

ORIGINAL ARTICLE

P16^{INK4a} expression as a potential prognostic marker in cervical pre-neoplastic and neoplastic lesions

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Abstract

An immunohistochemical analysis with monoclonal antibody p16^{INK4a} was performed in formalin-fixed, paraffin-embedded samples of 60 cases. The aim was to investigate in biopsies the expression of p16^{INK4a} of normal uterine cervical tissue, pre-cancerous and cancerous lesions, and their relation with human papilloma virus (HPV) and HIV status. Three parameters were evaluated: percentage of p16^{INK4a} positive cells, reaction intensity, and cell staining pattern. All of these parameters were statistically different when compared among different histological groups. However, logistic regression model showed that the reaction intensity was the best indicator of the expression of p16^{INK4a}. This expression increases from normal to invasive squamous carcinoma. Sixty-six percent of the patients with CIN grade 1 (CIN1) expressed p16^{INK4a} (all these cases were infected with high risk HPV). Our study supports the hypothesis that p16^{INK4a} expression in pre-cancerous lesions and cancers can be used to identify HPV-transformed cells. Of great interest for routine diagnostic use is the fact that immunohistochemical testing for p16^{INK4a} seems to be capable of identifying HPV-positive cells and potentially recognizing those lesions with an increased risk of progression to high-grade lesions.

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Introduction

There is epidemiological evidence that persistent infection, high viral load [8,24], and integration of oncogenic types of human papilloma virus (HPV) in the host genome [32] play a preponderant role in the development of squamous uterine cervix carcinoma.

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Although oncogenic HPV has been detected in almost all pre-neoplastic and neoplastic uterine cervical lesions [31] and its presence is considered an important causal factor, other genetic and epigenetic factors may be necessary to increase the risk of transition from cervical infection to cancer [14].

More than 100 types of HPV have already been identified, of which approximately 30 types are associated with ano-genital infections [27]. Of these, 15 types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) have a high oncogenic risk [17] and can be detected in almost all cervical cancers [31]. HPV 16 and 18 are the most prevalent, representing 59.8% and 15%, respectively, of the viral types involved in invasive cancer [17]. HPV16 has also been demonstrated to be the single most important factor linked with disease progression [27].

HPV DNA integration occurs within the viral E1–E2 region, thereby disrupting E2 and triggering uncontrolled expression of the transforming genes E6 and E7 [26]. HPV E7 interacts with, and neutralizes, the function of pRB pocket proteins, p107 and 130 [5]. This interaction, analogous to cyclin-dependent-kinase-mediated phosphorylation, induces pRB degradation, resulting in liberation of activated E2Fs and stimulation of entrance into the S phase, causing high levels of expression of the cyclin-dependent kinase inhibitor p16^{INK4a} [6, 16]. The keratinocytes transformed by the oncogenic HPV genes E6 and E7 are not cellular clones with completely malignant phenotypes, but require additional mutagenic agents [4]. Induction of the p16^{INK4a} protein occurs during immortalization in HPV-positive pre-malignant lesions, an early event resulting in tumorigenesis [18].

Alterations in protein p16^{INK4a} expression have been described in many cancers [1] due to mutation, homozygotic deletion, or gene hypermethylation [7,9]. Several papers have evaluated the role of p16^{INK4a} as a diagnostic marker of cervical neoplasia [9,10,12,13,18,19,22,23,28,29]. Recently, some authors have shown that p16^{INK4a} expression is associated with disease progression [19,30].

Morphological criteria are important for diagnosis of cervical intraepithelial neoplasia (CIN) [20,21], although it is not possible to differentiate those that will regress or persist from those that will progress into invasive cancer. Owing to the limitations of current conventional examinations, there is a need to identify new, specific, and more sensitive markers to determine the presence of high-risk HPV types and functional activity of these viral oncogenes [3,12,23,32].

Our objective was to investigate, through immunohistochemistry, the expression of p16^{INK4a} in biopsies of normal uterine cervical tissue, pre-cancerous and cancerous lesions, and their relation with HPV and HIV status.

Materials and methods

Informed consent for this study was obtained from 60 patients selected from women who presented an atypical transformation zone (ATZ) and an atypical cytology result or negative cytology with persistent ATZ (≥ 6 months), between October 1999 and March 2001 at the State Center for Oncology – CICAN (State Health Authority and at the Outpatient Clinic of Gynecology (Infectious Diseases/AIDS Unit) of the Professor Edgar Santos Hospital, Federal University of Bahia (UDAI-HUPES-UFBA) in the city of Salvador, Bahia. The mean age of the patients was 37 years (ranging from 19 to 82).

