Chapter 6

The Diagnostic of Cervical Carcinoma: From Theory to Practice

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Additional information is available at the end of the chapter

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Abstract

Human papillomaviruses (HPV) are naked particles composed of 72 subunits, each formed by 2 structural proteins designated L1 and L2 (L = late). HPV does not grow outside of squamous epithelium cells, in which it infects the suprabasal prickle cell layer. The viral double-stranded DNA (vDNA) has about 8 kilobase pairs (kbp) and also encodes several non-structural polypeptides, designated E1–E7 (E = early). At least 3 early oncoproteins (E5, E6, and E7) induce host cell proliferation, driving them into permanent division. During long-term latency, the circularized HPV DNA may get integrated into the host cell DNA molecule. The circular HPV DNA is then interrupted, usually within the E2 open reading frame (ORF), which then cannot exert its regulatory (feedback) effect on the early gene expression. The increased expression of E6/E7 proteins seriously affects the regulation of host cell division mainly via dysregulation of the functions of p53 and Rp proteins. HPV infects the female genital tract representing the main cause of cervical dysplasia and subsequent squamous cell carcinoma (SCa). The HPV isolates exist mainly in the form of amplified DNAs; based on the similarity and/or variations (dissimilarity) of their L1 capsid polypeptide sequence, 96 human genotypes were included into five genera of the *Papillomaviridae* family. The clinically most important genotypes that cause lesions at mucosal membranes and/or on the skin, belong mainly to the Alphapapillomavirus genus. The genotypes, associated with severe dysplastic changes and/or cervical cancer, were designated as high risk (HR-HPV). The prevalence of the integrated HPV DNA sequence over the episomal molecules appears in a proportion smears-graded LSIL (low-grade squamous intraepithelial lesion). Later on, carrier cells revealing the integrated HPV genome expression the oncoproteins (E6/E7) clearly prevail especially in HSIL (high-grade squamous intraepithelial lesion) smears and in the cervical cancer itself. What is crucial for the modern diagnostic of cervical dysplasia, is the p16/INK4A (inhibitor kinase) polypeptide, which itself represents a form of cell defense against the viral oncogenic proteins. The p16 antigen shows a continuous parabasal staining in the CIN I lesion. If dysplastic cells occupy at least one half (or two thirds) of squamous epithelium, the

designation CIN II/HSIL is correct, and at the stage of CIN III/HSIL, dysplastic cells replace the entire squamous epithelium. Another frequently used immunohistochemical marker of intraepithelial cervical dysplasia so far is the Ki-67 antigen, which occurs in the nuclei of proliferating and/or repeatedly dividing (immortalized) cells. Women revealing p16-positive ASCUS (atypical smear cells of unknown significance) as well as those showing LSIL (low-grade cytological changes) should be examined for the presence of the HPV DNA. The detection of HPV DNA alone, that is, in the absence of cytological screening, has a relatively lower prediction value, though the HR HPV positive DNA test in the absence of morphological alterations may in part predict the possible progression into malignancy. Nevertheless, only the combined cytological as well as molecular follow-up (cervical smear examined for cytology as well as for HPV DNA) is regarded for the most reliable diagnostic approach.

Keywords: Human papillomavirus, Cervical intraepithelial neoplasia (CIN), Cervical dysplasia, Squamous cell carcinoma, p16/INK4A Polypeptide, Ki-67 polypeptide, Laboratory diagnostic, PA smears, Squamous intraepithelial lesion (SIL), Liquid-based cytology (lbc), Cervical biopsy, Histology, HPV DNA testing

1. Introduction

Virus infection had been for a long time anticipated in the pathogenesis of cervical cancer. The suspected role of herpes simplex virus 2 (HSV 2) early protein as suggested by Aurelian et al. [1] was not confirmed, since several carefully designed prospective and/or serological studies following the significance of elevated anti-HSV 2 antibody levels did not confirm any association. Although certain isolated DNA fragments coming from the HSV genome may transform the tissue culture cells of rodent origin, their relevance in the induction of human cervical carcinoma has not met any further approval [2, 3]. Contemporarily, the human papillomavirus (HPV) has been accepted to represent the most probable infectious agent in respect of cervical cancer [4]. During the last decades, a great bulk of convincing data has accumulated in favor of the latter assumption, which has become widely accepted. Thus, based on substantial clinical and biological evidence, strong relationship exists between HPV infection and the development of cervical cancer. Nevertheless, in line with a few but independent observations, a hypothesis has repeatedly emerged saying that HSV 2 co-infection might represent an accelerating cofactor for cervical carcinoma formation [5]. Novel reports from Brazil and/or some other comparative studies on the given issue, which had been performed simultaneously among Indonesian and Swedish population, confirmed the possible role of HSV 2 in the former but not the in the latter geographical area [6].

Our paper reconciles the role of latent HPV infection (mainly based on the detection of corresponding DNA sequences and/or their fragments) in the stratified squamous epithelium cells of oral, vaginal, and/or anal mucosa (especially at their transition sites to cylindrical epithelium, such as uterine cervix) and/or in the keratinized squamous skin epithelium cells. Regarding to the role of the HPV genome in the pathogenesis of cervical cancer, we shall point at the importance of a complex diagnostic approach overcoming the possible barriers between

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scientific disciplines such as virology, pathology, and molecular biology on one hand and explain the need of the rapid application of novel achievements in diagnostic pathology, biochemistry, cytology as well as in the clinical practice. Despite of the difficulties of any interdisciplinary approach, the introduction of molecular virology (such the HPV DNA test) and immunohistochemical procedures (p16 and Ki-67 antigen detection) into diagnostic pathology and/or cytology when done in close cooperation with gynecologists has led to a dramatic decrease of the frequency of cervical cancer in several European countries, Slovakia not excluding. Reporting the frequency of positive cervical smears was registered in the terms of the conventional Papanicolaou (PA) test, which is still most suitable for large-scale screening. **Table 1** summarizes six annual reports of one of several Alpha medical Company Ltd Diagnostic Centres, which were destined for domestic insurance companies. The categories of classification of cervical dysplasia in given statistics do not correspond to the Bethesda classification system [7, 8]. According to the latter, atypical squamous cells of unknown significance (ASCUS) should be distinguished from non-neoplastic reactive changes of cervical squamous epithelium cells using the criteria summarized in **Table 2**. The squamous intraepithelial lesion (SIL) can be recognized by the presence of dysplastic cells, which show either mild (low grade) or more severe (high grade) alterations (**Table 3**). The original cytological nomenclature used to describe the appearance of cells in conventional PA smears before the introduction of Bethesda classification system has been repeatedly compared by several authors [9, 10] with the recently adopted definitions. Based on these data, we proved the individual categories of the so-called MKCH classification system (Slovak abbreviation for the expression International Classification of Diseases). To achieve an upmost precise interpretation, we used terms essentially resembling to the CIN (cervical intraepithelial lesion) classification, namely a similar three-degree scale (I—low, II—medium, and III—high). Evaluating in detail each protocol from the archives of the Pathology Diagnostic Centre of Alpha medical Company in Martin coming from a single-year period, that is, the year 2015 (compare **Table 4**), we found that the medium-grade cervical dysplasia category from the MKCH statistics in fact encompassed as many as 64% of smears-graded LSIL, while the proportion smears scored HSIL in the same category was approximately 6%. Noteworthy, another 6% fall into the group of patients with negative smears and the rest protocols revealed the diagnosis of ASCUS and/or ASC-H (in 24%). It should be mentioned that out of 516 samples of the given MKCH category, 110 had been repeatedly tested patients (these women were re-examined within 4–6 months intervals, i.e., at least twice during the same year). Thus, the MKCH category "medium-grade cervical dysplasia" consisted of 516 smears coming from 457 women. In the group of repeatedly examined patients, 10% underwent spontaneous healing during the relatively short 1-year follow-up period (data not shown). The repeatedly negative smears came mainly from patients, which were at first examination scored ASCUS. Unfortunately, their HPV DNA status is not always determined, even though in this group ("medium-grade cervical dysplasia") during the year 2015, altogether 162 of smears were tested HPV DNA, from which 74 were positive (a proportion of 39.5%), while 13 were HPV type 16 positive and 6 were HPV type 18 positive. While only 32% of screened patients were examined by cytology as well as for HPV, many HPV tests were made in the absence of cytology and/or *vice versa* (compare paragraphs 4 and 5, see also **Table 12**).

Diagnosis	Patient number		Per cent	Modified[4]
	Positive	Total examined		
Low-grade cervical dysplasia[*,1]	3070[**]	206,317	1.5%	3943 (1.9%)
Medium-grade cervical dysplasia[2]	1456		0.7%	Cancelled
High-grade cervical dysplasia[3]	263		0.13%	466 (0.23%)
ASCUS (includes also ASC-H, from low and medium dysplasias)[1,2]				230 (0.13%)
Carcinoma *in situ*	36		0.02%	No change
Cervical (spinocellular) carcinoma	16[***]		0.007%	No change

[1]Corresponds to LSIL (88%) and/or ASCUS (12%).[2]Corresponds to LSIL (60%), ASC-H/ASCUS (18%) and/or to HSIL (10%).[3]Corresponds to HSIL.[4]Modified according to above mentioned[1,2,3], remarks (see text for details).[*]Designation of given statistical categories according to author´s translation (from Slovak).[**]Positive smears after excluding the negative ones or those referred to as reactive changes.[***]Including 5 cases showing invasive growth into distant into (surrounding) tissues.[#]From report of Pathology Diagnostic Centre, Alpha medical Company Ltd, Martin (Slovakia) for domestic authorities (with permission).

