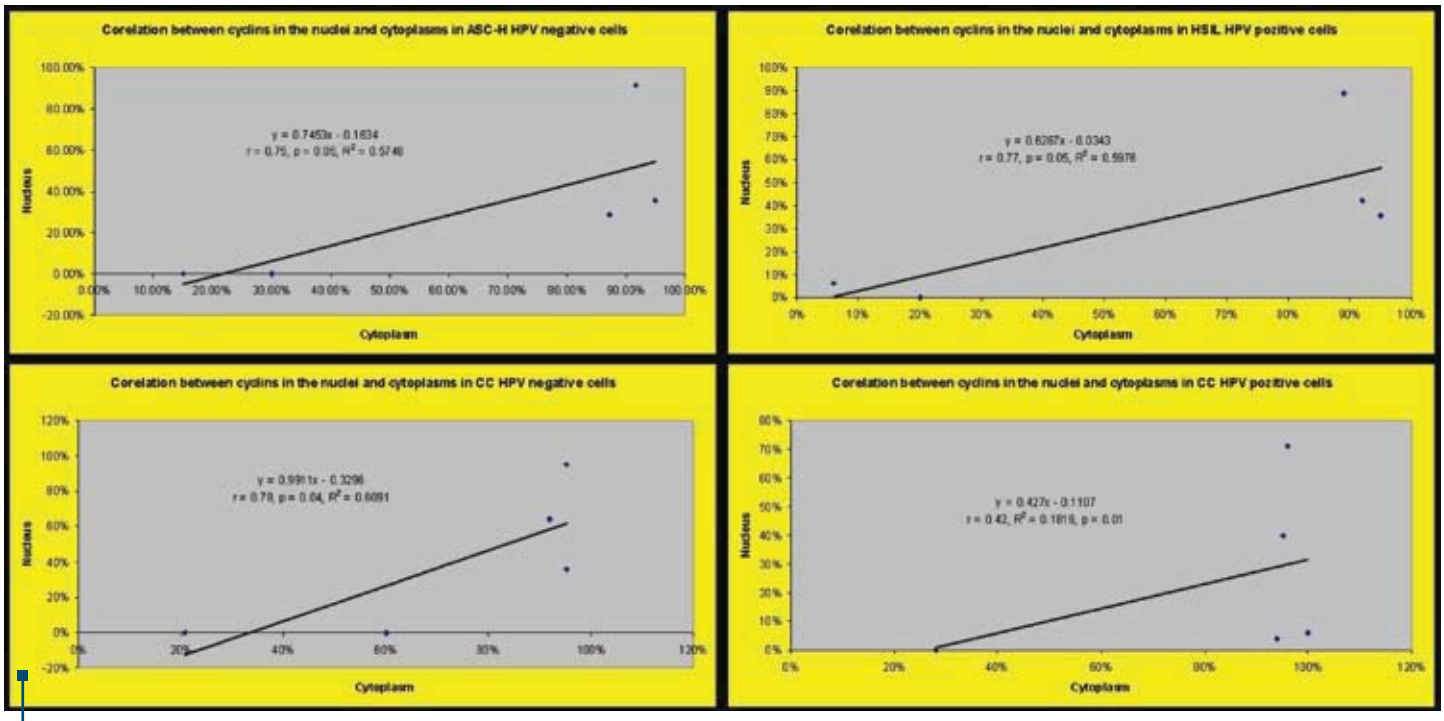


Chart 3. Corelation between cyclins in cytoplasma and nuclei of ASC-H and CC lesions in HPV positive and negativ cells

ming pre MRF. Analysis of cyclins B in cervical lesions shows an increase of complex cyclin B-p34cdc2 in high-risk HPV H-SIL lesions[15].

The results obtained by immunofluorescence on cell cultures in terms of pattern of epithelial expression of cyclins D, E, A and B demonstrates the existence of cytoplasmic and nuclear intensity variations between the investigated lesions. Cases of cancer lesions and HPV infection are distinguished by the high intensity of cyclin B in the nucleus, predominantly in the dividing cells.

Conclusions

The analysis of this cyclin intensity in epithelial cell cultures from biopsies performed on cases with different degrees of cervical lesions allowed the finding of observations that may have medical application both in diagnosis and in therapy. Highlighting by immunofluorescence, with an emphasis on S phase and M phase cyclins, respectively cyclin A and B, in severe cervical lesions can justify the ability of these cyclins as markers for the diagnosis of high-grade cervical lesions/cancers. Just like p16ink14 marker, cyclins A and B could be determined in samples of cervical cells collected in liquid medium. Immunocytochemical determination of cyclins complements the immunocytochemical investigations to determine p16ink4a marker in order to differentiate cervical lesions in terms of evolution to cervical intraepithelial neoplasia/cancer[17,18,19]. This research provides data relevant for the pharmaceutical industry that could initiate research on the discovery of specific anti-cyclin drugs, drugs that, if applied locally, could block the evolution of cell cycle progression to mitosis, thus stopping the evolution to cervical cancer. ■

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