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The Immunohistochemical Assessment of HPV Related Adenocarcinoma: Pathologic and Clinical Prognostic Significance

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Abstract: Although several epidemiologic studies have confirmed the association between high-risk human papillomavirus (hr-HPV) and adenocarcinoma of the cervix, there are few papers focusing on the molecular immunophenotype of the HPV related cervical adenocarcinoma and its precursor lesions. The present study is aimed to assess the immunohistochemical expression of p16, p53, cyclin D1, EGFR, and COX-2 in benign and malignant lesions of cervical glandular components, and consequently the identification of the relationship between these markers and the HPV L1 capsid protein. We investigated 7 cases of endocervical adenocarcinoma *in situ* (AIS), 8 cases of adenosquamous carcinoma, 15 cases of invasive adenocarcinoma of endocervical type, and 5 cases without malignant lesions (normal and/or benign endocervical epithelium). The tissue fragments underwent standard laboratory procedures for the histopathological and immunohistochemical exams. For each marker, the semi-quantitative assessment was performed using appropriate scoring systems.

Our results showed that: (i) the combination of L1 capsid protein and p16 can predict the progression risk of precursor lesion of endocervical adenocarcinomas; (ii) p53 - COX2 - p16 co-assessment is useful as a panel of relevant biomarkers for L1 – p16 association; (iii) EGFR increases according to the progression in lesions severity; (iv) cyclin D1 is a reliable marker for the invasive capacity. Further studies are necessary to quantify the value of these markers, as prognostic factors in HPV related cervical adenocarcinoma.

Keywords: HPV, cervical adenocarcinoma, adenosquamous carcinoma, immunohistochemistry.

INTRODUCTION

The incidence of adenocarcinoma of the cervix, represented by a heterogeneous group of neoplasms, that displays a variety of histologic patterns, has changed, with several papers reporting increased rates of adenocarcinoma among young women, with a correspondent modified proportion between adenocarcinoma and squamous cell carcinoma [1-4]. Epidemiologic studies have shown a strong association between high-risk human papillomavirus (hr-HPV) and adenocarcinoma of the cervix [5-8], with HPV-16 being the most important type and HPV-18 playing a greater role in endocervical adenocarcinoma than in squamous cell carcinoma [9]. Despite these data, some investigators have revealed that uncommon types of endocervical adenocarcinoma are hr-HPV- negative [5, 8, 10]. However, the immunoexpression of HPV L1 capsid protein was demonstrated only in the early productive phase of cervical carcinogenesis (low grade squamous dysplastic lesions), but not in high grade squamous lesions, squamous cell carcinoma, and cervical adenocarcinoma [11]. HPV L1 quantification is further complemented by the assessment of tumor supression protein p16 as a valuable biomarker of squamous and glandular types of cervical carcinoma providing supplementary information about HPV related precursor lesions and malignancies progression [9, 11, 12].

An overview of HPV related cervical adenocarcinoma literature identified limited data regarding its immunohistochemical phenotype expressed by a large panel of molecules involved in carcinogenesis progression.

Cyclin D1 is responsible for the cellular proliferation initiation and progression through the G1-S cell cycle phases by forming a complex with the cyclin-dependent kinases. Cyclin D1 exhibits a distinct expression pattern in endocervical adenocarcinoma, suggesting a localised alteration in cell cycle regulation at the tumor-stromal interface [13, 14].

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The tumor suppresor gene p53 which plays a major role in cell cycle control and growth arrest following DNA damage is overexpressed in cervical adenocarcinoma and its overexpression is correlated with a poor prognosis [15]. Although HPV 16 and 18 are major etiopathogenic factors in endocervical glandular malignancies, the involvement of HPV infection might be a separate event in the development of endocervical adenocarcinoma [16], with p53 mutation representing a late event in endocervical carcinogenesis [17].

The epidermal growth factor receptor is overexpressed in cervical adenocarcinoma and adenosquamous carcinoma due to its involvement in cell cycle control, apoptosis, angiogenesis, and regulation of invasive and metastatic potential [18, 19], with a weaker expression when compared to its value in squamous cell carcinoma [18, 20]. Despite the presence of EGFR overexpression, the EGFR gene activating mutations are absent [19]. Moreover, EGFR is associated with HPV infection, but not correlated with HPV type [18].

Cyclooxigenase-2 (COX-2), a key enzime involved in the synthesis of prostaglandins and also in carcinogenesis, exhibits an enhanced expression in cervical adenocarcinoma [21, 22], as a probable result of deregulation of the EGFR signaling pathway [21].

In the context of current research developments, the aim of our study was to comparatively assesss the immunohistochemical expression pattern of p16, p53, Cyclin D1, EGFR, and COX-2 in benign and malignant lesions of cervical glandular structures, in order to identify the pathological link connection of these molecular markers to HPV L1 capsid protein.

MATERIALS AND METHODS

The study group comprises 35 cases selected from the files of the Pathology Laboratory of the "Elena Doamna" Obstetrics and Gynecology University Hospital of Iasi, Romania. The study was approved by the Ethics Committees of University of Medicine and Pharmacy "Grigore T. Popa" and "Elena Doamna" University Hospital, based on the informed consent (signed by the patients) for

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research use of samples after the pathological exam requested for the diagnosis.

