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Immunofluorescence expression of Ki-67, p53 and cyclin inhibitors ($p16^{ink4a}$, p21 and p27) in low-grade cervical lesions *versus* high-grade cervical lesions. Research study on cell cultures

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Abstract

Objective: The aim of this research was to assess the immunofluorescence expression (IFE) of cell cycle regulators $p16^{ink4a}$, p21, p27, in association with proliferation and prognosis factors Ki-67, p53 respectively, in cell cultures, obtained from different types of cervical intra-epithelial lesions. The final purpose was to distinguish a best marker able to identify with high accuracy the high-grade squamous intra-epithelial lesion. *Materials and Methods*: The study was carried out on 68 epithelial cell cultures. Three senior specialists have analyzed 500 cells/case individually. The statistic analysis for correlation between used markers has been performed. *Results*: The study batch revealed a very low expression of investigated parameters (<1%) in negative cases for malignancy and intraepithelial lesion (NMIL), a progressive exponential expression in low-grade squamous intraepithelial lesion (LSIL), and a very high expression in high-grade squamous intra-epithelial lesion (HSIL) and invasive squamous cervical carcinoma (ISCC). Ki-67 and p53 were overexpressed in nuclei both in LSIL and HSIL. A slightly direct correlation between p21 and Ki-67 (r=0.35, p<0.001) was observed in HSIL. Statistically significant correlations were noticed between some markers: $p16^{ink4a}$ and p27 (r=0.4, p=0.03), $p16^{ink4a}$ and Ki-67 (r=-0.4, p=0.002). *Conclusions*: The most reliable parameters for assessing HSIL and ISCC proved to be Ki-67 and $p16^{ink4a}$. Both were with percentages and intensity of IFE around 100% and higher immunoexpression within the nucleus of cell cultures. Our study reveals that p27 cyclin inhibitor was not reliable in differentiating between LSIL and HSIL.

Keywords: immunofluorescence expression, cell cycle regulators, cyclin inhibitors, cell culture, high-grade squamous intra-epithelial lesion, low-grade squamous intraepithelial lesion.

Introduction

Human high-risk papillomaviruses (hr-HPVs) are the most important factors, which in situation to act within the nucleus of the cervical cells develop cervical carcinogenesis process. The relationship between E6 and E7 oncogene proteins of hr-HPVs and the components of the cell cycle switches the normal regulatory mechanism of cells to a proliferative process that leads to carcinogenesis [1]. The initiation of neoplastic transformation processes begins when the human papillomavirus (HPV) does exist within the nuclei of infected epithelial cells [2]. According to Zu Hausen, mentioned by Knebel MD (2009), this fact distinguishes between transient and transforming HPV infection during the carcinogenesis process [3].

The regulation of cell cycle progression is done by proteins, which are classified in the next five classes: cyclins, cyclin-dependent protein kinases (CDKs), proteins belonging to the Rb family, cyclin-dependent protein kinase activating kinases (CAKs) and cyclin-dependent protein kinase inhibitors (CKIs) [4].

The evolution of the cell cycle starts with the joint between cyclins and CDKs and is followed up by the phosphorylation of the Rb protein by specific cyclins. The CAKs and CKIs are responsible to regulate the cyclin/CDK complexes. The cell cycle progression is blocked by a family of proteins – CKIs –, which has the property to bind to specific CDKs. As regards the CKIs, these are classified into two subclasses as follows: kip/Cip and INK4. The kip/Cip class includes p21, p27, and p57 cyclin inhibitors while INK4 class contains p15, $p16^{ink4a}$, p18 and p19 proteins [3].

The mechanism by which human papillomaviruses (HPVs) interferes with normal cell cycle is represented

by the binding of the E6 oncoprotein of hr-HPV (most probably 16 or 18 types) to the p53, respectively the binding of E7 to pRb tumor suppressor proteins [5, 6]. In this manner, a proliferative process is initiated increasing the number of abnormal cells that lead to cervical cancer. The CDKs and CDKIs are both involved in the up and down regulation of the normal cell cycle.

The E6 oncoprotein binds p53 and causes the down regulation of the cell cycle. P53 acts as a tumor suppressor protein and has the capacity to induce the expression of p21 [7]. The blockage of the p53 functions leads to inactivity of p21–CDKI [8].

The overexpression of $p16^{ink4a}$–CDKI is related to the negative feedback induced by this in the situation when E7 hr-HPV oncoproteins bind pRb [9]. This condition disturbs the functional activity of pRb. The $p16^{ink4a}$ down regulates the cell cycle by inhibiting the creation of complexes between cyclin D1 and CDK4 or CDK6 [10]; so, the phosphorylation of pRb is blocked. The $p16^{ink4a}$–CDKI is a very important regulator of G0–G1 transition [11]. This role becomes ineffective in the presence of E7, which is competitive with E2F factor for the same site of pRB. The binding of E7 to pRb blocks the activity of CDKI [12]. In this manner, the release of E2F from pRB allows continuing the mechanism of DNA replication and consequently cells proliferation [13].

The purpose of this study was to characterize the expression of $p16^{ink4a}$, p21, p27, p53 and Ki-67 in precancerous and cancerous cervical lesions by using the immunofluorescence technique in cell cultures from cases with different types of cervical lesions. We performed immunofluorescence analysis of p53, Ki-67 and p16, p21, p27 cyclin inhibitors aiming to characterize the immunofluorescence pattern of aforementioned cell cycle regulators and to report their relationship with the histological degrees of cervical lesion.

We decided to carry out this study on cell cultures setting up as purpose to enhance the field of immunofluorescence expression of investigated factors inside the abnormal cells, both in nucleus and cytoplasm with a deeper analysis of the expression of abovementioned regulator factors. The main results of our study serve to improve the knowledge useful for oncological drugs discovery and to ensure the choice of the most appropriate marker for detection of high-grade cervical lesions.