Prior to colposcopy, ecto- and endocervical scrapes were made for detection and typing of HPV by PCR (PCR-based line blot assay, first generation, Roche Molecular Systems). Biopsies were performed by colposcopy, and tissue samples were fixed in 4%-buffered formalin, embedded in paraffin, cut as 4- μ m-thick sections, and stained for hematoxylin–eosin and immunohistochemistry. Three pathologists examined all slides (CQ, ES, and LF) and, in case of disagreement, a fourth pathologist (VA) was consulted. WHO classification was used for the histologic diagnosis, in which morphological lesions ascribed to infection by HPV are classified as CIN grade 1 (CIN1).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections (4 μ m thick) were deparaffinized in xylene and rehydrated through a series of graded ethanols (100–70%). For antigen retrieval, slides were pretreated by boiling (90–95 °C) in citrate buffer (pH 6.0) for 60 min in a pressure cooker. The slides were then incubated in methanol containing 0.3% H₂O₂ to inhibit endogenous peroxidase activity. After being washed, sections were incubated overnight at 4 °C with primary antibody anti-human p16^{INK4a}/MTS1 (Code MTM-E6H4, MTM Laboratories, Germany) at a dilution of 1:200 (0.6 μ g/ml) in 53022DAKO diluent. After being washed in PBS, the slides were incubated for 30 min with secondary antibody α IgG mouse coated in dextran polymer with several molecules of peroxidase (DAKO Envision System). After being washed, color development was achieved with 3,3-diaminobenzine (DAB) as chromogen and hematoxylin counterstaining. Formalin-fixed, paraffin-embedded sections from invasive uterine cervix squamous cell carcinoma biopsies served as positive controls because all slides were run in the same batch, including slides incubated with non-immune serum, from the same source as the primary antibody as negative controls.

The reaction was considered positive when a chestnut-brown color was seen in the nucleus and cytoplasm. Three parameters were evaluated: percentage of p16^{INK4a}-positive cells, reaction intensity, and cell staining pattern:

The percentage of positive cells was evaluated in the highest expression area (“hot spot”) and graded as follows: negative (grade 0) = no cells stained; less than 50% of positive cells (grade 1), 50–80% of positive cells (grade 2), and more than 80% of positive cells (grade 3).

The intensity of the reaction was scored as negative (0), weak (1), moderate (2), and strong (3).

The cellular reaction pattern considered the intensity of cytoplasmic expression of p16^{INK4a}. In all positive cases, nuclear staining was observed. However, cytoplasmic expression varied and was classified as 1 when weak, and as 2 when strong.

Statistical tests

Non-parametrical procedures of Kruskal–Wallis (Software: SPSS for Windows – Version 11.0), Dunn and Holmes, Mann–Whitney’s test, Fisher’s Exact test, and Univariate Logistic Regression were used [2,11]. Univariate logistic analysis was used to validate the results of p16 by comparing the percentage of positive cells, intensity, and cellular pattern of the reactions.

Results

According to histopathological examination, the 60 cases were classified as follows: 11 patients were normal/negative for neoplasia (including squamous metaplasia), 15 cases were classified as CIN1, 10 cases as CIN2, 15 cases as CIN3, and nine cases as invasive squamous cell carcinoma. Among the cases diagnosed as CIN or carcinoma, 90.6% were HPV-DNA positive (Table 1). Of the patients who tested positive for HPV, two types of low-risk HPV were identified: types 6 and 54, as well as 14 types considered to be of high oncogenic risk, types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and

73, and one considered to be of probable high risk, type 53. Only one patient had HPV6 isolated. Of the 60 patients in this study, 20 were HIV-positive, and all of them tested positive for HPV-DNA.

Immunohistochemical evaluation

We observed a significant difference between the histological diagnoses and percentage of positive cells, reaction intensity, and cellular reaction pattern of p16^{INK4a} ($p < 0.005$) (Fig. 1).

Percentage of cells positive for p16^{INK4a}

Table 2 depicts the percentage of cells positive for p16^{INK4a} according to histologic diagnosis. Only one out of 11 cases diagnosed as cervicitis was positive for p16^{INK4a}, whereas 100% of the cases with invasive carcinoma were p16^{INK4a}-positive. The percentage of positive cells tended to increase following the severity of CIN.