The specimens were obtained from endocervical curettage, polipectomy or total hysterectomy. Within the study group, there were 7 cases of endocervical adenocarcinoma *in situ* (AIS), 8 cases of adenosquamous carcinoma, and 15 cases of invasive adenocarcinoma of endocervical type. We also included in the study group 5 cases without malignant lesions (normal and/or benign endocervical epithelium), corresponding to hysterectomy specimens performed for uterine leiomyomata. One case with AIS was diagnosed within an endocervical polyp. The adenocarcinomas of usual endocervical type were classified as well-differentiated (11 cases) and moderately differentiated (4 cases). The depth of invasion was assessed through the percentage of carcinomatous involvement of the wall.

All tissues were fixed in neutral-buffered formalin, routinely processed and paraffin-embedded. Serial sections of 4 μm were dewaxed and stained with Hematoxylin–Eosin, or furthermore prepared for immunohistochemistry. Table **1** summarizes the characteristics of the primary antibodies, and the antigen retrieval technique. After blocking the endogenous peroxidase and non-specific binding, the samples were incubated with the primary antibodies for 30 minutes, at room temperature, followed by the amplification with the appropriate secondary antibody and the Streptavidin–Biotin– Peroxidase HRP complex (code K5001, DAKO, Denmark). Finally, the sections were developed with 3,3'-diaminobenzidine tetrahydrochloride chromogen (DAB, code K5001, DAKO, Denmark), counterstained with Lillie's modified Hematoxylin, dehydrated with ethanol and permanently coverslipped. Positive and negative controls were simultaneously run.

Supplementary, the patients with malignant endocervical glandular lesions (AIS, endocervical and adenosquamous carcinoma) underwent genotyping for HPV 16/18, by *in situ* PCR, because of the abnormal glandular lesions identified in liquid-based Pap test.

The semi-quantitative assessment was performed using different scoring systems, as follows.

For HPV L1 capsid protein, the presence of at least one epithelial cell with strong nuclear staining represented the criterion for the immunopositive detection [23].

The semi-quantitative evaluation of p16 and COX-2 used two criteria: the percentage (P) of positive cells (0 – no staining; 1 for less than 1% positive; 2 for 1-10% positive; 3 for 11-33% positive; 4 for 34-66% positive; and 5 for more than 66% positive) and the intensity (I) of staining (0 – no staining, 1 – weak, 2 – moderate, 3 – strong), resulting a P+I score (from 0 to 8 points) [24, 25].

The evaluation of p53 was made considering only the percentage of positive cells: 0 for up to 5%, 1 for 5-25%, 2 for 26-50%, 3 for 51-75%, and 4 for more than 76%. The intensity of the reaction was not considered [15].

The EGFR immunohistochemical expression was quantified as: 0 for no staining, or membrane staining in <10% of the neoplastic cells; 1 for weak complete and/or incomplete membrane staining in >10% of the neoplastic cells; 2 for moderate complete and/or incomplete membrane staining in >10% of the neoplastic cells; 3 for strong complete and/ or incomplete membrane staining in >10% of the neoplastic cells.

For cyclin D1, the assessment was based on the proportion of positive tumour cells, and the reaction was considered as 0 for negative stain, 1 for less than 50% (focally positive stained) and 2 for more than 50% (diffusely positive stained) [14].

RESULTS

The cases with normal and benign endocervical epithelium presented focal immature squamous metaplasia, tunnel clusters or Nabothian cysts. Two of the seven cases diagnosed as AIS also had a low grade squamous intraepithelial lesion (LSIL), with evidence of cytopatic HPV effect (koilocytes). The microscopical examination revealed widely spaced or densely arranged glandular pattern in endocervical adenocarcinomas and associated focal solid growth pattern in moderately differentiated adenocarcinomas (four cases). The architectural pattern showed cribriform and papillary features. The cytology was characterized by simple or stratified columnar epithelial cells exhibiting pleomorphism, marked atypia, with elongated, hyperchromatic nuclei, and evident mitotic figures. The adenosquamous carcinomas presented a well-differentiated squamous component, with keratin "pearls" or individual cell keratinization. The invasion pattern was represented by cellular nests, aggregates or tumor cords which infiltrated the cervical wall.

All patients with AIS were positive for HPV 16/18. Twelve patients diagnosed with endocervical adenocarcinoma of the total of fifteen and six of the patients diagnosed with adenosquamous carcinoma of the total of eight had HPV 16/18 positive test.

The HPV L1 capsid protein immunoexpression was positive in benign glandular epithelium in a single case. The HPV L1 capsid protein was absent in all cases of AIS, endocervical adenocarcinomas, and adenosquamous carcinomas. Although L1 expression was absent in AIS cases, it was detected in the nuclei of the LSIL component (two cases). The positive reaction was confirmed by the strong staining of the nucleus surrounded by cytoplasm, with no background. The immunoexpression was focally detected in the nuclei of the glandular epithelial cells and also from the squamous component.