Materials and Methods

The present study was conducted by the Obstetrics and Gynecology Department from "St. Pantelimon" Emergency Clinical Hospital, Bucharest, Romania, in partnership with Department of Molecular and Cellular Biology and Department of Pathology from "Carol Davila" University of Medicine and Pharmacy, Bucharest, during two years, from November 1, 2008 until October 31, 2010. The funds for the study were supported from a National Research Grant concerning the early detection of cervical cancer lesions. The study followed the ethical recommendations of the Research Department of "Carol Davila" University of Medicine and Pharmacy, Bucharest. All necessary investigations related to this research (with the goal of finding new markers able to early detect precancerous cervical lesions) have been performed by getting both the patients' agreement and that of the Ethic Committee of "St. Pantelimon" Hospital, Bucharest.

The research study is based on 80 cases of women aged between 25 to 65 years submitted for investigations with the purpose of detecting early pre-cancerous or cancerous cervical lesions. All these 80 cases were investigated by Pap test (Bethesda system reported), Human Papillomavirus Test, colposcopy, histopathological diagnosis (Hematoxylin–Eosin staining) of the cervical biopsies and all accepted to collected small pieces of cervical tissue for cell cultures in research purpose. We deem necessary to emphasize that our research should just stand for the starting point of a large and complex experimental activity. One of the biotechnologies, which we decided to explore, was immunofluorescence assay on epithelial cervical cell culture as regards the immunoexpression of cell cycle regulators such as $p16^{ink4a}$, p21, p27, p53 and Ki-67 cyclins. Our research was accomplished in more significant steps. As starting point, we selected cases with abnormal cytologies at Pap test, colposcopic lesions who had indication for biopsy diagnosis and HPV test done. For a clear understanding of our complex work, we mention that the pieces picked up by cervical biopsies have been further used for many other research investigations. The sample pieces were selected from lesions suspected at colposcopic image; the measure of the cervix biopsy tissue was at least minimum 2 mm diameter for one piece, from each quarter portion of the cervix. If the pieces of biopsies for other investigations – such as histopathological and immunohistopathological diagnosis – were put in boxes with formalin, the pieces for the present research were immediately shipped to laboratory in a transport medium (Modified Eagle's Medium Dulbecco 2% with antibiotic contain) in thermally isolated bags. Therefore, we got more information about the same case.

Thus, the performed investigations on the same subject as regards the present study have been: Pap test, colposcopy, human papillomavirus test, biopsy for histopathological diagnosis, cell culture of cervical pieces with immunofluorescence expression (IF) for cyclins aforementioned.

We obtained a primary cell culture by cultivating explants (small fragments of tissue) picked up by biopsies under colposcopic view of suspected sites of abnormal lesions. The second step was allocated to those necessary procedures concerning the achievement of a stable and durable cell culture, made up of a greater number of cells. Keratinocytes restriction medium was used as the method for cultivation. In the third step, immunofluorescence techniques with monoclonal anti-cyclins inhibitors antibodies were carried out on all cell cultures, at passage 2. Step 4 was performed in order to gain accurate data related to cyclin inhibitors proteins immunofluorescence expression on epithelial cells in different types of cervical lesions, from benign cases to invasive cancer. To achieve this goal, we focused our attention to cytokeratin expression (marker of epithelial cells). This step was done with the purpose of establishing with accuracy the epithelial origin of culture cells. The use of anti-pancytokeratins antibodies allowed us to recognize highly conserved sequences present in all types of cytokeratins to determine the epithelial nature of CulCel [14]. The

material obtained was incubated with monoclonal primary antibodies achieved in rabbit both for anti-cyclins inhibitors proteins (p16^{ink4a}, p21, p27), and for anti-p53, anti-Ki-67 too. The technique used was indirect immunofluorescence (DakoCytomation, Glostrup, Denmark) with primary antibodies (Santa Cruz Biotechnology California, USA) on 1:50 dilution. Anti-rabbit secondary antibody were used, goat produced, coupled with Alexa Fluor 488 or Fluor Alexa 555 (Invitrogen, USA). Nuclei highlighting was performed using DAPI dye (Sigma Chemical, St. Louis, MO, USA). The examination of cell cultures prepared from cervical biopsies with varying degrees of lesions was performed by Nikon TE300 microscope equipped with Nikon DX1 image acquisition system, 40×, 60× and 100× PlanApo Nikon objectives. The interpretation was made by comparing image acquisition parameters of cultures incubated with primary antibodies fluorescently labeled to those without incubation with primary antibodies. Aperture and sensitivity of the capture device remained fixed in all cases varying only exposure times. The longest period of exposure was established as that to which no residual fluorescence occurs in the negative control sections (Figure 1).

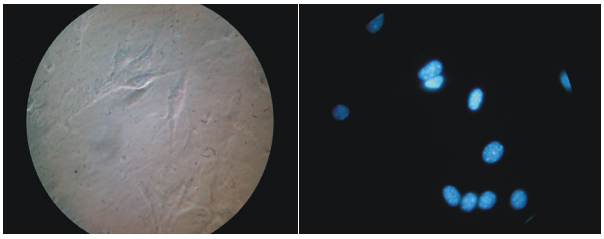

Figure 1 – ***Suggestive image for the control of the experiment study. The background of the immunofluorescence assay is black under the lack of binding by the antibody; at right: the coloration with DAPI of the nucleus of the cells.***

Exposure time of preparations incubated with primary antibodies was not higher than that previously established for negative control experiment (Table 1).