Table 1. Frequency of cervical dysplasia and/or cervical cancer by screening of cytological smears from 1 January 2010 to 31 December 2015[#].

Atypical cells of unknown significance (ASCUS)	Reactive changes due to inflammation
Size of nuclei increased significantly (three times)	Slightly enlarged nuclei (up most two times)
Cells revealing doubled nuclei may be present	
Moderately dense chromatin staining	Slight hyperchromatic staining only
The fine granular chromatin is dispersed throughout nucleus	
Nuclei may be slightly elliptic, but regularly are nearly round shaped	
The nuclear/cytoplasm ratio (N/C) in moderately increased	
	Atrophic or shrink nuclei
	The cytoplasm may be vacuolated
	Polymorphonuclear leukocytes are present
	Bacteria may be present
Koilocytes (proving HPV) can be recognized	
Hyperchomatosis, dense chromatin granules.	
The nuclear membrane has irregular appearance	
A faint perinuclear ring may be visible	

* Based on the data from corresponding manuals [11, 12].

Table 2. Definition of ASCUS versus reactive changes in cervical smears[*].

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LSIL	HSIL
Nuclei relatively enlarged (over three times)	Nuclei increased in size, shrink cytoplasm
	Dysplastic cells frequent, smaller in size
	The small dysplastic squamous cells form larger aggregates
The N/C ratio increased not only due to larger nuclei but also by loss of cytoplasm	
The size of nuclei varies, multiple nuclei* (larger polykaryocytes)**	
Evident hyperchromatosis, dark nuclei	
Chromatin shows irregular distribution	
Relatively fine nuclear granules	Coarse grainy chromatin
Occasionally irregular nuclear membrane	Undulate nuclear membrane
Nucleoli may be visible	Nucleoli are not seen
	The cytoplasm may show keratin granules

* Based on data from corresponding manuals [11, 12].

Table 3. Definition of low-grade versus high-grade squamous intraepitelial lesion (SIL)*.

Diagnosis	Patient number		Per cent
	Positive	Total examined	
Low-grade squamous intraepithelial lesion*	1324	37,414	3.54%
High-grade squamous intraepithelial lesion**	151		0.4%
Atypical cells of unknown significance***	231		0.621%
Carcinoma *in situ*	3		0.04%
Cervical carcinoma (SCa)[1]	2		0.005%

* LSIL ** HSIL *** ASCUS.
[1] Squamous cell carcinomas.

Table 4. The frequency of cervical dysplasia and/or cervical cancer by screening of cytological smears from 1 January 2015 to 31 December 2015.

Alternatively, we analyzed a proportion of still available original protocols from a limited number of women (300 at random chosen patients were re-evaluated out of 1425, i.e., 20.6%), in order to assess the estimate number of LSIL and HSIL cases in result of cancelling the undesired category of "medium-grade dysplasia". Despite of some doubts concerning the precision of such calculations, we could demonstrate the decreased incidence as well as a significantly lower morbidity for cervical carcinoma within the last 6-years period. Regarding

to the frequency of 7.5 positive smears out of 100,000 samples enrolled, and comparing this number with the overall morbidity rate of 15.4/100,000 as reported for Slovakia in 1999 [13], the estimated decrease of cervical carcinoma cases should be much higher than 50%. Namely, the former number reflects the relative proportion of positive samples out of the total enrolled, while the latter represents the frequency of disease in whole women population (either in the fertile and/or post-fertile age). The estimated proportion of women examined in comparison with the total woman population in given age might range from 10 to 20%. Even if this proportion of followed may not be correct, the decreased morbidity for cervical cancer in Slovakia during the last decade might be at least tenfold. Furthermore, **Table 1** also shows that at least 6% (by minimal rate 263/3070) but not more than 12% (by maximal rate 466/3943) of smears which had been scored LSIL progressed into the stage of HSIL. For the above-mentioned minimal rate, the intermediate MKCH category was not taken into account; for the maximum rate, it has been split (modified) into corresponding Bethesda categories as explained above. Thus, regardless to any imperfections of both calculations, which may arise from comparison of the Tables 1 and 4, our data point at an increased incidence of newly emerging LSIL cases. Namely, the average positive rate among the smears, which was in the range of 1.5–1.9% during the last 6-years period, has reached 3.5% in the year 2015 (an increase of 180%). As already suggested in our previous paper [14], and confirmed by others, the probability of transition from LSIL and/or ASC-H cases into HSIL strongly depends on the presence of HPV as detected by one of the available HPV DNA tests [15, 16]. The great majority of the ASCUS cases and up to 85% of LSIL cases (especially in the absence of HPV DNA) might undergo spontaneous healing as shown in next paragraph.

2. The role of human papillomavirus (HPV) in the pathogenesis of cervical dysplasia and cancer

The first mammalian papillomavirus (PV) was described by Shope and Hurst [17], who characterized the transmissible nature of cutaneous papillomas arising in wild cottontail rabbits. The narrow host range of PVs in culture to sites with stratified squamous epithelia that is either cornified (skin) or non-cornified (mucosa) was overcome by introducing molecular technics such as the DNA extraction, the polymerase chain reaction (PCR), and vDNA sequencing allowing to identify the genes of the PV DNA regarding to the function of corresponding proteins. The papillomaviruses (PVs) comprise a group of non-enveloped epitheliotropic DNA viruses that predominantly induce benign lesions of the skin (warts) and mucous membranes (condylomas). Some PVs have also been implicated in the development of epithelial malignancies, especially in the cancer of uterine cervix, certain tumors of urogenital tract, and upper airway cancers [18]. Due to given relationships, an increasing amount of information has accumulated from sequencing results of various PV DNAs. Later on, the PVs were classified according to the host species they infect, so that the PVs of human origin were designated as human PVs (HPV). Officially recognized by the International Committee on the Taxonomy of Viruses (ICTV), the former *Papovaviridae* family now falls into two separate families, *Papillomaviridae* and *Polyomaviridae* [19]. The reason being the missing helicase motif

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in the HPV E1 protein, a domain stretching longer than about 230 amino acids (aa) within the analogous non-structural HPV polypeptide, which otherwise has some similarity with the SV40 T-antigen, the parvovirus NS1 protein, and with a planarian virus-like element [20].

Among the most extensively studied HPV genomes, nearly 100 genotypes were described based on the at least 90% nucleotide homology of sequence encoding one of 2 structural capsid proteins (the L1 protein sequence). The L1 ORF is the most conserved gene within the genome and has therefore been used for the identification and classification of new HPV types corresponding to later characterized species is shown in **Table 5**. It should be mentioned that sorting into species and genus has some theoretical and/or scientific importance, but for practical reasons, the old genotype classification is still in use (**Table 6**). A new HPV isolate is recognized as such, if its complete genome has been cloned and sequenced to determine in which extent the L1 ORF differs from the closest known HPV genotype. If a more than 10% bp difference can be found that is a distinct genotype. Differences between 2 and 10% homology define a subtype and <2% difference reveals a variant. The closely related HPV types HPV-2 and 27, HPV-6 and 11, and HPV-16 and 31, which cause common warts, genital warts, and cervical cancer, respectively, are excellent examples of numerous consistencies between phylogeny and pathology. Furthermore, the HPV genotypes could have been distinguished as high risk (HR) and low risk (LR) according to their ability to transform cells, due to their relationship to cervical dysplasia and/or cancer, and according to their frequency. The squamous carcinoma (SCa) cells and/or the cells in *epidermodysplasia veruciformis* (EV) harbor multiple genome copies of specific HPV types, especially HPV5 and HPV8, but also of HPV14, HPV20, and a few others [21–23]. Their transcripts have been described in several the so-called EV-associated SCa cells [24]. In 2009, HPV5 and HPV8 were classified by IARC as "possibly carcinogenic" in EV patients [25].