The HPV infection status revealed by the expression or absence of L1 respectively was correlated to p16, a marker which reflects

Table 1. Antibodies and Antigen Retrieval Technique

ANTIDOBY	SOURCE	CLONE	DILUTION RANGE	ANTIGEN RETRIEVAL
L1 protein	Viroscreen Virofem Diagnostics GmbH, Germany	VAHK1006	RTU	HIER, 20 minutes
P16	Santa Cruz Biotechnology, USA	2D9A12	1:100	HIER, 30 minutes, pH 6
P53	DAKO, Denmark	DO-7	1:50	HIER, 30 minutes, pH 9
Cyclin D1	DAKO, Denmark	DCS6	1:40	HIER, 30 minutes, pH 9
EGFR	DAKO, Denmark	E30	1:50	PIER, 5 minutes, RT
COX-2	DAKO, Denmark	CX-294	1:100	HIER, 30 minutes, pH 9

HIER, Heat-induced epitope retrieval; PIER, Proteinase-induced epitope retrieval; RT, room temperature; RTU, ready-to-use.

Table 2. Correlation Between HPV L1 and p16 Immunoexpression in the Study Group

LESION SUBGROUP (no. cases)	HPV L1(+)/p16(+) (no. cases, %)	HPV L1(+)/p16(-) (no. cases, %)	HPV L1(-)/p16(+) (no. cases, %)	HPV L1(-)/p16(-) (no. cases, %)
Benign endocervical epithelium (n = 5)	1 (20%)	0	0	4 (80%)
AIS (n = 7)	0	0	5 (71.4%)	2 (28.6%)
Endocervical adenocarcinoma (n =15)	0	0	15 (100%)	0
Adenosquamous carcinoma (n = 8)	0	0	8 (100%)	0

HPV oncogenic potential. The association L1(+)/p16(+) was found only in one case of benign lesions. L1(-)/p16(+) was present in five cases of AIS, and in all cases of endocervical adenocarcinoma and adenosquamous carcinoma. For L1(-)/p16(-), there were four cases of benign lesions and two cases of AIS. None of the patients presented the relationship L1(+)/p16(-) (Table **2**).

The score values achieved in the semi-quantitative assessment of p53, cyclin D1, EGFR, p16, and COX2 were summarized in Tables **3**-**6**, each table corresponding to a subgroup of lesions, as follows: benign endocervical epithelium (Table **3**), AIS (Table **4**), endocervical adenocarcinoma (Table **5**), and adenosquamous carcinoma (Table **6**).

The p16 expression was observed in the epithelial cells nuclei of benign lesions (one case), from AIS (five cases) and from all cases of invasive malignant lesions (Fig. **1**-**2**), with varying degrees of staining intensity and area extent (Tables **3**-**6**).

The staining pattern of p53 was predominantly nuclear. In the subgroup diagnosed with benign lesions (Table **3**), four cases were p53 negative, and one case presented a positive reaction in less than 25% of cells (score 1). A weak p53 positivity (score 1) was noted in three cases of AIS (Table **4**). All cases of endocervical adenocarcinoma and adenosquamous carcinoma, except for one from each category, exhibited p53 immunoexpression (Table **5**, Table **6**). The tumor cells showed immunopositivity in more than 76% of cells (score 4) in one case of endocervical adenocarcinoma (Fig. **3**) and in two cases of adenosquamous carcinoma (Fig. **4**).

The EGFR immunoreactivity was predominantly membranar, with focal cytoplasmic positivity.

A large staining heterogeneity was observed, with positive cells admixed with negative cells. However, the proportion of cases with a more intense immunoexpression of EGFR registered a progressive intensity correlated to the severity of lesion. Accordingly, a moderate immunostaining was observed in two cases diagnosed with adenosquamous carcinoma and in one case diagnosed with endocervical adenocarcinoma (Fig. **5**, Tables **5**-**6**). A weak immunostaining was also more frequent in carcinomas than in AIS and benign lesions (six and three cases for endocervical and adenosquamous carcinomas respectively, in comparison with one and two cases for benign epithelium and AIS respectively) (Table **3**, Table **4**).

Cyclin D1 was diffusely positive in the cytoplasm and nuclei of only two cases showing immature metaplasia (Table **3**). The immunoreactivity was patchy (Fig. **6**) also in two cases of AIS (Table **4**), eight cases of endocervical adenocarcinoma (Fig. **7**, Table **5**) and three cases of adenosquamous carcinoma (Fig. **8**, Table **6**). Eighteen of the total thirty-five cases were completely unstained.

COX2 was detected in all *in situ* and invasive carcinomas (Tables **4**-**6**), with a higher total score and a more diffuse immunopattern in endocervical adenocarcinomas (Fig. **9**) and adenosquamous carcinomas than in AIS (Fig. **10**). The epithelial cells cytoplasm in immature squamous metaplasia and normal glandular epithelium of two cases of benign lesions were stained in 10-33%, exhibiting a weak or moderate intensity (Table **3**). COX2 was expressed in both epithelial tumor cells and inflammatory cells, as well as in glandular and squamous components in adenocarcinomas and adenosquamous carcinomas.

DISCUSSION

Although numerous researches regarding cervical adenocarcinoma have been recently performed, to our knowledge, there are few released papers focused on a global immunoprofile of the molecules involved in the HPV related precursor lesion progression and cervical adenocarcinoma development.

For this reason, our study aimed to assess the immunohistochemical pattern of p16, p53, cyclin D1, EGFR, and COX-2 in benign and malignant lesions of cervical glandular structures, in order to identify possible correlations between these markers and the HPV infection status.