Table 1 – ***Standardized protocol for cervical epithelium culture***

Standardized protocol for cervical epithelium culture. Department of Molecular and Cellular Biology, "Carol Davila" University of Medicine and Pharmacy, Bucharest		
Steps	***Primary culture***	***The subculture***
		It is done after 5–6 days after inoculation, when the formed colonies have 50–75 cells/colony.
1.	The biopsy piece must be removed from its transportation device. It must be washed two or three times, with 5 mL sterilized PBS, with 50 µg/mL Gentamicin + 5 µg/mL Amphotericin.	The medium is aspirated.
2.	The piece is placed with the epithelium facing down into a Petri plate.	The plate is washed two times with PBS.
3.	With a scalpel (blade no. 22), it must be scratched as much as possible the muscular tissue and the stroma, leaving only a thin piece of epithelial tissue.	On each plate, it is added Trypsin solution, enough to cover the plate. Afterwards, it is incubated at 37^{0}C until it is detached.
4.	The epithelial tissue is grinded with a pair scissors.	It is transferred into a spin. The plate is washed with culture medium and it is added over the cells suspension.
5.	It must be added 10 mL of Trypsin solution preheated at 37^{0}C. Afterwards it must be transferred into a 50 mL spin by using a dropper. The spin must contain a sterilized tube for the magnetic agitator.	For 5 minutes, it is centrifuged at 250 g.
6.	Further, another 5–10 mL of Trypsin solution are added.	The supernatant is aspired and it is resuspended in complete medium KSM.
7.	It must be incubated at 37^{0}C, for 30–40 minutes – mild agitation.	After being counted inside a counting chamber, the plates are inoculated at a density of 12×10^4 cells/cm^2.
8.	The suspension must be left for 2–3 minutes for the decantation process.	–
9.	The suspension must be moved through a sieve that has 40 µm, then it is transferred into a 50 mL spin.	–
10.	It must be added 10 mL complete medium with 10% fetal serum.	–
11.	The cells must be centrifuged at 250 g for 5 minutes.	–
12.	The cells are resuspended in 10 mL PBS.	–
13.	The cells must be centrifuged at 250 g for 5 minutes.	–
14.	The cells are resuspended in 10 mL PBS.	–
15.	The cells must be centrifuged at 250 g for 5 minutes.	–
16.	Afterwards it must be added 10 mL complete medium KSM.	–
17.	After being counted inside a counting chamber, the plates are inoculated at a density of 12×10^4 cells/cm^2.	–

The quantification of IF expression in nucleus and cytoplasm of epithelial cell cultures was done in conformity with data exposed below. The working methodology included both computing the presence percentages of IF expression within the nucleus and cytoplasm of the 500 cells investigated for each cell culture obtained from each case enrolled as well as evaluating the intensity of IF inside the morphologic structure of examined cells. In order to quantify the percentages of IF expression on epithelial cell cultures, we introduced, by similarity, a score alike with the well-known one used by Eleutério J Jr *et al.* (2007) in immunohistochemistry diagnosis [15]. Thereby, in our study, the immunofluorescence score for cyclin inhibitors p16^{ink4a}, p21, p27, p53 and proliferative marker Ki-67 was appreciated as:

- Score 1 – absent: <1% of cytoplasmatic and nuclear positivity PIF*;
- Score 2 – sporadic: 1–10% of cytoplasmatic and nuclear positivity PIF*;

▪ Score 3 – moderate: 10–30% of intense cytoplasmatic and nuclear positivity PIF*;

▪ Score 4 – diffuse >30% of intense cytoplasmatic and nuclear positivity PIF*;

▪ Score 5 – over immunofluorescent express: 100% of nuclear and cytoplasmatic positivity PIF*.

(PIF* – Percentage of immunofluorescence expression).

As regards the intensity of cell immunofluorescence cyclins expression, it was quantified from '1+' to '4+'; a grade '1+' was assigned to diffuse and low intensity of immunofluorescence expression, a grade '2+' to medium positive immunofluorescence intensity, a grade '3+' and grade '4+' to high, and strong intensity respectively, in order of ascending strength of immunofluorescence expression intensity.

Counting was done by three researchers: one senior specialist in molecular cell biology, and two senior specialists in pathology field. The result was an average of these three counters.

We correlated our research results data about cyclins IF expression on epithelial culture cells of cervical lesions with HPV results test, cytologic and histopathological (HE staining) diagnosis results recorded in the information system of our Research Grant – Department of Obstetrics and Gynecology, "St. Pantelimon" Hospital (Programme Partnerships in Priority Domains; Biotechnologies; National Centre of Management Programme – Research Programme Folder No. 3368/61–44/2007–2010). The HPV test was performed in Laboratories of Hyperclinic MedLife, Bucharest – grant agreement – by Linear Array HPV Genotyping methodology.

We notice that the present work used a methodology which relying on the characteristics of basic research focused on the highlight of cyclins' activity expressed by immunofluorescence on cell epithelial cervix culture. The recorded data allowed us to perform a more profound analysis with clinical connection possibility.

Descriptive statistics was used for uniform distributed data of the study batch for mean, median and standard error. For correlation between parameters, statistical analysis has been done using the Student's *t*-test, "paired two samples for mean" variant, one-group two tails, from the Analysis Tool Pack of Microsoft Excel 2007, running under Windows Vista. The Pearson correlation index shows the existence of a linear relation between variables for $r \geq \pm 0.4$, assuming equal variances among symmetrical data. A value of $p<0.05$ was considered statistically significant.

Results

From 80 cases enrolled, we obtained only 68 cell cultures since a part of them was unsuitable for immunofluorescence technique.

According to investigated parameters, the above-mentioned cases of the present research are belonging to the following classification: negative for malignancy and intraepithelial lesion (NMIL) (n=15) [22%], positive for low-grade intraepithelial lesion (LSIL), respectively CIN1 (n=31) [45%], and high-grade squamous intraepithelial lesion (HSIL) [32%], respectively CIN2+ [CIN2, CIN3 (n=14) (21%), and invasive cervical cancer (ICC) n=8 (12%]). As regards the presence of the HPV infection, the cases were negative at HPV test for all NMIL cases, while all LSIL, HSIL and ICC cases investigated were positive for high-risk (hr)-HPV test (see Tables 2 and 3, which are depicting the classification in a synthetic manner).