Intensity of the expression of p16^{INK4a}

Table 3 shows the increasing reaction intensity of p16^{INK4a} from normal cases (only one case was weakly positive) to invasive carcinoma, in which we observed moderate to strong intensity.

Cellular reaction pattern of p16^{INK4a}

Table 4 shows that the intensity of cytoplasmic expression of p16^{INK4a} increases from cases without neoplasia to cases with invasive cancer. Only one case without CIN was weakly positive. On the other hand, in all invasive carcinomas, cytoplasmic reaction was grade 2. Nuclear staining showed little variation (data not shown).

Univariate logistic analysis showed that the intensity of the reaction is the best parameter to evaluate the positivity of p16^{INK4a}.

The non-parametric test of Dunn and Holmes compared all pairs of histologic diagnoses. These results showed that the pair negative/CIN1 and the pair CIN1/CIN2 were statistically different. However, no significant difference was observed between the pairs CIN2/CIN3 and CIN3/carcinoma.

Spearman’s correlation between intensity reaction of p16^{INK4a} and HPV positivity was statistically significant ($r = 0.32$; $p = 0.0117$). According to Mann–Whitney’s test at a 5% level of significance, there is a difference concerning p16^{INK4a} immunostaining between HPV oncogenic-positive and -negative patients ($p = 0.0126$). However, this test did not show differences concerning p16^{INK4a} immunostaining between HIV-positive

Table 1. Correlation between histopathological and HPV results

DNA–HPV			
Histology	<i>n</i>	HPV +	HPV –
Normal	11	5	6
CIN1	15	13	2
CIN2	10	10	0
CIN3	15	13	2
Cancer	9	5	4
Total	60	46	14

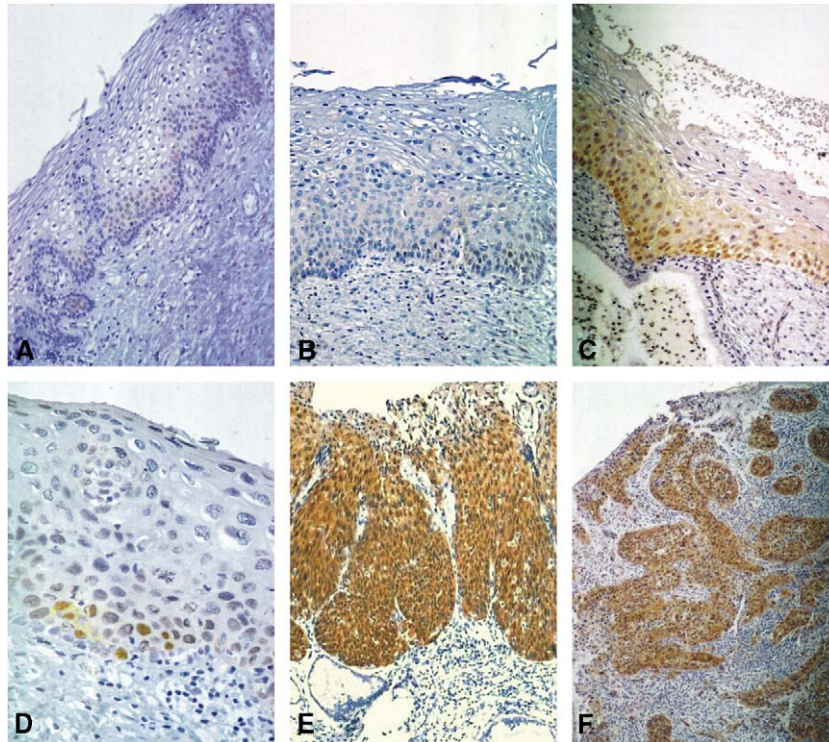


Fig. 1. (A) Normal epithelium, negative. (B) CIN1 negative. (C) CIN1, moderate expression of p16INK4a, in between 50% and 80% of cells. (D) CIN2, weak expression, mainly in nucleus, in less than 50% of cells. (E) CIN3 and (F) invasive cancer, strong nuclear and cytoplasmatic expression of p16INK4a, in more than 80% of cells.