Table 3. The Semi-quantitative Assessment of p53, Cyclin D1, EGFR, p16, and COX2 in Benign Lesions

CASE	p53 SCORE	CYCLIN D1 SCORE	EGFR SCORE	p16			COX2		
				SCORE					
				PS	IS	Total	PS	IS	Total
1.	0	2	0	0	0	0	0	0	0
2.	1	1	0	0	0	0	0	0	0
3.	0	0	0	2	1	3	3	2	5
4.	0	2	1	0	0	0	2	1	3
5.	0	1	0	0	0	0	0	0	0

Table 4. The Semi-quantitative Assessment of p53, Cyclin D1, EGFR, p16, and COX2 in Adenocarcinoma *In Situ*

CASE	p53 SCORE	CYCLIN D1 SCORE	EGFR SCORE	p16			COX2		
				SCORE					
				PS	IS	Total	PS	IS	Total
1.	1	0	0	2	3	5	2	2	4
2.	0	0	0	0	0	0	3	2	5
3.	1	1	1	2	2	4	3	2	5
4.	0	0	0	1	2	3	3	2	5
5.	0	0	0	2	3	5	2	1	3
6.	0	0	1	0	0	0	3	2	5
7.	1	1	0	2	2	4	2	1	3

Table 5. The semi-quantitative Assessment of p53, Cyclin D1, EGFR, p16, and COX2 in Endocervical Adenocarcinoma

CASE	p53 SCORE	CYCLIN D1 SCORE	EGFR SCORE	p16			COX2		
				SCORE					
				PS	IS	Total	PS	IS	Total
1.	2	0	1	3	3	6	4	2	6
2.	3	0	1	4	3	7	4	2	6
3.	1	0	1	4	3	7	5	3	8
4.	4	0	2	3	2	5	3	1	4
5.	3	1	1	5	3	8	4	2	6
6.	1	0	0	3	3	6	5	2	7
7.	3	1	0	4	3	7	5	2	7
8.	1	1	0	4	3	7	4	2	6
9.	3	1	1	5	3	8	4	2	6
10.	1	1	0	4	3	7	3	2	5
11.	2	1	0	5	3	8	4	1	5
12.	0	0	0	5	3	8	4	3	7
13.	1	1	0	3	2	5	4	2	6
14.	3	1	1	5	3	8	5	2	7
15.	3	0	0	5	3	8	4	2	6

The L1 capsid represents the main target of the cellular immune response to HPV infection and consequently is expressed in the early productive phase of cervical carcinogenesis (LSIL) [11]. The studies performed on cervical squamous cell carcinoma and its precursor –namely intraepithelial neoplasia – revealed a progressively decrease of HPVL1 expression, in parallel with the increase of the cervical lesion severity [11, 23]. Our results showed that the HPVL1 capsid protein immunoreactivity was occasionally detected in the epithelial cells from a case belonging to the subgroup diagnosed with benign lesions, and absent in all cases of AIS, endocervical adenocarcinoma, and adenosquamous carcinoma. Although L1 expression was absent in AIS cases, it was detected in the nuclei of the LSIL component. These findings suggest that the expression of capsid protein occurs as an early event in endocervical carcinogenesis, despite the lack of specific HPV morphological features in glandular cells in contrast with infected squamous cells. Our findings are consistent with previous studies on cervical squamous precancerous lesions and carcinoma which concluded that the deficiency of L1 expression has a predictive value for an increased risk of malignant transformation of cervical dysplasia [11, 26].

The p16 value as a biomarker of the E7-driven oncogenic activity of HPV has been already proven, in keeping with its expression

Table 6. The Semi-quantitative Assessment of p53, Cyclin D1, EGFR, p16, and COX2 in Adenosquamous Carcinoma

CASE	p53 SCORE	CYCLIN D1 SCORE	EGFR SCORE	p16			COX2		
				SCORE					
				PS	IS	Total	PS	IS	Total
1.	3	0	0	3	3	6	4	2	6
2.	4	1	2	4	3	7	5	2	7
3.	4	0	1	4	2	6	4	2	6
4.	3	1	2	5	3	8	4	2	6
5.	2	0	1	5	3	8	3	1	4
6.	0	0	0	5	3	8	3	2	5
7.	2	0	0	4	2	8	4	2	6
8.	3	1	1	5	3	8	4	1	5

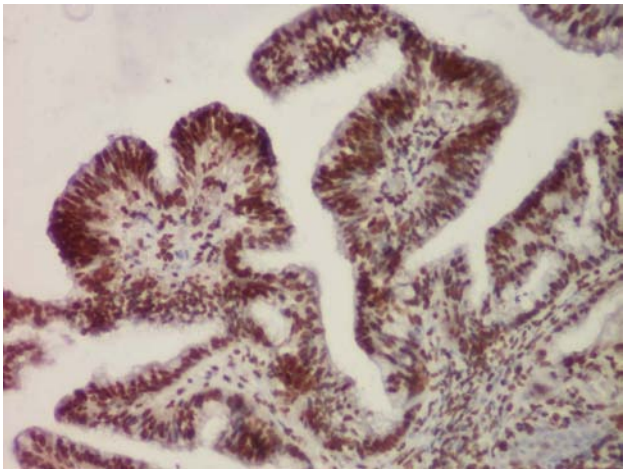

Fig. (1). Endocervical adenocarcinoma, diffuse strong nuclear tumor cells p16 immunoexpression (x10).