Table 2 – ***Immunofluorescence expression of p16, p21, p27, p53 and Ki-67 in nucleus and cytoplasm of cell cultures of cervical lesions – percentage and intensity of immunofluorescence expression***

Cyclins	Lesions							
	Cytologic and histopathologic diagnosis Negative for malignity HPV results test				*Cytologic and histopathologic diagnosis Positive for malignity HPV results test*			
	NMIL hr-HPV negative n=15 22%		*LSIL–CIN1 hr-HPV positive n=31 45%*		*HSIL–CIN2 HSIL–CIN3 hr-HPV positive n=14 21%*		*HSIL–ICC hr-HPV positive n=8 12%*	
	N	C	N	C	N	C	N	C
P16	0.4%	0.8%	27% 3+	100% 1+; d 3+; pfc	100% 3+	100% 1+; d 3+; pfc	100% 3+	100% 1+; d
P21	0.2%	0.8%	5% 3+	100% 3+; pnf	83% 1+ 17% 3+	100% 1+; d	75% 1+ 25% 3+	100% 1+; d
P27	0.2%	0.8%	100% 3+	100% 1+; d 3+; pnf	100% 3+; inf 1+; d	100% 3+; pnf 1+; d	100% 3+	100% 1+; d 3+; pnf
P53	0.7%	0.9%	100% 3+	100% 2+; pnf 1+; pfc	10% 4+ 90% 1+	100% 1+; d	15% 4+ 85% 1+	100% 2+; pnf 3+; pnf 1+; d
Ki-67	0.8%	0.9%	31% 4+ 58% 2+	100% 1+; d	93% 4+	100% 1+; d	100% 4+	100% 1+

Abbreviations: NMIL – Negative for malignancy and intraepithelial lesion; LSIL – Low grade intraepithelial lesion; HSIL CIN2, CIN3 – High-grade squamous intraepithelial lesion; ICC – Invasive cervical cancer; hr-HPV – High-risk human papillomavirus; N – Nucleus; C – Cytoplasm; n – No. of cases; 1+ – Low intensity; 2+ – Medium intensity; 3+ – High intensity; 4+ – Very high intensity (overexpressed); pnf – Perinuclear focus; inf – Intranuclear focus; pfc – Peripheral cytoplasmic focus; d – Diffuse expression.

Table 3 – ***Score value of the expression of regulator cell cycle: p16^{ink4a}, p21, p27, p53 and proliferative marker Ki-67 – immunofluorescence assay in nucleus and cytoplasm of cell cultures of cervical lesions in cell cultures***

Cyclins	Lesions							
	Cytologic and histopathologic diagnosis Negative for malignity HPV results test				*Cytologic and histopathologic diagnosis Positive for malignity HPV results test*			
	NMIL hr-HPV negative n=15 22%		*LSIL–CIN1 hr-HPV positive n=31 45%*		*HSIL–CIN2 HSIL–CIN3 hr-HPV positive n=14 21%*		*HSIL–ICC hr-HPV positive n=8 12%*	
	Score		Score		Score		Score	
	N	C	N	C	N	C	N	C
P16	1	1	3	5	5	5	5	5
P21	1	1	2	5	4 2	5	4 5	5
P27	1	1	5	5	5	5	5	5
P53	1	1	5	5	2 4	5	2 4	5
Ki-67	1	1	3 4	5	4	5	5	5

Abbreviations: NMIL – Negative for malignancy and intraepithelial lesion; LSIL – Low grade intraepithelial lesion; HSIL CIN2, CIN3 – High-grade squamous intraepithelial lesion; ICC – Invasive cervical cancer; hr-HPV – High-risk human papillomavirus; N – Nucleus; C – Cytoplasm; n – No. of cases; Score 1 – absent: <1% of cytoplasmatic and nuclear positivity PIF*; Score 2 – sporadic: 1–10% of cytoplasmatic and nuclear positivity PIF*; Score 3 – moderate: 10–30% of intense cytoplasmatic and nuclear positivity PIF*; Score 4 – diffuse: >30% of intense cytoplasmatic and nuclear positivity PIF*; Score 5 – immunofluorescent overexpression: 100% of nuclear and cytoplasmatic positivity; PIF* – Percentage of immunofluorescence expression.

The observational criteria used for the analysis of the cyclins inhibitors immunofluorescence expression p16^{ink4a}, p21, p27 associated to p53 and proliferative marker Ki-67 allowed to distinguish some particular features concerning these investigated regulators of the cell cycle. The variability of immunofluorescence expression of the investigated cell cycle regulators inside different degrees of cervical infected HPV lesions is shown in the Tables 2 and 3.

Among investigated cell cycle regulators immunofluorescence expression of Ki-67, followed up p53 is overexpressed to 100% percentage in nucleus both in LSIL–CIN1 and HSIL–CIN2+ too. For the same cervical lesions, the intensity of immunofluorescence expression of Ki-67 and p53 in nucleus was identified with high-level 3+ or 4+ (Figures 2–6).

In cytoplasm, the percentage of cells with immunofluorescence expression for cell cycle regulators p16^{ink4a}, p21, p27, p53 and proliferative marker Ki-67 is very high but with lower intensity such as 1+, or rarely 2+ (Figures 7–10). Only p53 immunofluorescence image is expressed at nucleus bound with 3+ intensity in cell cultures from HSIL and ICC cases.

As regards the p16^{ink4a} immunofluorescence expression, this is strongly expressed within the nucleus in cell cultures obtained from HSIL–CIN2+ in contrast to LSIL–CIN (Figures 11 and 12). On the other hand, the immunofluorescence expression of p16^{ink4a} in cytoplasm has a high percentage in the cell cultures investigated but with low and diffused intensity.

The high immunofluorescence expression of p16^{ink4a} in nucleus of the high-grade intraepithelial lesions allows considering p16^{ink4a} a very good marker for detecting these types of cervical lesions.

The results of our research shown that p27 could identify the existence of the lesion inside cervical tissue but without the possibility to discriminate between low- or high-grade cervical lesions.