Table 2. Correlation between histological diagnosis and percentage of cells positive for p16^{INK4a} staining

Histological diagnosis	Percentage of cells positive for p16 ^{INK4a}							
	0		1		2		3	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Normal	10	90.9	0	—	1	9.1	0	—
CIN1	5	33.4	2	13.3	2	13.3	6	40.0
CIN2	1	10.0	2	20.0	5	50.0	2	20.0
CIN3	1	6.6	0	—	7	46.7	7	46.7
Carcinoma	0	—	0	—	1	11.1	8	88.9

0, no cells stained; 1, less than 50% of positive cells; 2, 50–80% of positive cells; 3, more than 80% of positive cells.

Table 3. Correlation between histological diagnosis and reaction intensity for p16^{INK4a} staining

Histological diagnosis	Reaction intensity (p16 ^{INK4a})							
	0		1		2		3	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Normal	10	90.9	1	9.1	0	—	0	—
CIN1	5	33.3	4	26.7	3	20.0	3	20.0
CIN2	1	10.0	4	40.0	4	40.0	1	10.0
CIN3	1	6.6	0	—	10	66.7	4	26.7
Carcinoma	0	—	0	—	4	44.4	5	55.6

0, negative; 1, weak; 2, moderate; 3, strong.

Table 4. Correlation between histological diagnosis and reaction pattern for p16^{INK4a} staining

Histological Diagnosis	p16 ^{INK4a} cellular reaction pattern					
	0		1		2	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Normal	10	90.9	1	9.1	0	—
CIN1	5	33.3	4	26.7	6	40.0
CIN2	1	10.0	4	40.0	5	50.0
CIN3	1	6.6	1	6.6	13	86.8
Carcinoma	0	—	0	—	9	100.0

Cytoplasmic expression: 1, weak; 2, strong.

and -negative patients (p 0.462) or between HPV oncogenic types and number of types (p 0.1777).

Normal squamous and glandular epithelia adjacent to the lesions did not express p16^{INK4a}.

Discussion

Using immunohistochemical analysis, we determined the presence of p16^{INK4a} in histologic samples of cervical biopsies from 60 patients with ATZ and atypical cytology or negative cytology, considering the following parameters: percentage of cells positive for p16^{INK4a}, reaction intensity, and cellular reaction pattern.

Our results show that the expression of p16^{INK4a} increases from normal to invasive squamous carcinoma in the uterine cervix and reinforce that it might be a useful prognostic marker for risk of developing cervical cancer in women infected with HPV. All cases of invasive cancer in this study had p16^{INK4a} expression, and only two patients with high-grade lesions (CIN2 and 3) did not express p16^{INK4a}. Importantly, 66% of the patients with CIN1 expressed p16^{INK4a}, and 40% of them had high frequency of positive cells with strong intensity of expression of the protein in 20% of the cases. All cases that expressed p16^{INK4a} in CIN1 group were infected with high risk HPV. These findings suggest that among CIN1 patients, there is a subgroup that may be at an increased risk of progressing to invasive cancer and should be followed up more closely. According to previous reports in the literature, pRB inactivation via the p16/cdk-cyclin/RB pathway and increase in p16^{INK4a} expression in HPV-transformed cells is an important mechanism for cervical carcinogenesis [9,13,18,22,23]. Also reported is an increased p16^{INK4a} expression virtually in all cases of cervical dysplasia, with no expression observed in normal or inflammatory epithelia of cervical mucosa [12]. Our finding of

expression of p16^{INK4a} in cases of CIN1 is in accordance with von Knebel Doeberitz et al., who reported that approximately 60% of low-grade lesions strongly stained for this marker in the basal layer of the squamous epithelium. Those authors also reported that lack of p16^{INK4a} expression occurred in high-risk HPV infection cases that showed viral replication characteristics (koilocytes) [29]. A recent study with CIN1 found that cases with diffuse p16^{INK4a} staining had a significantly higher tendency to progress to a high-grade lesion than that of p16^{INK4a}-negative cases [19]. This suggests that part of the low-grade lesions showed deregulated expression of the viral oncogenes in the basal or parabasal compartments. Therefore, lesions with increased p16^{INK4a} expression may probably exhibit an aberrant pattern of the E6–E7 genes, while p16^{INK4a}-negative lesions shall not express these viral oncogenes in basal cells and, thus, have little risk of progression [29]. Klaes et al. found 100% of p16^{INK4a} expression in CIN1 associated with high-risk HPV. They explained that this elevated rate was due to the fact that all cases came from cones, and the lesions were clinically more advanced than simple acute infections [13].