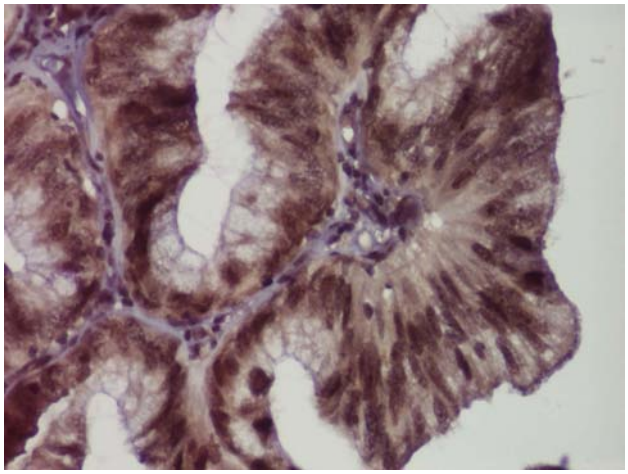

Fig. (3). Endocervical adenocarcinoma, strong nuclear tumoral cells p53 immunopositivity (x20).

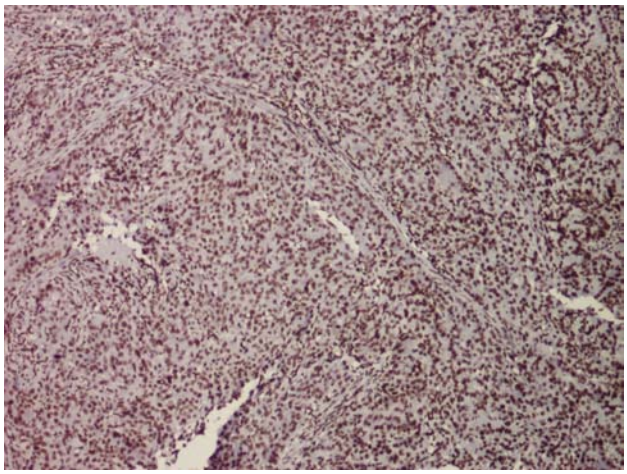

Fig. (2). Adenosquamous carcinoma, diffuse strong nuclear p16 immunoexpression in both glandular and squamous components (x4).

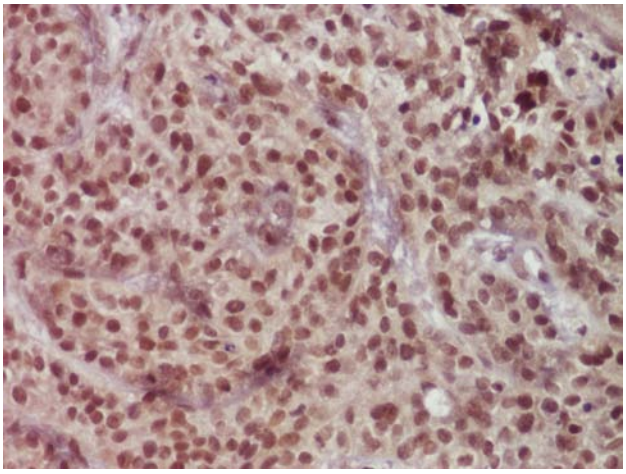

Fig. (4). Adenosquamous carcinoma, homogenous strong nuclear tumoral cells p53 immunoreactivity (x20).

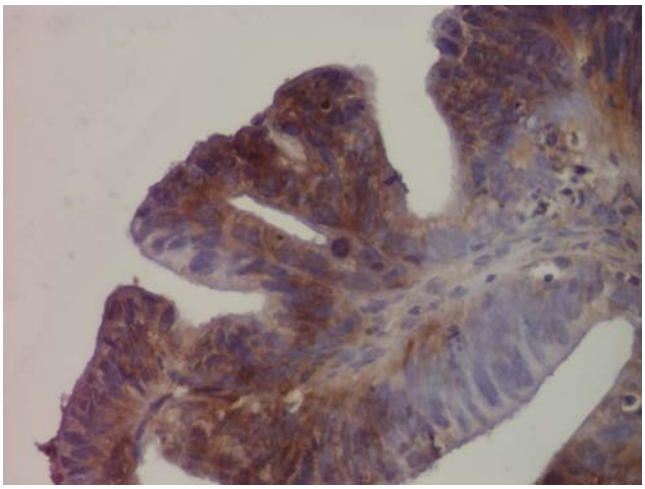

Fig. (5). Endocervical adenocarcinoma, moderate focal cytoplasmic EGFR positivity (x20).

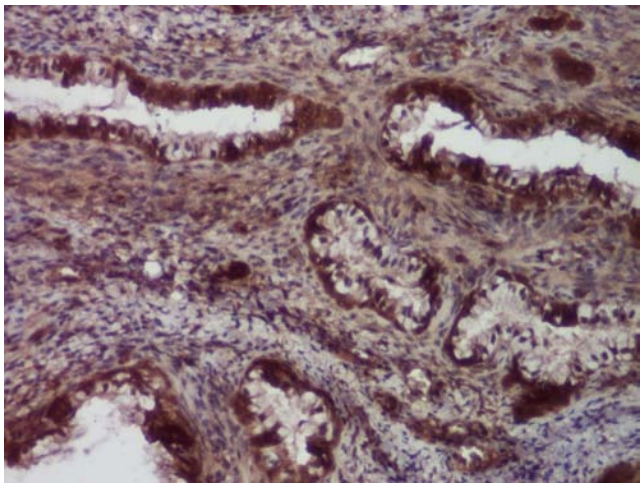

Fig. (6). Adenocarcinoma *in situ*, patchy cytoplasmic and nuclear cyclin D1 expression (x10).