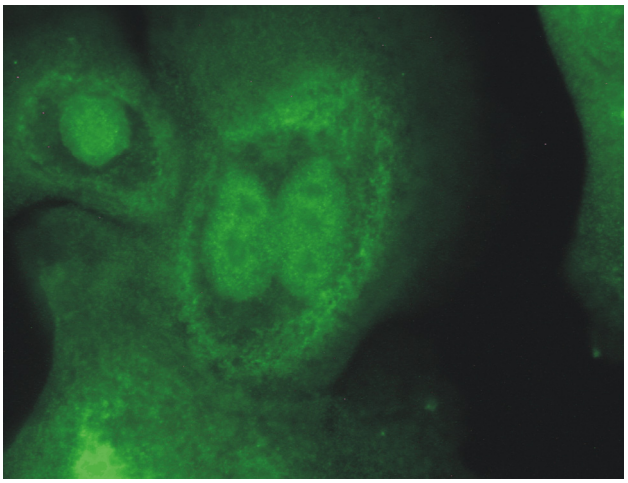

Figure 2 – ***Immunofluorescence expression of p53 in cell cultures of LSIL, ob. 100×.***

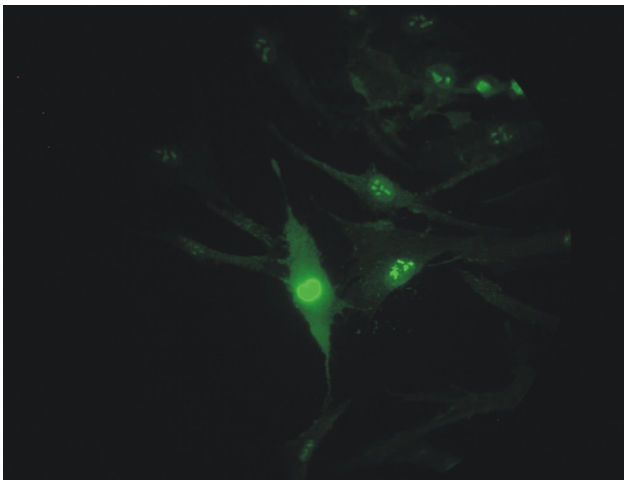

Figure 3 – ***Immunofluorescence expression of p53 in cell cultures obtained from cervical explants from a case with cervical cancer, ob. 60×.***

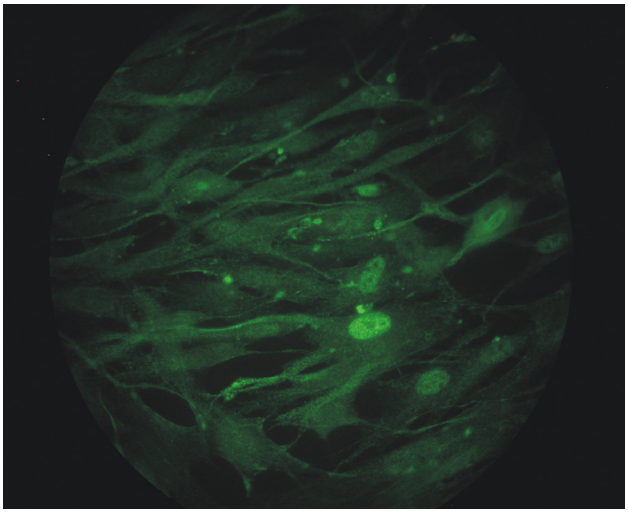

Figure 4 – ***Immunofluorescence expression of p53 in cell cultures obtained from cervical explants from a case with cervical cancer, ob. 60×.***

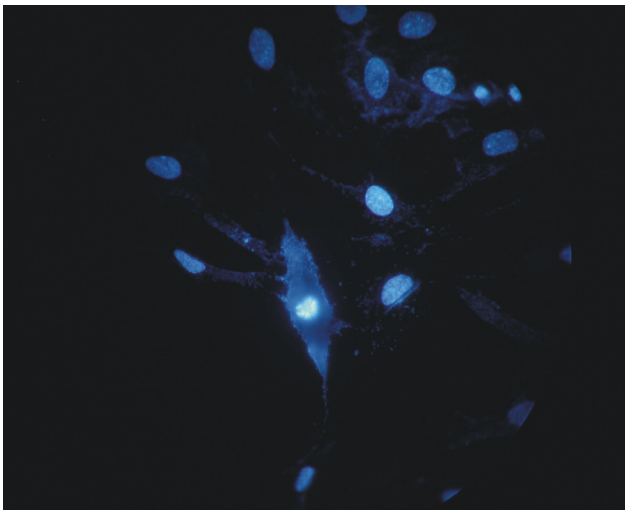

Figure 5 – ***Immunofluorescence expression of p53 in cell cultures obtained from cervical explants from a case with cervical cancer, ob. 60×; nuclei of the cell cultures have immunofluorescent expression with DAPI.***

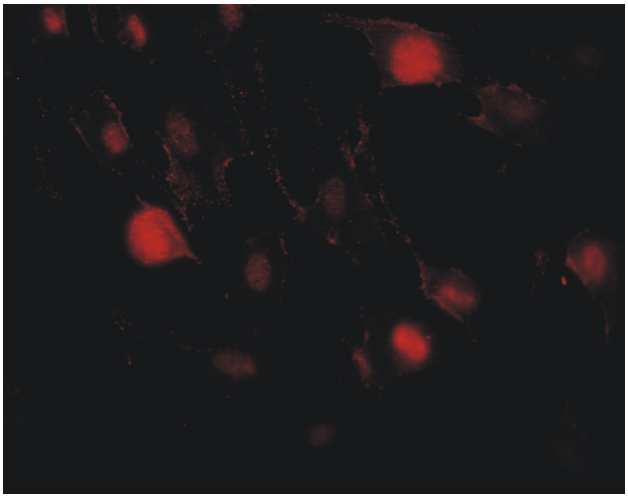

Figure 6 – ***Immunofluorescence expression of Ki-67 in cell cultures obtained from cervical explants negative for intraepithelial cervical lesions, ob. 40×.***