Genus	Properties	Species	Genotype(s)	Clinical significance
Alphapapillomavirus	Mainly	*1*	HPV32, HPV42	Benign lesions, oral or genital mucosa, LR
	mucosa but	*2*	HPV3, HPV10	genotypes, benign cutaneous or mucosal
	also skin		HPV28, HPV29	lesions,
	lesions;		HPV78, HPV94	
	conserved E5	*3*	HPV61, HPV72,	LR genotypes, mucosal lesions
	ORF within		HPV81, HPV83,	
	the ERL* ;		HPV84	
	HR genotypes	*4*	HPV2, HPV27,	Skin warts (common), frequently benign,
	immortalize		HPV57	LR genotypes,
	keratinocytes,	*5*	HPV26, HPV51,	HR genotype, mucosa lesions
			HPV69, HPV82	
		6	HPV30, HPV53,	HR genotypes, mucosa lesions,
			HPV56, HPV66	some are LR genotypes

Genus	Properties	Species	Genotype(s)	Clinical significance
		7	HPV18, HPV39,	HR genotypes, mucosal lesions
	E5 ORF is		HPV45, HPV59,	
	different		HPV68, HPV70,	
		8	HPV7, HPV40,	LR genotypes, butcher warts, skin and
			HPV43	mucosa
		9	HPV16, HPV31,	HR genotypes, mucosal lesions
			HPV33, HPV35,	
			HPV52, HPV58,	
			HPV67	
		10	HPV6, HPV11,	LR genotypes, rarely verrucous carcinoma
			HPV13, HPV44,	
			HPV73, HPV74	HR genotypes, mucosal (cervical) lesions
		11	HPV34, HPV73	(dysplasia and carcinoma)
		13	HPV54	LR genotype, mucosal lesions
		15	HPV71	LR genotype, mucosal lesions
Betapapillomavirus	Latent	1	HPV5, HPV8,	Mainly benign cutaneous lesions
	infection		HPV12, HPV14	
	possible in		HPV19, HPV20	
	general		HPV21, HPV25	
	population		HPV36, HPV 47,	
			HPV93,	
		2	HPV9, HPV15,	Commonly associated with EV,
			HPV17, HPV22,	mucosal lesions and detection of vDNA in
			HPV23,HPV37,	immunosuppression,
			HPV38,HPV80	most frequently LR genotypes
		3	HPV 49, HPV75,	LR genotypes, benign cutaneous
			HPV76	
		4	Candidate types 60	Pre-malignant cutaneous
		5	and 88	Pre-malignant cutaneous
Gammapapillomavirus		1	HPV4, HPV65	Cutaneous lesions, intracytoplasmic
			HPV95	homogenous inclusion bodies
		2	HPV48	Cutaneous lesions
		3	HPV50	Cutaneous lesions
		4	HPV60	Cutaneous lesions

Genus	Properties	Species	Genotype(s)	Clinical significance
		5	HPV88	Cutaneous lesions
Nu-papillomavirus	Several not assigned ORFs,	1	HPV41	Bening as well as malignant cutaneous
Mu-papillomavirus	Human PV, inclusion bodies specific	1 2	HPV1 HPV63	Heterogenous cytoplasmic inclusions, filamentary cytoplasmic inclusions, LCR/URR has 982 bp

[1] According to deVilliers et al. [30]; [2] epidermodysplasia verruciformis. * ERL = the DNA segment between E and L ORFs.

Table 5. Classification of papillomaviruses and the list of their important genotypes.

Disease	Frequent association	Rare association
Skin infection		
Deep plantar warts	1, 2	4, 63
Common warts	2, 1	4, 26, 27, 29, 41, 57, 65, and 77
Butcher's warts	7, 2	1, 3, 4, 10, and 28
Epidermodysplasia verruciformis (ER)*	2, 3, 5, 8, 9, 10, 12, 14, 15, and 17	19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, and 70
Skin carcinoma associated with ER	**5** and **8**	
Anogenital infection		
Condyloma acuminatum	6, 11	30, 42, 43, 44, 45, 51, 54, 55, 70
Intraepitelal dysplasia (CIN I a CIN II/III)	6**, 11**, **16**, **18**	(**16**, **18**, 74, 86) 6, 11, **26, 31, 33, 35, 39**, 42, 43, 44, **45, 51, 52**, 53, **56, 58, 66, 73**, and **82**
Carcinoma *in situ*; and invasive carcinoma	**16, 18, 31, 45**	**33, 35, 39, 51, 52, 56, 58**, 59, **66, 67, 68**, 73, and 82
Recurrent laryngeal papilloma	6, 11	
Conjuctival papilloma	6, 11, and **16**	

* Congenital skin lesion with high sensitivity to HPV infection.
** Mainly in CIN I/LSIL, CIN II and/or non-invasive forms CINIII/HSIL and/or CIN III+/HSIL.
# According to Bonnez [31].

Table 6. Clinical relevance of high-risk (in bold) and low-risk HPV genotypes according to their frequency.

The HPV virions are small non-enveloped capsids 55 nm in size and of icosaedral symmetry. They are composed of 72 subunits formed by 2 structural proteins (L1 and L2, L = late), which are synthesized at the late intervals of productive replication cycle. The viral double-stranded DNA (vDNA) has about 8 kilobase pairs (kbp); it encodes 7 or 8 non-structural polypeptides, designated E1–E8 (E = early). The transcription of viral mRNA is directed clockwise under the control of two promoters, namely the early promoter (P_{97}) and the late promoter (P_{670}) sequence. In between the initiation codon for the transcription of E1 polypeptide ORF (open reading frame) and the stop codon for the L2 capsid protein ORF, an approximately 1.1 kbp long control repeat (LCR) is situated that contains the origin of vDNA replication as well as several binding sites for the binding of viral E2 and/or E1 regulatory proteins (**Table 7**). In addition to the motifs for down-regulation of the productive replication cycle (on the basis of a feedback mechanism), the LCR (also referred to as the URR, upstream regulatory region) contains enhancer sites for attachment of cellular transcription cofactors promoting the binding of cellular RNA polymerase to accomplish viral mRNA synthesis.

Protein	MW	Properties and function
E1	68–76 kDa	Binds to the regulatory long control repeat (LCR/URR) upstream from the E6/E7 ORF promoters. Forms a heterodimer with E2, associates with H1 histones and with cyclins, especially with cyclin E.
E2	40–58 kDa	Activates viral mRNA transcription, binds to the LCR/URR sequence, associates with the E1 protein; acts as cofactor of vDNA replication; operates at distribution of newly copied vDNA molecules during cell division (also in latency). Suppresses the expression of E6/E7 proteins and interacts with the L2 capsid polypeptide (especially at productive virus replication). Induces apoptosis.
E3	10–17 kDa	Function unknown.
E4		Associates with the L1 capsid polypeptide and facilitates virion formation (during productive replication); co-localizes with cytokeratins being produced in the medium and upper spinous layers.

[1] Long control repeat/upstream regulatory region.

Table 7. Basic functions of HPV-coded early (non-structural) polypeptides involved in the replication and latency.

Protein	Molecular weight	Properties and function
E5	10 kDa	Increases and prolongs the activities of receptors interacting with external growth factors such as EGF and/or PDGF2 by binding to their cytoplasmic domain. Inhibits the acidification of endosomes, binds to ATPase within the membranes of vacuoles and increases the stability of the engulfed EGF/EGFR complexes. Activates cellular transcription factors such as AP1* and signal transmission mediating proteins such as c-Jun** and/or c-Fos.
E6	16–18 kDa	Binds to the pivotal cell division regulator p53 (cellular anti-onc protein), enhances its degradation by ubiqitination (most efficiently acting are the E2 from HPV16 and/or HPV18 genotypes).
E7	10–14 kDa	Binds to the retinoblastoma (Rb) protein (its p107 and p130 forms) , which regulate cellular DNA transcription via binding or release of the transcription initiation factor E2F (in response to phosphorylation of the Rb associated *cdk* complex). Activates mainly cyclins A and E,

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Protein	Molecular weight	Properties and function
		especially influencing the translocation into nucleus of the transcription cofactor AP1. Activates the histone deacetylase (HDAC) acting at the level of the so-called epigenetic regulation of transcription by removing its blockade.

[1] Epidermal growth factor [2] Platelet-derived growth factor, * Activator protein 1, * Cellular ju-na-na *** Cyclin-dependent kinas.

Table 8. Basic functions of the HPV-coded early (non-structural) oncoproteins.

The E2 polypeptide was initially described as a transcriptional activator [26] capable to initiate viral transcription through the E2 recognition elements located within early promoter (for HPV16, it is the above mentioned P_{97}, while alternatively, for HPV18, it is the P_{105}). As described below in more detail, tumorigenesis is mediated by an integrated HPV DNA fragment, encompassing the E6 and E7 genes exerting transformation activity (**Table 8**). During productive (vegetative) virus replication in human squamous epithelial cells, the essential activities displayed either by the HPV type 16 P_{97} or by the HPV type 18 P_{105} promoters can be repressed by the full-length E2 polypeptide, which binds to one of the four E2 binding sites upstream of either P_{97} or P_{105} [27]. The E2 protein also facilitates long-term persistence of HPV genome in host cells by episomal maintenance providing a mechanism ensuring the distribution of viral genome within the dividing epithelium and its segregation into daughter cells [28, 29]. This can be achieved due to the association of E2 with the mitotic spindles, when it interacts with condensed mitotic chromatin and thereby ensures that the viral genome (which duplicates within the dividing host cell during cellular DNA synthesis) gets attached to nuclear envelope at anaphase and reforms during telophase. The duplication of viral genome during mitosis is accomplished by means of the cellular DNA polymerase, which becomes modified by another early HPV-coded protein, the El polypeptide. The E1 is required for both the initiation and elongation of viral DNA synthesis being accomplished by the cellular DNA polymerase complex by means of its ATPase and DNA helicase activities. The E2 can complex with E1 to strengthen its affinity for binding to the origin of vDNA replication. The papillomavirus E2 protein has several well-characterized regulatory functions affecting viral transcription, viral DNA replication as well as long-term plasmid maintenance. In contrast to the duplication of HPV DNA, which is the main strategy to ensure long-term persistence of viral genome, in the course of acute virus replications, the necessary point is to generate many genome copies which will be packaged into virions. Why that process occurs only in the terminally differentiated cells of the upper squamous epithelium layer and in the benign vegetative vDNA replication, is not known. The switch in question may involve the presence or absence of cellular cofactors expressed in the differentiating keratinocytes, i.e. only within the cytokeratin forming upper squamous epithelium layer. As described later, these cells, if previously proliferating papilloma cells. The mechanisms regulating the switch from plasmid maintenance to vDNA replication not known. This switch may envolve the presence or absence of cellular cofactors expressed in the differentiating keratinocytes, thus is occurs only within the cytokeratin forming upper squamous epithelium layer. As described later, these cells, if previously infected within the lower squamous epithelium layer, then show typical morphol-