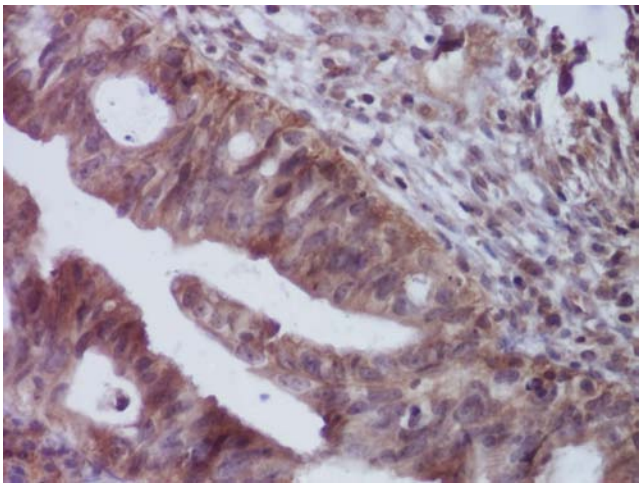

Fig. (7). Endocervical adenocarcinoma, diffuse cytoplasmic cyclin D1 immunoreactivity, with focal nuclear expression (x20).

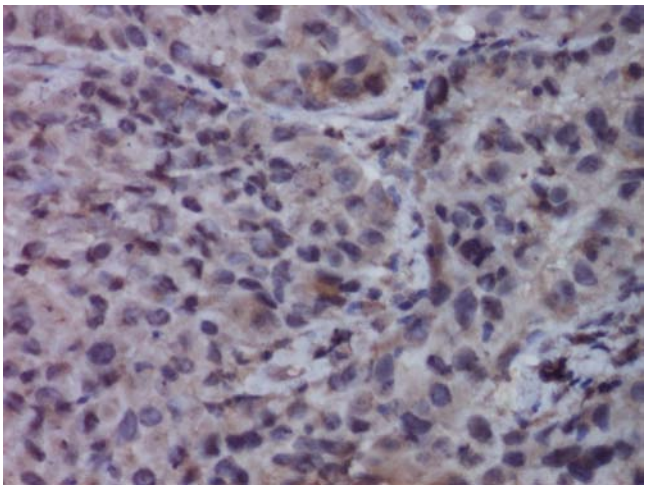

Fig. (8). Adenosquamous carcinoma, homogeneous faint cytoplasmic cyclin D1 expression (x20).

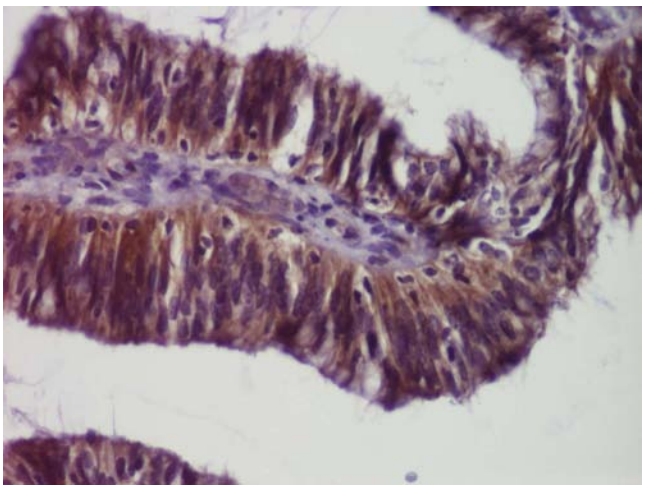

Fig. (9). Endocervical adenocarcinoma, strong diffuse cytoplasmic COX2 immunostaining (x20).

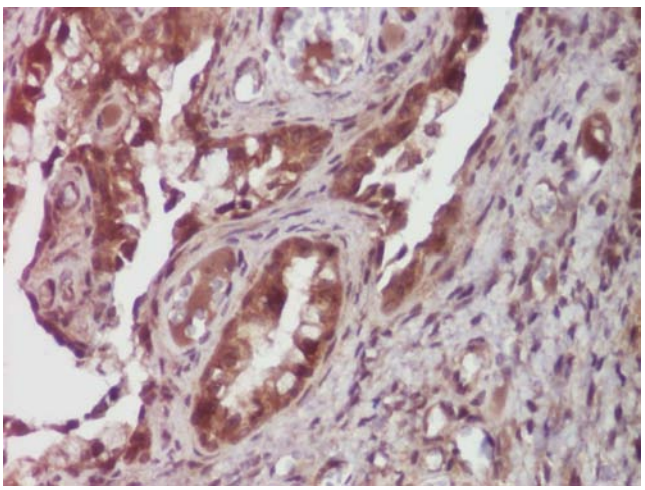

Fig. (10). Adenocarcinoma *in situ*, moderate cytoplasmic COX2 expression (x20).

in almost all high-grade squamous lesions, squamous carcinoma, high grade-dysplastic glandular lesions, and adenocarcinomas of the cervix [27-31]. E7 is responsible for the main converting and immortalizing activity that characterizes the high-risk types of HPV. The binding site for retinoblastoma gene (Rb) provides E7 the cell proliferation regulation capacity by variable phosphorylation degrees during the cell cycle [32]. Moreover, the retinoblastoma protein (pRb) intervenes in the cell cycle activation by inhibiting the transcription of the inhibitory gene of cyclin-dependent kinase p16(INK4A). Subsequently, the blocking of pRb function results in the overexpression of p16INK4A in the corresponding cells.