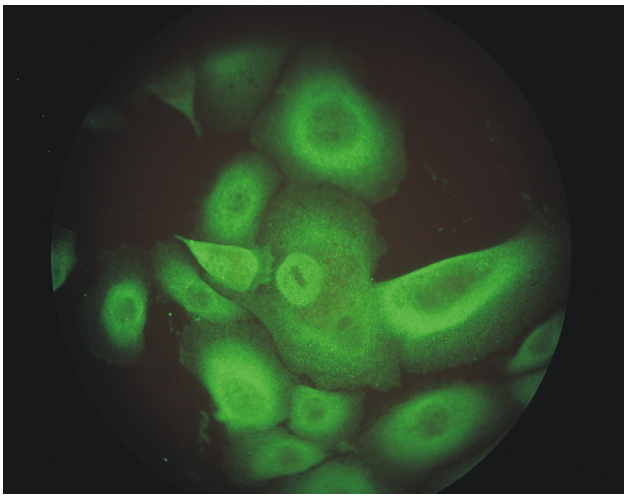

Figure 7 – ***Immunofluorescence expression of p21 cyclin inhibitor in cell cultures of LSIL, ob. 100×.***

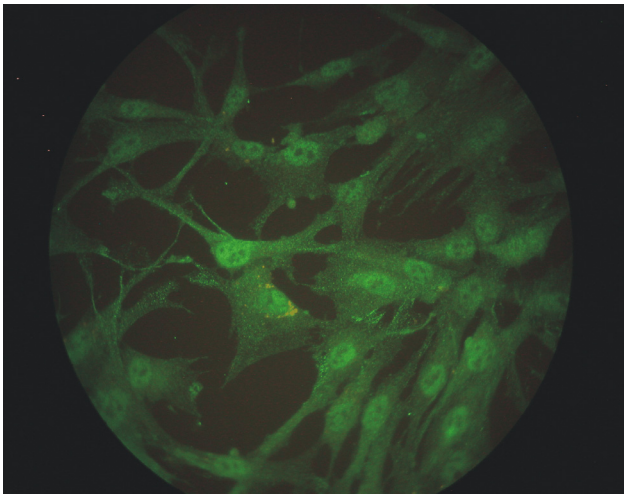

Figure 8 – ***Immunofluorescent expression of p21 cyclin inhibitor in cell cultures obtained from cervical cancer explants, ob. 100×.***

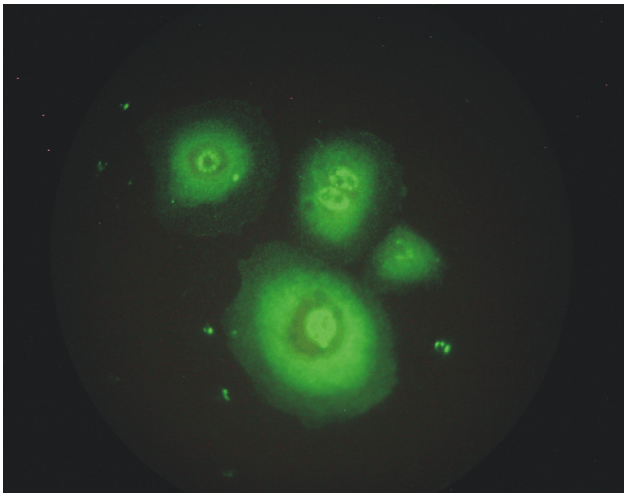

Figure 9 – ***Immunofluorescent expression of p27 cyclin inhibitor in cell cultures of LSIL, ob. 100×.***

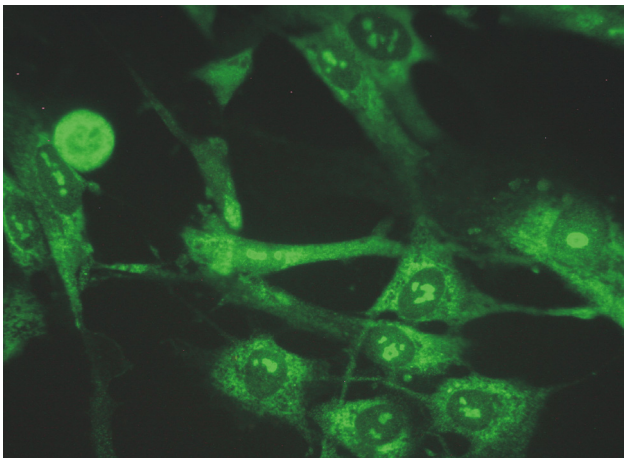

Figure 10 – ***Immunofluorescent expression of p27 cyclin inhibitor in cell cultures of invasive cervical cancer explants, ob. 100×.***

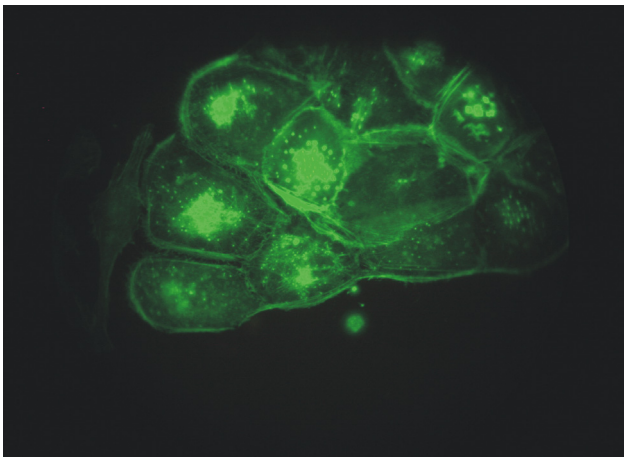

Figure 11 – ***Immunofluorescent expression of p16^{ink4a} cyclin inhibitor in cell cultures of LSIL, ob. 100×.***