ogy, the so-called koilocytes. Clearly, the relatively increased levels of HPV proteins such as E1 or E2 (or their modifications) may change the appearance of terminally differentiating keratinocytes. One might anticipate that the vegetative DNA replication occurs bi-directionally, through a theta structure intermediate or by a rolling circle mode, which is the principle of vDNA replication in general. Finally, the virion assembly must take place in the nuclei of terminally differentiated keratinocytes, which also contributes to koilocyte formation. The nascent capsids might randomly attach to the HPV DNA, and are further stabilized by the formation of disulfide bonds between conserved cysteines on adjacent L1 monomers acquiring resistance to proteolytic digestion. Taken together, as result of productive replication, the virions form large aggregates within the nuclei of infected keratin forming upper squamous epithelium cells, which then regularly show koilocytosis [32], but rarely reveal cytoplasmic inclusion bodies (see **Table 5** for details).

The newly produced HPV virions do not appear outside of squamous epithelium cells, and its productive replication is closely bound to the suprabasal, mainly medium and upper slayer of dividing keratinocytes. At infection, the HPV virions preferentially bind to heparan sulfate proteoglycans (HSPGs) on the basement membrane or to the basal stem cells, which may be exposed to environmental influence at sites of epithelial trauma or permeabilization. The differentiating squamous epithelium cells cannot become infected. To achieve selective infection of basal differentiating squamous epithelium cells, the initiation of infection preferentially requires attachment to the basal stem cells layered at the basal membrane of stratified squamous epithelium [33–35]. The basement membrane-bound virion undergoes a conformational change of its L2 capsid protein that exposes a highly conserved N-terminal peptide motif to cleavage by furin or the closely related pro-protein convertase [36]. There is a remarkably long delay of 1–3 days between the capsid cell surface binding and its penetration resulting in the onset of viral genome transcription. Itself the internalization of capsids as starts from the attachment to cell surface until the uncoating process begins lasts at least 2–4 h and is very asynchronous. The endocytosis as well as the consequent transmembrane trafficking of HPV capsids is not fully understood. Penetration of engulfed capsids may be initiated either from the acidified late endosomes, in which the L2 conformation change takes place, or by clathrin-dependent uptake [37]. The penetrated virions are transported from the internal membrane surface by involutions called vilopodia, namely from their leading edge to the central cell body via actin-directed retrograde flow [38]. Classical observations testify that there is extremely difficult to isolate and propagate any HPV types in conventional human cell cultures [39].

During the last decade, several details of HPV induced continuous host cell proliferation have been elucidated. It became clear that three non-structural HPV-coded oncoproteins, namely E5, E6, and E7 (**Table 8**) are involved in host cell immortalization, which later on, under the conditions of continuous host cell division results in malignant transformation. It should be mentioned that the transformation as such is a multistep process, launched by the virus-coded oncoproteins as suggested the "hit and run" hypothesis. The E5 protein enhances the sensitivity of HPV carrier cells to external proliferative stimuli, such as the epidermal growth factor [40]. It also binds to the growth factor receptors, for example, to the platelet growth factor

receptor (PDGFR), and activates the signal transmission in a ligand-independent manner [41, 42].

Because the integrated vDNA regularly encompasses just the ORFs of E6 and E7 oncoproteins along with the closely positioned LCR sequence, a continuing and increased expression of E6/E7 proteins occurs which seriously affects the regulation of host cell division in direction of its down-regulation [43, 44]. The E6 polypeptide binds the pivotal cell division inhibiting p53 protein [45], while E7 polypeptide binds the retinoblastoma (Rb) protein and the cyclin inhibitory proteins p27 and p21 [46]. The HPV-coded oncoproteins E6/E7, when expressed in significant amounts, continuously drive the host cell from the phase G1 to phase S (synthesis), in which the replication of cell DNA proceeds in an unlimited manner. Noteworthy that immortalization and the process of cancerogenesis are not the same, since the latter is much more complex being related to several chromosomal alterations and to increased number of *c-myc* gene copies (probably arisen due to repeated host cell DNA sequence transpositions), a finding even proportional to the grade of HPV driven dysplasia [47]. In low-grade dysplasias (such as CIN I/LSIL), only a restricted number of the HPV oncogenes is expressed.

Nevertheless, E6 and E7, which have several other activities (**Table 8**), appear to be the main drivers for the progression to high-grade dysplasia and later on to cancer, by orchestrating a series of pathogenic changes. Both are transcribed from the same major early promoters located within the LCR region of their genome. As a rule, the expression of the episomal (not yet integrated) HPV genome is restricted to E6 and E7 polypeptides, which are present in the parabasal, lower layer of squamous epithelium. Here, the viral genome undergoes a regulated duplication along with the host cell proliferation, which is under physiological conditions regulated by corresponding stimuli. The E6 protein is expressed at substantially lower levels than E7, and in early LSIL lesions, since the level of E7 may be more limited than that of E6. Noteworthy, both genes are expressed from a single promoter (latency associated and different from the above-mentioned vegetative ones); their transcripts are alternatively spliced by a post-translation mechanism determining as well as regulating their relative level within the carrier host cell. Integration of the HPV DNA fragment into cellular DNA via non-homologous recombination represents a key change towards immortalization that appears to stabilize the high expression of E6/E7, which, in turn, becomes frequently associated with more severe lesions. Integration may still not occur in great majority of CIN I/LSIL lesions, but is present by an increased rate in HSIL/CIN II and/or CIN III lesions, and is the most frequent in pre-invasive cancer (Ca *in situ*). As a rule, whoever, the integrated E6/E7 ORF containing the HPV DNA fragment is at high probability found in the CIN III lesions. The frequency of viral DNA integration may vary with the HPV genotype, being more frequent for the high-risk (HR) genotypes such as HPV18 and/or HPV16.

The integration of vDNA and/or its fragment occurs due to interruption of its sequence, which can appear at many sites throughout the genome, but is found preferentially at fragile genomic sites, which undergo nicking and cutting in association recombination and/or translocation events of the host cell genome. In certain cases, practically the rest of whole genome may be deleted, so that only the E6 and E7 ORFs remain intact and ready for the transcription along with the nearby located LCR sequence, containing the crucial promoter and enhancer signals

lying upstream of the integration site [48]. In a given *in vitro* immobilized keratinocyte culture, the integration process usually involves only one locus or a few loci. The E6/E7 ORF transcription slowly increases due the loss of the feedback block provided by the viral E1 and E2 proteins, which ORFs had been either deleted or at least disrupted. This situation permits the constant expression of high levels of the undesired E6 and E7 mRNA molecules [49]. Consistent with the multistep nature of tumorigenesis, cervical cancers may show additional cytogenetic alterations as compared with adjacent high-grade dysplastic and/or carcinoma *in situ* lesions.

Low-grade dysplasia may be caused by infection with either low-risk (LR) or high-risk (HR) HPV. Persistent (i.e., long-term) infection with a HR HPV type, which occurs in a minority of infected women, is the most important risk factor for developing CIN III or pre-invasive cancer (CIN III+). However, the magnitude of the risk depends not only on the given HPV type, but even more on a HR variant within the given type (compare **Table 6**). In practical terms, HPV persistence usually means that the same HPV genotype can be recovered from at least two or more subsequently taken genital samples obtained over a period of 4–12 months. Persistent infection may clear spontaneously, but less likely it does so in the course of longer duration. Taken together, only some persistent infections progress to CIN III, and subsequently to invasive cancer, but the HR HPV16 infections so such outcome much more likely than other HPV types. The distinct biologic effects of HR E6 and E7 may present at least a partial explanation of the differences in the likelihood of low-grade dysplasia progression, which presence may not be consistently associated with the same probability of the progression to high-grade lesion. Thus, most genital HPV infections are self-limited, and the majority would clear within 12 months.

Taken together, the HPV genome persists within transformed host cells in two different forms: as a non-integrated (episomal) circularized full-length vDNA and, less frequently, as a linear and integrated vDNA sequence. There should be mentioned that during long-term latency, the integration of HPV DNA may occur due to the linearization of the persisting circular vDNA molecule. At integration, the HPV DNA chain gets either interrupted or partially deleted, preferentially at nt 3362–3443 of the E2 ORF [50, 51], usually within the E2 gene ORF [52]. The mixed (episomal as well as integrated) vDNA distribution pattern seems the most prevalent physical state of HPV16 DNA found in ASCUS-graded smears (atypical cells of unknown significance), but can also be detected in cervical scrapings which do not reveal dysplastic changes. This indicates that HPV infection may not always cause dysplasia of the squamous epithelium cells. The prevalence of the integrated HPV DNA sequence over the episomal molecules then appears in a proportion of ASCUS-graded smears (sometimes characterized by ASC-H cells) and in a proportion of LSIL smears, but later on becomes clearly prevalent in the HSIL-grade smears and, of course, in cervical cancer. Women with the prevalence of integrated HPV DNA were almost 10 years older than those with a predominating episomal HPV DNA pattern, which points to a higher risk of HPV infection in women aged over 35 years [53].