Strictly regarding the localization pattern of p16 immunostaining, our data revealed a moderate or strong expression restricted to nucleus, without cytoplasmic staining described by other researchers [31, 33]. p16 was positive in one case with immature squamous metaplasia of both the endocervical surface and the glandular epithelium, and strongly positive in five cases of AIS and in all cases of endocervical adenocarcinoma and adenosquamous carcinoma. These results are in agreement with the literature data, several papers showing that p16 expressed diffuse immunoreaction in most neoplastic cervical epithelia, and no or only focal positivity in non-neoplastic lesions [27, 31, 34, 35]. Two cases of AIS were p16 negative, despite the fact that the patients were HPV 16/18 positive. The lack of protein p16 expression can be the result of the hypermethylation of the p16INK4a promoter and it is not correlated to HPV status or to the lesion grade, as has been already mentioned in previous studies [27, 28, 31, 34, 35, 36].

Recent studies reported the importance of association of p16 and HPVL1 immunodetection in the prediction of cervical disease progression [11, 26, 37]. As we have already mentioned, the protein capsid L1 is expressed in the early, productive phase of cervical carcinogenesis and is progressively lost in the later phases, when p16 gets overexpressed [38]. In our study, the association L1(+)/p16(+) was found only in one case of benign lesions, with immature squamous metaplasia and tunnel clusters. The significance of this finding is that the lesion is still productive (L1+), but the pRb pathway is probably inhibited (p16+). L1(-)/p16(+) pattern of expression was registered in five cases of AIS, and in all cases of endocervical adenocarcinoma and adenosquamous carcinoma. These lesions can be interpreted as proliferative entities (L1-), with an inhibited pRb pathway, with p16 positivity. They probably represent high-risk glandular lesions, as previously demonstrated for squamous precursor lesions of the cervix [37]. There were four cases of benign lesions and two cases of AIS with negative expression of both L1 and p16. This finding was expected in benign lesions, but a careful evaluation is mandatory in such AIS cases, mainly because the patients were positive for HPV 16/18. As already demonstrated for squamous intraepithelial lesions [37], we can assert that the combination of these two biomarkers can predict the progression risk of precursor lesion of endocervical adenocarcinomas.

It has been already demonstrated that p53 regulates the cell proliferation in cervical neoplasia, through stimulation of other specific cell cycle control genes [15]. The staining pattern of p53 was predominantly nuclear in our study. In the benign lesion subgroup, 4 cases were p53 negative and 1 case presented a p53 expression quantified as score 1, based on less than 25% of epithelial cells were stained positively for p53. In three of five cases of AIS a weak positive reaction of p53, also assessed with score 1, was noted. Within the malignant lesion subgroups, the p53 immunoexpression was present in the majority of cases (14 of 15 endocervical adenocarcinomas and 7 of 8 adenosquamous carcinomas). The score values range the entire scale, one case of endocervical adenocarcinoma and two cases of adenosquamous carcinoma exhibiting a strong reaction, corresponding to the maximum score value, namely 4. A consensus of our results with one of the previous published work was noticed, with 70% of adenocarcinoma and 20% of AIS p53 immunoexpression [17]. Oppositely, other studies reported lower or absent p53 protein expression in AIS or high-grade dysplastic glandular lesions [16, 39] or even a lack of correlation of p53 immunoexpression with the malignant development [39].

The p53 immunoexpresion, as a reflection of the protein conformational changes, reflects a key point in the late stages of carcinogenesis sequence, with evident p16 overexpression parallel features.

Although a controversial matter [40-42], this parallelism can have clinical prognostic significance in the evaluation of HPV related cervical adenocarcinoma, revealing p53 as a supplementary biomarker of the association of protein capsid L1 and p16 in our opinion.

It has been already demonstrated the HPV proteins role in EGFR expression [43], which is correlated with HPV infection status. The EGFR immunoreactivity has a parallel expression with the increase in severity of intraepithelial lesions, without an identifiable relation to the HPV type [44-46]. HPV E5 protein causes the overexpression of EGFR, through inhibition of internalized EGFR degradation [47]. HPV E6/E7 protein complex may increase the EGFR levels and disrupt the growth rate of cervical carcinoma cell lines [45, 46]. Moreover, a possible difference between the squamous carcinomas and adenocarcinomas were identified based on their genetic alterations [19].

In our study, only two cases of adenosquamous carcinoma and one case of endocervical adenocarcinoma presented a moderate EGFR staining, quantified as score 2. A weak immunostaining, corresponding to the score 1, was also more frequently observed in carcinomas than in AIS and benign lesions (6 and 3 cases for endocervical and adenosquamous carcinomas respectively, compared with one and two cases for benign epithelium and AIS), as already reported in previous studies [20, 48, 49] which revealed EGFR overexpression in adenosquamous carcinomas. In conclusion, our results demonstrate the EGFR immunoexpression increase according to the progression in lesions severity, based on the HPV 16/18 etiopathogenic context.