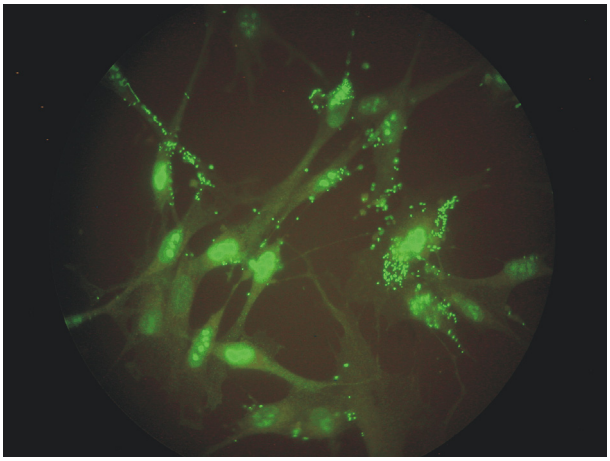

Figure 12 – ***Immunofluorescent expression of p16^{ink4a} cyclin inhibitor in cell cultures in cervical cancer explants, ob. 100×.***

The score value has allowed us to observe the immunofluorescence expression of cyclins inhibitors p16^{ink4a}, p21, p27, and of proliferative marker Ki-67 and p53 too, within the nucleus and cytoplasm of the cell cultures of cervical lesions investigated. Therefore, p16^{ink4a} showed both in nucleus and cytoplasm an ascending score evaluated as 1 to 5 in concordance with the higher grade of cervical lesion (Table 3). The similar variable growing score was observed too, for the other investigated regulators of cell cycle, but with the mention that the score value for cyclin inhibitor p27 could not allow to differentiate the cervical lesion gravity. On the other hand, p21 score value evaluated by immunofluorescence expression on cell cultures was higher compared to the p21 expression in immunohistochemical assay on cervical tissue lesions as known from published data in the literature.

According to the results of our statistic analysis, we concluded about some obtained research data in relationship with the objective of the present study and other similar published studies.

The type of lesions distributed among selected cases in the study batch showed a predominance of LSIL with ~45% (Figure 13).

A very low expression of all cell cycle regulators (<1%) in NMIL, a progressive exponential expression of studied parameters in LSIL, and a very high expression of these markers in HSIL and ICC (Figure 14) were observed.

In ICC, a strong direct correlation, statistically significant (r=0.76, p<0.001), has been noted between the aggressive factors p21 and p53 (Figure 15). In this situation, they might be considered as predictive factors for the subsequently evolution of the tumor. However, because there is a low number of cases in the batch (n=9), further studies are required in order to support this ascertainment with strong evidence.

In HSIL, a reasonable reverse proportion, statistically significant (r=-0.43, p=0.003, n=14) was observed between the same markers (p21 and p53) (Figure 16). A slightly direct correlation between p21 and Ki-67 (r=0.35, p<0.001) was also noticed in HSIL.

In LSIL, the antibodies were independent (did not correlate to each other), being neither specific, nor sensitive; therefore, they cannot be used to assess the aggressiveness or behavior of the lesion.

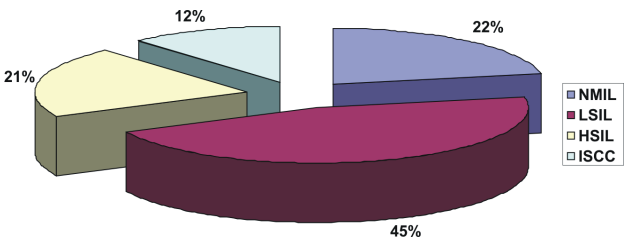

Figure 13 – ***Type of lesions distributed among selected cases in the study batch (n=68).***

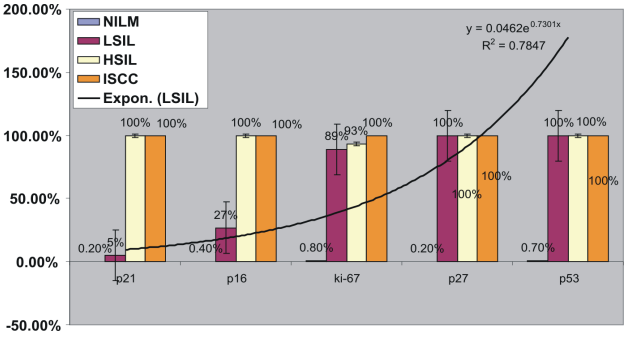

Figure 14 – ***Nuclear IF expression of studied antibodies in cell cultures.***

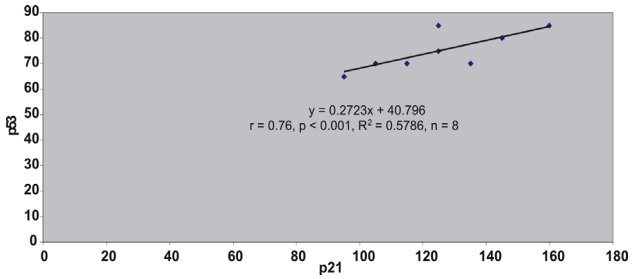

Figure 15 – ***Correlation between p21 and p53 in invasive carcinoma.***

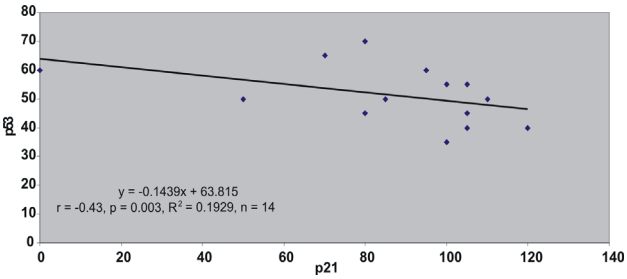

Figure 16 – ***Correlation between p21 and p53 in HSIL.***

In cases negative for intraepithelial lesions or malignancy, the studied markers have shown variable correlations, more or less statistically significant in the mathematical model (p16 and p27: r=0.4, p=0.03; $p16^{ink4a}$ and Ki-67: r=-0.4, p=0.002; $p16^{ink4a}$ and p53: r=0.23, p=0.001) and therefore with no significance from the biological point of view at this moment.

Discussion

The results of our research are partially similar to the data published in this field. An encountered difficulty in comparing our results was the relatively reduced number of reported results in the field. In order to compensate this limitation we deemed useful to weigh against closed investigation ways like immunohistochemical assay (but the last applied only on tissue sample).