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3. Overexpression of the p16/INK4A regulatory polypeptide in dysplastic and/or proliferating cells

The aim of our further considerations was to assess the role of p16/INK4A (inhibitor kinase) protein, which is, as a rule, overexpressed in cells revealing increased E7 polypeptide production. The p16/INK4A (inhibitor kinase) polypeptide is a cellular regulatory protein, which inhibits the cyclin-dependent kinases (especially cdk4 and cdk6) associated with cyclins D and/or E. These kinases, if activated, phosphorylate the retinoblastoma phosphoprotein (pRb) complex, which in turn, releases the transcription factor E2F, which is bound in inactive form in non-dividing cells. Under physiological conditions (in normal cells), the Rb protein liberated from the disrupted complex has a feedback effect on p16 expression [47]. Since the E2 polypeptide binds to LCR, the presence of E2 in cells carrying the episomal (i.e., non-integrated) HPV DNA may efficiently control the transcription of mRNA encoding the E6/E7 oncoproteins [54]. In contrast, in cells which carry the integrated HPV genome, the E2 polypeptide production stops, since the E2 ORF becomes disrupted. Therefore, the E2 but also the E1 proteins (both

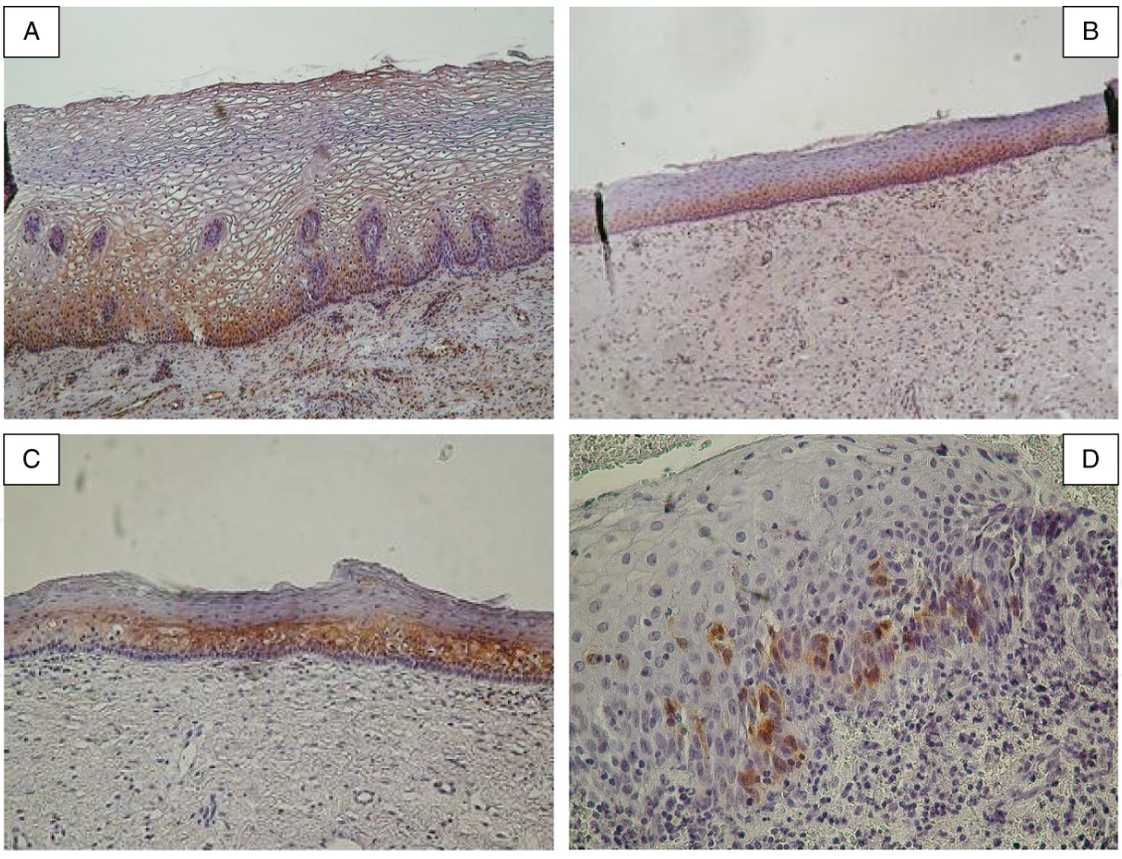

Figure 1. Different patterns of positive p16 staining in CIN I/LSIL. Upper line: continuous staining of p16 antigen of low (A) and/or high intensity (B) confined to the lower spinous layer and/or to the transit amplifying cells, which are situated in parabasal location just adjacent to the p16 negative basal stem cells. No dysplasia can be seen. Line below: staining of p16 antigen in a thin layer of dysplastic cells at parabasal area showing signs of HPV infection such as koilocytes (in the left, C). Bottom in the right (D): progressed dysplasia of the basal layer in part involving the stem cells, which cannot be clearly recognized.

closely involved in the maintenance of long-term latency) may be missing in dysplastic and/or HPV-transformed cells. Unlike to transformed cells, their production becomes down-regulated in the late phase of vegetative (productive) virus replication cycle. It can be stated that in HPV transformed cells, the expression of p16/INK4A protein increases proportionally to elevated levels of the increasing expression of the virus-coded E7 polypeptide, since both rise in the absence of E2 protein [55–58]. The p16/INK4 mRNA more stable and is present in higher levels in the cells in which the HPV DNA sequence had been integrated [59]. The quantification real-time–polymerase reaction (qRT PCR) is useful to identify the levels of transcripts encoding the p16 polypeptide in cervical smears of patients obtained for diagnostic purpose, an approach which is more tedious as the antigen staining, but is similarly sensitive and occasionally may yield more confident results. The p16 mRNA was present in 30% of LGSL but in 75% of HGSIL cases reaching a rate of 85.7% in squamous cell carcinoma cases [60].

The Bethesda scoring system, originally destined for vaginal/cervical smears [1988/1989], has been later adopted for the cervical biopsies at histologic examination. This has happened regardless to the fact that pathologists already had their own nomenclature, which is still in use for cervical biopsy grading widely known as cervical intraepithelial neoplasia (CIN). The dysplastic cells showing a diploid nuclear pattern are characterized by the loss of polarity, crowding, overlapping disorganization, and anisocytosis [61]. Therefore, the stage of CIN I dysplasia cannot be regarded for a truly neoplastic process. At cytological level, the dysplastic cells show altered nuclear-to-cytoplasm (N/C) ratio as well as wrinkling and thickening of nuclear membrane (**Table 4**) so that any CIN I grade changes may correspond to the entity of LSIL [62]. In mild forms of CIN I/LSIL, the dysplastic cells occupy the parabasal layer only but form a continuous zone within the lower third of cervical squamous epithelium, in the so-called lower squamous layer along with the transit amplifying cells, which early dysplasia can be better recognized by the p16 antigen staining (**Figure 1**). Summing up the results of p16 antigen staining in biopsy sections graded CIN I/LSIL, Yildiz et al. [63] could distinguish the continuous parabasal staining of higher intensity (**Figure 1B**), from parabasal staining of lower intensity (**Figure 1A**). In addition, they described the scattered form of positive p16 staining of single and/or small groups of upper squamous epithelium cells, which do not correspond to the suprabasal layer of lower squamous epithelium or the transit epithelium cells (**Figure 2D**). The production of p16 protein to an extent stainable with the commercially available anti-p16 monoclonal antibody (for example the CINTec histology kit) has been attributed to the stimulation of CDKN2A gene (encoding the p16 protein) by the stimuli activated via alternative receptor pathways regulating the transient reactive cell proliferation. This situation should be strongly distinguished from dysplasia, where the cell growth regulation undergoes some kind of dysregulation (for example, by the viral oncogenic polypeptide E7) that causes the switch to an autonomic growth due to repeated cell divisions. Thus, the increased p16 expression in single epithelium cells in a location where the E7 polypeptide may not be present may be related to HPV infection. Noteworthy, older textbooks before the introduction of p16 staining [64] referred to the histological picture of chronic cervicitis characterized by with hyperemia and round cell (lymphocyte) infiltration of the underlying connective tissue in addition to the reactivity and dilution would also influence the p16 antigen staining results ranging from clearly negative to false positive for above-mentioned reasons (**Figure 3**). During

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the last years, several big companies introduced automatic staining procedures, which on one hand standardized the staining intensity as well as its color, but on other hand, new staining variations appeared between different laboratories. Therefore, in cases suspicious for CIN I/LSIL, or if thep16 staining reveals a faint reactivity or shows a distribution other than parabasal, or does not correspond to a dysplastic cells area as seen in a parallel HE stained section, the