Although EGFR gene amplification was reported as an independent prognostic factor in cervical squamous cell carcinoma [19], this statement cannot be yet extended on adenocarcinomas. EGFR is a very useful tool that should be added to the panel of biomarkers used in monitoring the evolution of HPV related dysplastic glandular lesions and adenocarcinomas. Moreover, recent papers consider EGFR expression as beneficial in clinical trials which evaluate the efficacy of anti-EGFR therapies in advanced cervical cancers, including adenosquamous carcinomas [49, 50].

Recently, cyclin D1 was evaluated in normal cervix and endocervical adenocarcinomas – the latter in relationship with the epithelial-mesenchymal transition and the tumoral growth pattern [13, 14]. The authors reported the presence of cyclin D1 in normal and parabasal squamous cells of non-neoplastic mucosa, its lack of expression in AIS, and a focal labelling in the infiltrative areas of the moderately differentiated adenocarcinomas. The positive reaction along the deep border of the malignant glands, in parallel with the negativity of the rest of the tumor, and the correlation with specific markers for EMT indicate that cyclin D1 is up-regulated. This fact can be interpreted in the context of its role of mediator in the main biochemical pathways that regulate the cellular proliferation, apoptosis and invasive capacity. Consequently, cyclin D1 was associated with migration and invasion [51].

In our study, cyclin D1 was diffusely positive in the cytoplasm and nuclei of only two cases with immature squamous metaplasia and in normal endocervical epithelium. The higher expression noticed in the squamous metaplasia is indicative of important basal/reserve cells reactivity. The immunoreactivity was observed also in two cases of AIS, 8 cases of endocervical adenocarcinoma

and three cases of adenosquamous carcinoma, both in proper tumoral areas, and in tumoral invasion front. Because twelve of the total twenty-three cases of adenocarcinoma and adenosquamous carcinomas were completely unstained, we consider our results partially similar with the previous reports mentioned above, in which the cyclin D1 immunoexpression was negative in the main tumoral areas, being positive only at the invasion borders.

The interconnection between HPV infection and cyclin D1 activity has been already demonstrated [52, 53]. pRb is inhibited by HPV oncoprotein E7, and thus pRb avoids the normal requisite for cyclin D1-CDK complex to initiate the cell cycle. PRb inactivation down-regulates cyclin D1, additionally enhancing its activity in cellular proliferation, and up-regulates p16, although this is functionally inactive [13]. These events are also useful in understanding and achieving a manageable dissimilarity of the mechanisms of both main types of cervical carcinogenesis [19], with the contrast increasing of a tumor suppressor protein (p16) and decreasing of a proliferation marker (cyclin D1). The relationship between HPV infection and the peculiar behaviour of these two biomarkers has been already reported in cervical adenocarcinoma and squamous cell carcinoma of the head and neck [13, 54, 55]. These correlations emphasize the feasible usefulness of cyclin D1 in the clinical management of HPV related cervical adenocarcinomas.

In our study, COX2 was detected in all *in situ* and invasive carcinomas, with a higher total score and a more diffuse immunopattern in endocervical adenocarcinomas and adenosquamous carcinomas than in AIS. There were also two cases of benign lesions with a weak or moderate COX2 positivity. COX2 was expressed in both glandular and squamous epithelial tumor cells, and stromal inflammatory cells of adenocarcinomas. These results are in accordance with previous data, including also our report, in which COX2 was overexpressed in squamous cervical carcinomas, adenocarcinomas, as well as intraepithelial lesions [21, 56].

COX2 levels increase along with the increase in severity of the glandular neoplastic epithelial lesion. This observation sustains the important role of COX2 in tumor development and progression, correlated with the antitumor immunity [22, 57].

Our findings raise also the possibility of a direct association between HPV status and COX2. Thus, in the context of confirmed HPV 16/18 infection in patients with *in situ* and invasive adenocarcinoma, we observed a high correlation between COX2 and p16 status, the immunoexpression of these two biomarkers showing an approximately parallel increase, especially in AIS and endocervical adenocarcinoma.

As we have already mentioned above, the HPV E-oncoproteins plays an essential role in the pathobiology of p53, EGFR, p16, in the cervical carcinogenesis pathogenic mechanism. It is well known that L1 viral capsid protein is considered a major target of the cellular immune response [58]. The loss of L1 immunoexpression indicates a poor local defense status, as a result of COX2 involvement in host-antitumor immunity. For these reasons, COX2 can be added to the protein panel which already proved their role in the management of pathologic and clinical behavior of the HPV related adenocarcinomas.

CONCLUSION

In the HPV related adenocarcinoma, the concomitant evaluation of L1 capsid protein and p16 can predict the progression risk of precursor lesions of endocervical adenocarcinomas. p53-COX2-p16 co-assessment is useful as a panel of relevant biomarkers for L1 – p16 association. EGFR increases according to the progression in lesions severity, and cyclin D1 is a reliable marker for the invasive capacity.

Further studies are necessary to identify other mechanisms and the concurrent effects of these markers in the precursor lesions, onset, and development of cervical cancer.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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