Among our histologically normal cases, only one showed a weak positive reaction for p16^{INK4a}. The case came from a patient with AIDS, positive for HPV 68, 73, and 84, with a previous cytological diagnosis of low-grade intra-epithelial lesion. HPV 68 and 73 are both high-risk factors for development of cancer, and it is possible that biopsy failed to show an area of CIN1 or a more advanced grade that could have been present. The detection of sporadic focal positivity of p16^{INK4a} staining in a low proportion of cases of normal squamous mucosa also was noted by other authors [9,28,30].

Progression from low-grade to high-grade CIN is accompanied by integration of the viral genome to the host chromosome [25]. In our study, the only CIN2 case negative for p16^{INK4a} was positive for both HPV 53 and 16. Among CIN3 cases, only one case positive for HPV16 was negative for p16^{INK4a}, and all carcinoma cases were positive.

Among p16^{INK4a}-positive cases, we verified a direct relation between lesion severity and reaction intensity. In all p16^{INK4a}-positive cases, there was a similar intensity in nuclear reaction; however, intensity of cytoplasmic staining varied according to the grade of CIN. This may be reflected by an increased synthesis of this protein and its overexpression in the cytoplasm.

To verify p16^{INK4a} expression, we evaluated the frequency of positive cells, reaction intensity, and cell staining pattern. All of these parameters were statistically significant and different when compared among different histological groups. Nevertheless, logistic regression model showed that the reaction intensity was superior to any other analyzed parameter, thus

being the best indicator of the expression of p16^{INK4a}. Taking into account the group pairs of histological lesions and the evaluation of protein p16^{INK4a} expression by Dunn's and Holmes' tests, we might infer that there is a significant difference between normal and CIN1 groups, and between CIN1 and carcinoma but no difference between CIN1 and CIN2, and CIN3 and carcinoma. This indicates that p16 is useful for separating HPV-negative patients or patients infected with low-risk HPV from those infected with high oncogenic risk HPV in which oncogenesis has already begun. Our findings point out that p16^{INK4a} can be useful not only in separating low-grade lesions from high-grade ones, but also to suggest that low-grade lesions are at increased risk of progression to cancer because of possible genomic incorporation of oncogenic HPV. Our findings support the idea that the integration of HPV to the genome of the host cells can occur in some CIN1 cases, as previously reported [15].

The HPV prevalence observed was as expected in most cases, namely very high in HIV-positive group, irrespective of the histopathological results, but increasing with disease severity in the HIV-negative group. The only exception is the low frequency of HPV positivity in tissues from invasive carcinomas. We speculate that these tissues had extensive areas of necrosis or few areas of epithelium, which could explain these results. Simultaneous infection with more than one type of HPV is not associated with an increased risk of cancer, compared to infection with only one viral type [17]. We did not observe any difference in expression of p16^{INK4a} in relation to simultaneous infection of viral types in the samples. Isolated HPV16, which was the most prevalent one in our series when compared to the other types, showed no statistical difference in p16^{INK4a} expression.

Our study reinforces that p16^{INK4a} expression in precancerous lesions and cancers can be used to identify HPV-transformed cells. P16^{INK4a} expression is conspicuous and fulfills the requirements for good biomarkers, indicating aberrant expression of viral oncogenes in replicating epithelial cells and potential risk of developing cervical cancer [23].

Of great interest for routine diagnostic use is the fact that immunohistochemical testing for p16^{INK4a} to identify HPV-positive cells has the potential to recognize those lesions with an increased risk of progression to high-grade lesions. However, further studies of a prospective nature are needed to evaluate the clinical utility of p16 expression as a tumor marker in cervical carcinogenesis.

CIN1 has a high, spontaneous regression rate, and about 70% of the cases do not need treatment. Yet, these lesions are of greater diagnostic difficulty among CIN, being responsible for the greatest inter- and intra-observer discrepancies. In this scenario, immunohistochemistry with p16^{INK4a} could help with patient follow-

up, avoiding unnecessary treatment in women with p16^{INK4a}-negative CIN1.