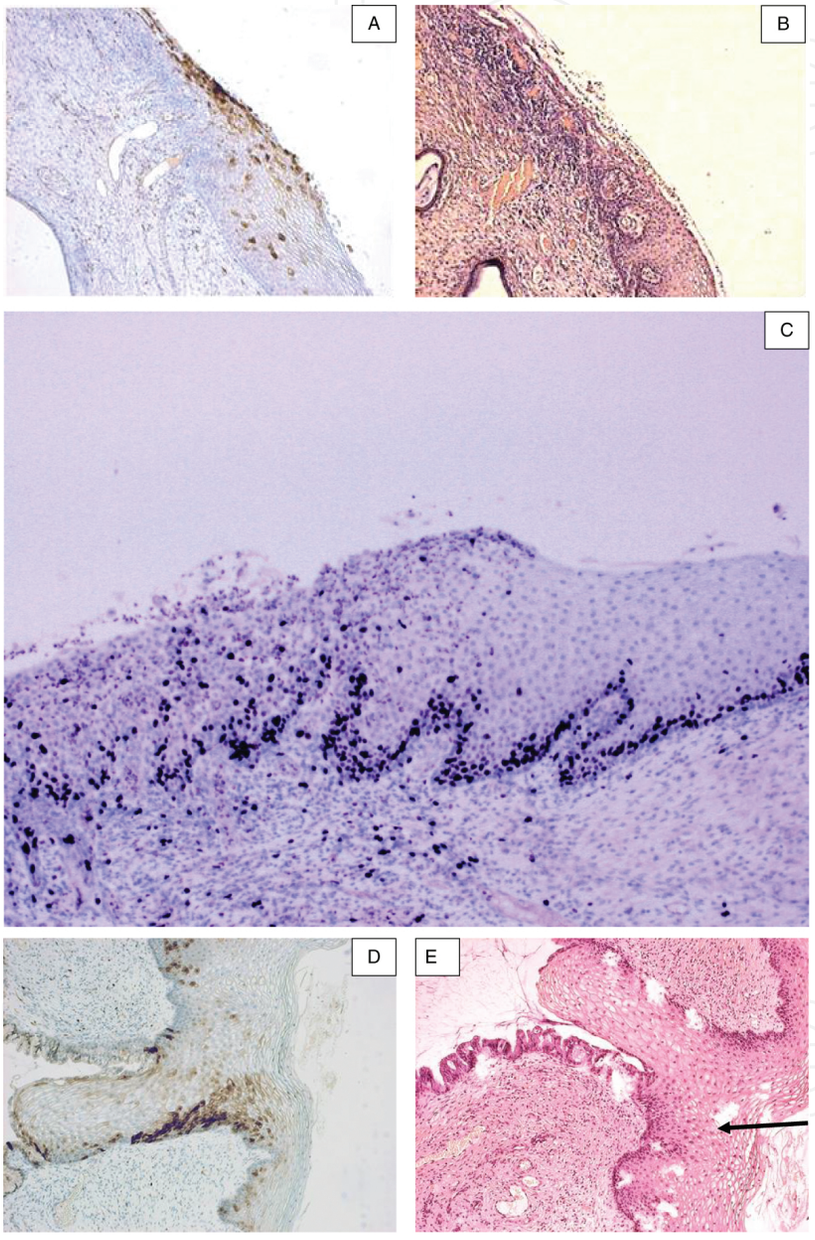

Figure 2. Metaplasia and/or focal dysplasia suspicious for CIN I /LSIL. Line above (A and B; C in the middle): transition of squamous epithelium into a metaplastic area. The cells positive for p16 antigen correspond to the foci of metaplasia (confluent staining), but single p16 positive squamous epithelium cells can be seen as well (A). The area of metaplasia is rich of scattered Ki-67 positive nuclei, while the regularly lined positive nuclei belong to the basal stem cells (C). The line below depicts a reactive proliferation of squamous epithelium (D and E) growing into the cervical gland at the squamocolumnar junction. The positive p16 staining is not precisely parabasal, but corresponds to an initial focus of dysplasia within the lower and/or medium squamous layers (shown by arrow at HE staining). The otherwise dispersed p16 antigen positive squamous cells may be unrelated to HPV infection.

prognostic value of positive p16 staining should be interpreted along with the outcome of the HPV DNA test.

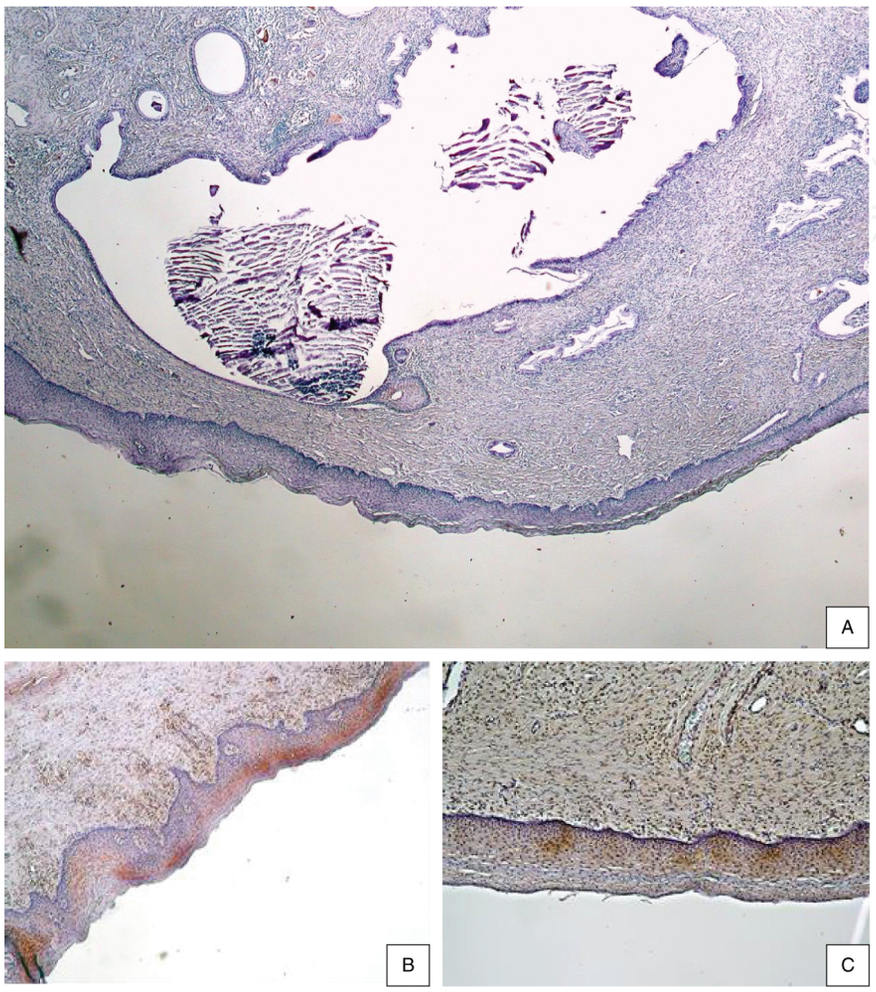

Figure 3. Examples of either negative (A, upper line) and/or midzonal (lower line, B and C) p16 antigen staining. Both examples show non-parabasal location of the p16 positive cells in the upper (rather than in the lower) squamous layer. The staining is of nearly confluent (B) or focal (C) distribution and often corresponds to the localization of koilocytes. Therefore, it is suggestive to accompany cases of productive (vegetative) HPV replication. Such pattern of p16 antigen staining may be suspicious of low-grade lesion (CIN I/LSIL), but should be combined with the HPV DNA test in order to obtain a precise diagnosis.

If dysplastic cells occupy at least one half (exceeding one third) of the original squamous epithelium thickness (but not the whole epithelium layer), the appropriate designation is CIN II. The p16 antigen staining is therefore useful to meet the diagnosis of CIN II/HSIL, since it allows to estimate the precise thickness of dysplasia involving the squamous epithelium (**Figure 4**). In contrast, at the stage of CIN III/HSIL, the dysplastic cells can fully replace the original epithelium (**Figure 5**). At progressed stage, the intensive staining of p16 polypeptide can be found within the nuclei of dysplastic cells as well as in their cytoplasm. The dysplastic cells in question show enlarged nuclei of ovoid shape, which are not equal in size. Since the presence of p16 antigen is a hallmark for distinguishing the immortalized and/or dysplastic

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cells, at first glance, the site of outgrowth and/or proliferation of squamous epithelium into cervical glands can be detected. The decreased availability of functioning Rb protein due to overexpression of E7 polypeptide, not only leads to increased p16 production, but also explains the higher frequency of nuclei positive for Ki-67 protein, a marker of cell division [65]. For more precise grading of the SIL lesions by the Ki-67 antigen staining, Kruse et al. [66] suggested to count the number of positive nuclei per 100 μm epithelium thickness starting from the parabasal zone (stratification index). The authors in question found a satisfactory correlation of 83% with the dysplastic squamous epithelium cells as seen in HE stained sections, but they also noticed that parallel sections may not fully correspond to the previously cut block level. Furthermore, as seen at our example of the CIN III/HSIL pattern shown on **Figure 5C** and **5D**, while the p16 antigen staining involves the whole dysplastic cell layer, in the Ki-67 stained section the calculation of stratification index may be less convenient. Taken together, staining of parallel sections for Ki-67 and p16 antigens confirmed the usefulness of both markers, even though the p16 marker is more suitable for practical reasons. On the other hand, the frequent zonal distribution of p16 reactivity fits well with the extent of dysplasia CIN I lesion encompassing less than one third of stratified epithelium. In the CIN II-graded lesion, the dysplastic cells encounter the half or nearly two thirds of squamous epithelium (**Figure 4**), while in the CIN III lesion a diffuse distribution of dysplastic cells involves the whole thickness of the original squamous epithelium layer. Therefore, at least in biopsies, the staining for p16 antigen alone has recently emerged as a reliable diagnostic marker suitable for quick orientation and relevant demonstration of the extent dysplasia as well as for the search of potentially invasive growth of squamous cell carcinoma cells penetrating the basement membrane (**Figure 6**).