Almost all authors sustain that Ki-67 is a strong marker of cellular proliferation, which is overexpressed in high-grade cervical lesion, invasive cancer respectively [16].

As regards the immunofluorescence expression of p21, this was constantly found, both in nucleus and cytoplasm of all culture cells, but with low intensity level in nucleus of the cells within high intraepithelial cervical lesions. This observation is contrary to the results reported by other researchers in their studies that were performed using immunohistopathological staining on cervical abnormal tissue. In this situation, they found that the low expression of p21 is poorly correlated with the development of cervical cancer [17].

In our study, Ki-67 proved to be a good marker for the detection of high-grade cervical lesions, so it is a marker of cell proliferation. Despite published data of other authors who have demonstrated that the intensity of immunofluorescence expression of Ki-67 is evidently opposite to the immunofluorescence expression of p21, the results of our study shown that this two markers are very well expressed with comparable intensity of immuno-fluorescence expression for the same degree of cervical lesion. An explanation for this different result could be attributed to the enrichment of number of cells investigated, which was obtained from explants of abnormal tissue investigated. The increase of number of cells properly for each type of cervical lesion led to a higher accuracy of the diagnosis of intraepithelial cervical lesions. The role of the CDKI is to bind, inactivate and degrade the cyclin/CDK complex. It is known that in the presence of hr-HPV the E7 oncoprotein inactivates p27 and in this manner disassociates it from the cyclin–CDK complexes. The published data have revealed that p27 is less expressed when cell division occurs [18]. In situation of cancer progression, both p21 and p27 are suppressed and cause cell cycle arrest [19].

By its functions, p53 plays critical roles in the cell cycle regulation and it is involved both in cell cycle arrest, DNA repair, and apoptosis, respectively [20]. Our work have shown that in high intraepithelial lesions there is a strong direct correlation, statistically significant (r=0.76, p<0.001) between the aggressive factors p21 and p53. The explanation for this obtained data is argued by the direct influence of p53 function to the activity of cyclin-dependent kinase inhibitor p21. In normal epithelial cervical cells, p53 is responsible for the activity of p21 and leads to the cell cycle arrest, so preventing tumor development [21].

On the other hand, our statistical analysis revealed a slightly direct correlation between p21 and Ki-67 (r=0.35, p<0.001). The intensity of immunofluorescence expression is higher for both regulators when the degree of cervical lesion grows.

As regards the cyclin inhibitor $p16^{ink4a}$, this has a relevant high intensity of immunofluorescence expression in nuclei of the cells belonging to high intraepithelial cervical lesions and invasive cervical cancer. Our previous published data shows that $p16^{ink4a}$ is a very good marker able to distinguish positive cases for cervical intra-epithelial lesions using immunohistochemical or immuno-cytological investigation methods [22].

A previous study published by Klaes R *et al.* using the technique of immunofluorescence on cervical tissue has showed that $p16^{ink4a}$ is overexpressed in almost 100% of high-grade dysplastic cervical lesions while in normal tissue cervix $p16^{ink4a}$ is not found to be immuno-expressed [23].

Although by far it was not an objective of this study to compare the methods (immunofluorescence *versus* immunohistochemical staining) as well as their afferent materials some differences between used criteria should be emphasized. The first criterion is that this work was performed on an enriched number of epithelial cells (cell cultures); the second criterion is that this study was conducted on epithelial cells with the lack of configuration of the epithelium layers but with respect for nucleus and cytoplasm of the epithelial cells, while other studies were conducted on samples (cervical biopsies) with respect to the morphohistological layers of cervical tissue investigated. This stands for an explanation of partially different results of our research.

The present study highlights the significance of the immunofluorescence markers used on tissue biopsies, and concretely enhances the obtained accuracy as described in the study published by Boşoteanu M *et al.* regarding

the decisive role of histopathological diagnosis [24]. Only the identification of HPV infection is not enough to conclude about the degree of the cervical lesions because the detection of HPV infection cannot distinguish between transient and transforming HPV infections [25]. According to many published data, the HPV infection in the cervical pathology remains mainly transitory [26], so these cases have not the possibility to develop cervical cancer under assumed circumstances. Therefore, we continued the research and we proved in our study that the immunofluorescence expression of some cyclins is able to perform the difference between low and higher intraepithelial cervical lesion identifying the worst prognosis cases.

As regards the modality to collect the biopsies from cervical lesions, we shared the option of Boicea A *et al.* point of view that the cervical biopsies performed under colposcopy lead to a better performance of cervical diagnosis lesions [27].

Further, for medical practice, the use in the diagnose protocol of the immunological markers on cervical tissue biopsies will direct to a higher accuracy as concerns the identification of high intraepithelial cervical lesion able to evolve to cervical cancer.

Conclusions

The immunofluorescence technique on cell cultures of cervical explants is a good method which could be used to identify the regulators of cells cycle p53, proliferative marker Ki-67 and cyclins inhibitors p16, p21 and p27. This technique allows to more accurately distinguishing the most significant marker able to detect high-grade cervical lesions. The particular feature of the immunofluorescence expression in cell cultures is that all investigated regulators of cell cycle are very well expressed in cytoplasm and variably in the cell nucleus, correlated with the gravity of cervical lesion. The immunofluorescence assay on cell cultures shows that the most immunoexpressed markers for high-grade lesions squamous intraepithelial and invasive cervical cancer are Ki-67 and cyclin inhibitor $p16^{ink4a}$. They are immunoexpressed in the nuclei of the culture cells with percentage around 100% for Ki-67 and 100% for $p16^{ink4a}$ and with high-grade intensity 4+, 3+ respectively. The p27 cyclin inhibitor could not perform the difference between the low- and high-grade of intraepithelial lesions but warn that there is a cervical lesion. Immunofluorescence method, using cell cultures, is able to improve the possibility to distinguish with more accuracy low-grade intraepithelial cervical lesions from high-grade intraepithelial lesions.

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Contribution Note

All the authors had equal contributions in the elaboration of this original article.

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