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References

- [1] P. Cairns, T.J. Polascik, Y. Eby, K. Tokino, J. Califano, A. Merlo, L. Mào, J. Herath, R. Jinkins, W. Westra, J.L. Rutter, A. Buckler, E. Gabuelson, M. Tockman, R. Chok, L. Hedrick, G.S. Bova, W. Isaacs, W. Koch, D. Schwab, D. Sishansky, Frequency of homozygous deletions at p16/CDKN2 in primary human tumors, *Nat. Genet.* 11 (1995) 210–212.
- [2] G.M. Cordeiro, Modelos Lineares Generalizados, VII SINAPE, UNICAMP Campinas, 1986, 286pp.
- [3] S. Duensing, K. Munger, Centrosome abnormalities, genomic instability and carcinogenic progression, *Biochim. Biophys. Acta* 1741 (2001) 81–87.
- [4] M. Dürst, S. Seagon, S. Wanschura, H. zur Hausen, J. Bullerdiek, Malignant progression of an HPV16-immortalized human keratinocyte cell line (HPK1A) in vitro, *Cancer Genet. Cytogenet.* 85 (1995) 105–112.
- [5] N. Dyson, P.M. Howley, K. Munger, E. Harlow, The human papillomavirus-16E7 oncoprotein is able to bind to the retinoblastoma gene product, *Science* 243 (1989) 934–937.
- [6] M. Giarre, S. Caldeira, I. Malanchi, F. Ciccolini, M.J. Lea, M. Tommasino, Induction of pRb degradation by the human papillomavirus Type 16E7 protein is essential to efficiently overcome p16^{INK4a}-imposed G1 cell cycle arrest, *J. Virol.* 75 (2001) 40705–44712.
- [7] J.G. Herman, A. Merlo, L. Mao, Inactivation of the CDKN2/p16/MTS 1 gene is frequently associated with aberrant DNA methylation in all common human cancers, *Cancer Res.* 55 (1995) 4525–4530.
- [8] G.Y. Ho, R.D. Burk, S. Kein, A.S. Kadish, C.G. Chang, P. Palan, J. Basu, R. Tachezy, R. Lewis, S. Romney, Persistent genital human papillomavirus infection as a risk factor for persistent cervical dysplasia, *J. Nat. Cancer Inst.* 87 (1995) 1365–1371.
- [9] J.T. Keating, A. Cviko, S. Riethdorf, L. Riethdorf, B.J. Quade, D. Sun, S. Duensing, E.E. Sheets, K. Munger, C.P. Crum, Ki67, cyclin E and p16INK4a are complementary surrogate biomarkers for human papilloma virus-related cervical neoplasia, *Am. J. Surg. Pathol.* 25 (2001) 884–891.
- [10] S.N. Khleif, J. Degregori, C.L. Yee, G.A. Otterson, F. Kaye, J. Nevins, P.M. Howley, Inhibition of cyclin D-CDK4/CDK6 activity is associated with an E2F-mediated induction of cyclin-kinase inhibitor activity, *Proc. Natl. Acad. Sci.* 93 (1996) 4350–4354.