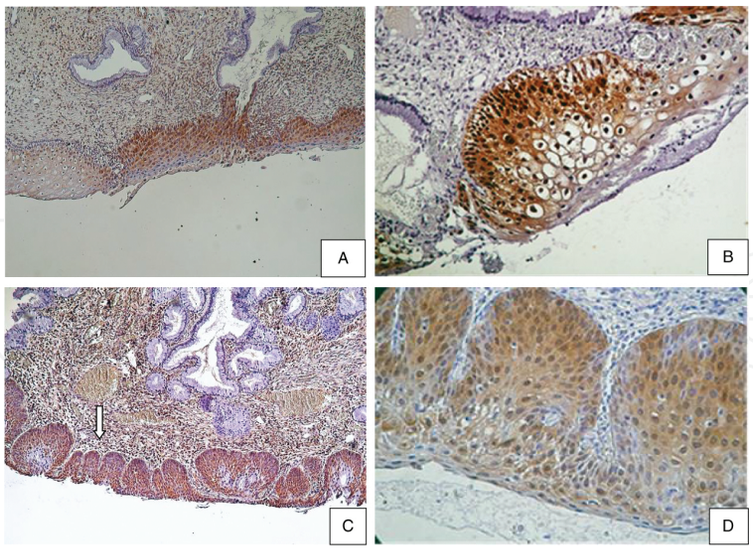

Figure 4. Examples of different CIN II lesions and of the corresponding p16 antigen staining. Upper line (A and B): confluent staining of p16 antigen at transition from stage CIN I (in the left half of the Figure) into stage CIN II (in the right half of same Figure 1A). Figure 1B: The p16 positive dysplastic cells involve the lower and the medium squamous layers but still not the whole epithelium; numerous koilocytes can be seen in the in-close vicinity of the p16 antigen positive dysplastic epithelium cells. Bottom line (C and D): Extensive dysplasia involves the lower and medium squamous cell layers, but is still absent at thin superficial layer of flat granular cells. The distribution of p16 antigen staining

corresponds to transition from CIN II into CIN III (the area enlarged at D is pointed by arrow). As shown in detail (D), a few koilocytes may be still found.

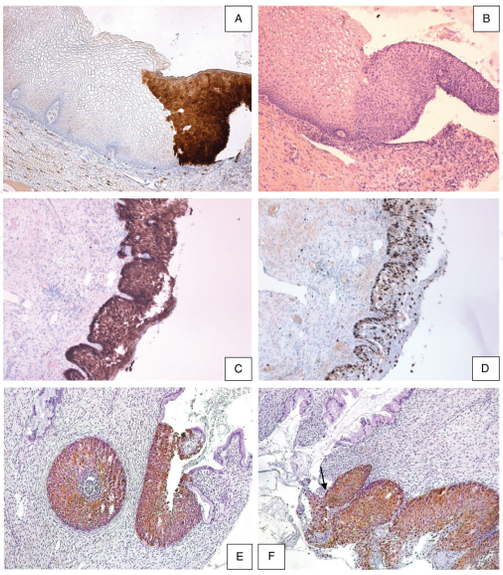

Figure 5. High degree of dysplasia (CIN III/HSIL) which occupies the entire epithelium layer and carcinoma *in situ*. Line above: Transition of squamous epithelium into dysplasia as shown by HE (B) and by p16 antigen staining (A). Middle line: The CIN III/HSIL as seen by p16 and Ki-67 antigen staining (C and D). While the p16 positive dysplastic cells occupy the whole epithelium, the nuclei of dividing cells are either round shaped and of regular size (basal stem cells closely at the basement membrane) or show an irregular ovoid shape of varying in size (dispersed at whole dysplastic layer but not each cell is positive). Bottom line: Carcinoma *in situ* proliferates into the surrounding connective tissue (cells p16 positive) and into a cervical gland replacing the cylindric epithelium but still keeping the basement membrane intact (E). The entirely dysplastic epithelium is positive for the p16 antigen; it shows loss of squamous differentiation along with extensive proliferative growth, but with the basement membrane preserved (F, no invasion at the arrow area).

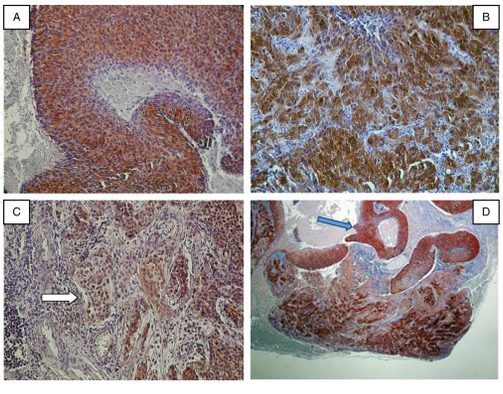

Figure 6. Carcinoma *in situ* (A) versus the invasive growth of spinocellular carcinoma (B) as related to the expression of p16 polypeptide. Dysplastic cells, forming the carcinoma *in situ* (A) as well as the non-differentiated malignant cells of invasive spinocellular carcinoma (B), are strongly positive for p16 and showing cytoplasmic as well as nuclear staining. After disrupting the basement membrane, groups of carcinoma cells grow not only within the surrounding connective tissue, but invade some capillaries and/or small venules as well as lymphatic vessels (C, arrow). At low power view, the origin of invasion can be recognized (D, arrow).

Nevertheless, any such comparisons may suffer from possible imprecision, since the repeatedly stained a tendency to worse grading at the primary evaluation of HE results. Namely, 3% of Ki-67 stained sections were graded CIN I rather than CIN II as described in HE stained sections. Alternatively, 14% cases graded CIN III in the HE stained sections were interpreted CIN II according to the Ki-67 staining. A similar tendency to worse grading from the evaluation, the HE stained sections only was found by us in p16 staining [14]. According to our experience, the viewing of parallel sections stained for p16 as well as Ki-67 antigen markers is of great help at confirming the extent of dysplasia seen in CIN II and/or CIN III HSIL-graded sections. In addition, it contributes to the correct evaluation of CIN I/LSIL lesions among which the reactive and other non-neoplastic staining patterns should be distinguished [67].

According to Dray et al. [68], the CIN II/HSIL- and/or CIN III/HSIL-graded lesions were found in 40.8% of cervical biopsies, while 14.3% showed the mild CIN I/LSIL lesion; the rest of 45% revealed a range of non-dysplastic (inflammatory or reactive) changes. In the latter group, the focal and weaker midzonal or superficial p16/INK4A immunostaining, suggestive of episomal HPV infection, was noted in 10% of biopsies. As shown in our statistics concerning biopsies in the year 2015 examined at the Pathology Diagnostic Center in Martin (Pathology 2, bottom line in **Table 11**), the definitely positive CIN I/LSIL lesions were seen in 36% of biopsies (90 out of 250). These lesions showed parabasal dysplasia within the lower third of squamous epithelium as well as koilocytes among adjacent squamous cells rarely involving the upper squamous layer (**Figure 1**). A proportion of suspicious CIN I/LSIL cases revealed focal dysplasia which, as a rule, correlated with the non-continuous p16 antigen positivity of squamous epithelium (**Figure 2**). The CIN II- and/or CIN III-grade HSIL lesion was detected in 37% of biopsies (89/250). The former showed dysplasia along with positive p16 staining involving about the half of squamous epithelium (**Figure 4**), while by the latter, the entire squamous epithelium layer was strongly positive for the p16 antigen (**Figure 5**). In CIN II cases involving more than one third of squamous epithelium, in the lower as well as in upper squamous layers occasional koilocytes found (**Figure 4B** and **4D**). In clearly CIN III-graded cases, the p16 positive dysplastic cells not only have replaced the whole squamous epithelium but also showed extensive proliferative growth either into the cervical glands or into the underlying connective tissue still with the basement membrane preserved (**Figure 5E** and **5F**). Nevertheless, similar proliferative growth might be occasionally found in CIN II/LSIL-graded cases (**Figure 4A–4C**), but very rarely, even in the CIN I/LSIL focal lesion. (**Figure 2D** and **2E**). In general, the increasing incidence of the combined p16/Ki-67 staining indicates more severe lesions: It may be positive by 26.8% of normal histology (missing dysplasia) cases, by 46.5% in CIN I histology, by 82.8% of CIN II, and/or by 92.8% of CIN III-graded histology [69]. In our hands, squamous epithelium showing no dysplasia was found in 55 out of 250 cases (20%) in biopsies of the Pathology Department 2, but only 10% of biopsies in the Pathology Department 1 (**Table 11**). The findings in the absence of dysplasia fall into at least 2 groups (**Figure 3**): (1) p16 negative cases showing neither dysplasia nor p16 antigen presence, and (2) cases of positive p16 immunostaining in the non-dysplastic areas of squamous epithelium, especially in its midzonal location, which does clearly differ from the parabasal distribution of p16 antigen. The p16 positive staining in the absence of dysplasia should be always examined for HPV DNA presence, and otherwise, the diagnosis can be regarded either for incomplete or at

least imprecise from the point of view future prognosis. Although there is good evidence that p16/INK4A immunostaining correlates with the severity of cytological/histological abnormalities, the reproducibility might be limited also because of the insufficiently standardized interpretation of p16 staining results [70].