- [11] B.R. Kirkwood, *Essentials of Medical Statistics*, Blackwell Scientific Publications, Oxford, 1988 288pp.
- [12] R. Klaes, T. Friedrick, D. Spitkovsky, R. Ridder, W. Rudy, U. Petry, G. Dallenbach-Hellweg, D. Schmidt, M. von Knebel Doeberitz, Overexpression of p16 INK4a as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri, *Int. J. Cancer* 92 (2001) 276–284.
- [13] R. Klaes, A. Dener, T. Friedrich, R. Rudiger, S. Herrington, D. Jenkins, R. Kurman, D. Schmidt, M. Stoler, M. von Knebel Doeberitz, p16INK4a immunohistochemistry improves interobserver agreement in the diagnosis of cervical intra epithelial neoplasia, *Am. J. Surg. Pathol.* 26 (2002) 1389–1399.
- [14] P.A. Lazo, The molecular genetics of cervical carcinoma, *Br. J. Cancer* 80 (1999) 20008–22018.
- [15] H. Lehn, L.L. Villa, F. Marziona, M. Hilgarth, H.G. Hillemans, G. Sauer, Physical state and biological activity of human papillomavirus genomes in precancerous lesions of the female genital tract, *J. Gen. Virol.* 69 (1988) 187–196.
- [16] D.J. Mann, N.C. Jones, E2 P-1 but not E2 F-4 can overcome p16 in G1 cell-cycle arrest, *Curr. Biol.* 6 (1996) 474–483.
- [17] N. Muñoz, F.X. Bosch, S. Sanjosé, R. Herrero, X. Castellsagué, K.V. Shah, P.J.F. Snijders, C.J.L.M. Meijer, Epidemiologic classification of human papillomavirus types associated with cervical cancer, *N. Engl. J. Med.* 348 (2003) 518–527.
- [18] Y. Nakao, Y. Yang, M. Yokoyama, A. Ferenczy, S.C. Tang, M. M Pater, A. Pater, Induction of p16 during immortalization by HPV16 and 18 not during transformation, *Br. J. Cancer* 75 (1997) 1410–1416.
- [19] G. Negrri, F. Vitadello, F. Romano, A. Kasal, F. Rivasi, S. Giralano, C. Mian, E. Ergarter-Vigl, p16^{INK4a} expression and progression risk of low-grade intraepithelial neoplasia of cervix uteri, *Virchows Arch.* 445 (2004) 616–620.
- [20] C.M. Roteli-Martins, S.F.M. Derchain, E.Z. Martinez, S.A.C. Siqueira, V.A.F. Alves, K.J. Syrjänen, Morphological diagnosis of HPV lesions and cervical intraepithelial neoplasia (CIN) is highly reproducible, *Clin. Exp. Obstet. Gynecol.* 28 (2001) 78–81.
- [21] C.M. Roteli-Martins, V.A.F. Alves, R.T.M. Santos, E.Z. Martinez, K.J. Syrjänen, S.F.M. Derchain, Value of morphological criteria in diagnosing cervical HPV lesions confirmed by in situ hybridization and hybrid capture assay, *Pathol. Res. Pract.* 1997 (2001) 677–682.
- [22] T. Sano, T. Oyama, K. Kashiwabara, T. Fukuda, T. Nakajima, Immunohistochemical overexpression of p16 protein associated with intact retinoblastoma protein expression in cervical cancer and cervical intraepithelial neoplasia, *Pathol. Int.* 48 (1998) 580–585.
- [23] T. Sano, T. Oyama, K. Kashiwabara, T. Fukuda, T. Nakajima, Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions, *Am. J. Pathol.* 153 (1998) 1741–1748.
- [24] N.F. Schlecht, A. Trevisan, E. Duarte-Franco, T.E. Rohan, A. Ferenczy, L.L. Villa, E.L. Franco, Viral load as a predictor of cervical intraepithelial neoplasia, *Int. J. Cancer* 103 (2003) 519–524.
- [25] E. Schwarz, U.K. Freese, L. Gissmam, W. Mayer, B. Roggeback, A. Stremow, H. zur Hausen, Structure and transcription of human papillomavirus sequences in cervical carcinoma cells, *Nature* 314 (1985) 111–114.
- [26] C.J. Sherr, Cancer cell cycles, *Science* 274 (1996) 1672–1677.
- [27] K.J. Syrjänen, Natural history of cervical HPV infections and CIN, In: K. Syrjänen, S. Syrjänen (Eds.), *Papillomavirus Infections in Human Pathology*, Wiley, New York, 2000, pp. 142–166 (Chapter 6).
- [28] B. Tringler, C.J. Gup, M. Singh, S. Groshong, L. Shroyer, D.E. Heinz, K.R. Shroyer, Evaluation of p16^{INK4a} and pRb expression in cervical squamous and glandular neoplasia, *Hum. Pathol.* 35 (2004) 689–696.
- [29] M. von Knebel Doeberitz, New markers for cervical dysplasia to visualize the genomic chaos created by aberrant oncogenic papillomavirus infections, *Eur. J. Cancer* 38 (2002) 2229–2242.
- [30] J.-L. Wang, B.-Y. Zheng, X.-D. Li, T. Angström, M.S. Lindström, K.-L. Wallin, Predictive significance of the alterations of p16INK4a, p14ARF, p53 and proliferation cell nuclear antigen expression in the progression of cervical cancer, *Clin. Cancer Res.* 10 (2004) 2407–2414.
- [31] J.M. Walboomers, M.V. Jacobs, M.M. Manos, Human papillomavirus is a necessary cause of invasive cervical cancer worldwide, *J. Pathol.* 189 (1999) 12–19.
- [32] H. zur Hausen, Papillomavirus infections: a major cause of human cancers, *Biochim. Biophys. Acta* 1288 (1996) 55–78.