4. The diagnostic value of cervical smears in carcinoma prevention

As already mentioned in Introduction, due to examination of cervical smears in Slovakia during the last decade, the absolute number of cervical carcinoma cases as well as the morbidity rate has considerably decreased. Preventive cytological examination, which started in our country from 2006, and became especially frequent in the last 5–6 years (compare **Table 1**) in part explains this favorable development. As shown in **Table 4**, in just one out of 10 diagnostic centers serving for the population of approximately 2 million Slovak women in the age from 15 to 60 years, over 37,000 PA smears were enrolled from about 34,000 women were examined.

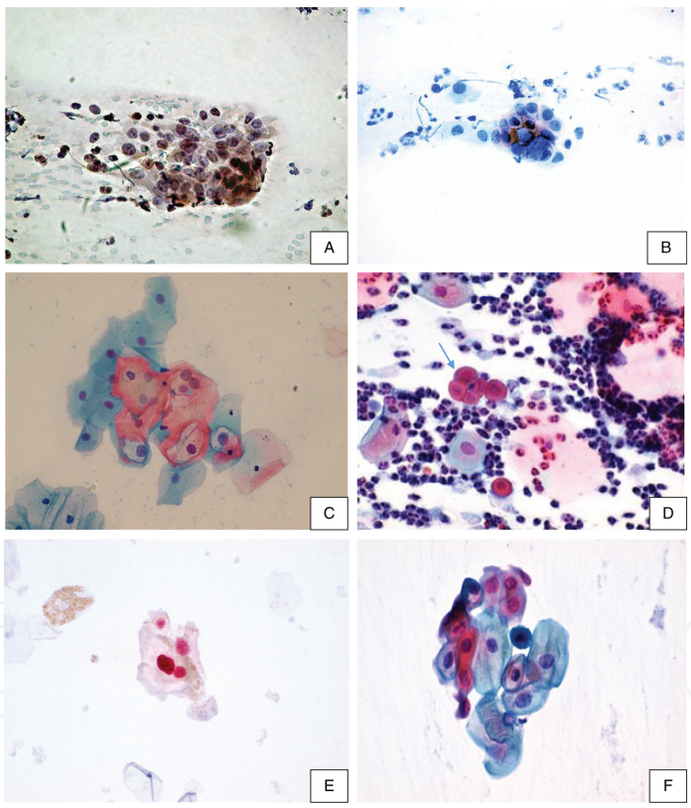

Figure 7. ASCUS- and/or LSIL-graded smears handled by the conventional PA method and stained for p16 antigen (A, B) in comparison with the appearance of atypical and/or abnormal squamous cells as seen after Liqui-PREP staining (C, D, E, and F). The upper line in the left depicts a group of atypical squamous epithelium cells with enlarged nuclei (A) in comparison with a group of abnormal cells-graded LSIL (B), both stained for p16 antigen. The middle line in the right (D) shows inflammation and metaplasia with a group of abnormal epithelium cells scored LSIL (pointed by arrow), while in the middle line left a group of atypical cells with enlarged nuclei can be seen (ASCUS) some of which being suggestive for LSIL (C) . The smear in the right, bottom line shows atypical cells along with the presence of koilocytes (F). In the bottom line left (E), one of the Ki-67 positive abnormal squamous cells shows faint p16 antigen staining in the cytoplasm (simultaneously stained for p16 as well as Ki-67 antigens).

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A positive rate of 4.6% experienced in our particular screening is in accord with the data previously reported from the US [16]. Despite of the success of mass screening based on the relatively simple PAP technique, improved cytological tests such as liquid-based cytology (Liqui-PREP) were introduced to achieve more precise reading. The new approach resulted in a significant decrease of low-quality samples [71], when allowing to identify and distinguish the atypical epithelium cells allowing to identify and better distinguish the atypical epithelium cells at their better visualization, which may be of great advantage especially for the recognition of LSIL cases (**Figure 7**).

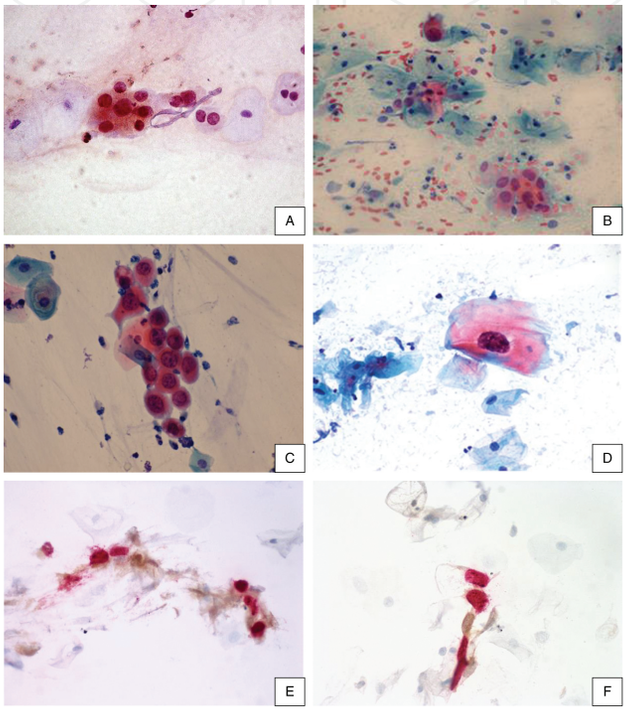

Figure 8. Examples of HSIL-graded smears: Above, in the left (A): The conventional PAP staining is of relatively good quality achieved using the CYNTec staining kit for p16 antigen detection (positive). The rest (B, C, and D) of smears were handled by liquid-based cytology and shows either groups or single dysplastic cells-graded HSIL (B, C) and/or HSIL+ (at D, a single dysplastic cell with an extremely large hyperchromatic nucleus is shown suggestive of carcinoma *in situ*). The bottom line (E and F) shows groups of distorted (shrunken) dysplastic epitelium cells stained with the anti/p16/anti-Ki-67 MoAb mix (nuclei dark purple, cytoplasm contains many p16 antigen brown granules).

As mentioned above, the Bethesda Committee classification [72] and the suggested definitions were later on slightly improved reaching the state which has become widely accepted [73]. Comparisons among laboratories showed that the diagnosis of ASCUS (atypical squamous cells of undetermined significance) may be often used just to avoid clear-cut decision making. Therefore, the principle has been be that this diagnostic category should not exceed 5–6% of the total number of smears investigated. As stressed by Geisinger et al. [74], the main criterion for the clear definition of SIL is the increased size of nucleus (<3 fold for ASCUS, >3 fold in LSIL), increased intensity of chromatin staining and its altered internal structure (finely granular chromatin structure and slight hyperchromasia in ASCUS, coarse chromatin and definitely visible hyperchromasia in LSIL reports claimed the relative value of p16 staining in

ASCUS smears, which may be positive in cases designated as p16 reactive ASC. The proportion of p16 negative smears was reported for the highest in ASCUS (40%) and the lowest in HSIL (5%) specimens [75, 76]. According to Grapsa et al. [77], the p16 staining is weak in LSIL (compare **Figure 7A**), but strong in HSIL cases (**Figure 8A** and **8B**). Thus, the faint p16 staining argues against the diagnosis of LSIL, while p16 overexpression along with high levels of p53 favors the process of malignant transformation of the atypical squamous epithelium cells.

According to Shin et al. [78], the p16 antigen was found in 66.7% of ASCUS and 70% of LSIL cases, confirming that detection of p16/INK4A protein can be used as adjunct test especially at liquid-based cytology. However, several authors noticed a high degree of false p16 reactivity within otherwise negative smears, especially in those containing atrophic cells [79]. They also stressed that in such smears, the number of single p16 reactive atypical cells per the total cell number of cells may not be significant. Such situation is less likely to appear in biopsy sections, where typical confluent distribution of p16 antigen positive cells at the parabasal epithelium layer is of essential help for correct interpretation of the result. In our previous paper [14], we focused our interest on unclear and/or ASCUS-graded smears, which were found HPV DNA positive by a probability of 8–10%, but have been p16 antigen positive with a higher probability ranging from 32 to 61%. The wide range of p16 antigen false positivity of atypical cells was found associated with the staining procedure itself, since the CYN-Tec cytology staining kit showed more clear-cut results, allowing to distinguish the proportion of really p16 antigen positive, but HPV unrelated atypical cells on hand, from artificially stained ones on other hand. It should be stressed that in the ASCUS-graded smears but also in a great proportion of LSIL scored smears the most important criterion still remains the evaluation of nuclear changes, such as the altered N/C ratio, which should be considered for each particular p16 reactive cell. The diagnosis of ASC gains some prediction value, if based on the presence of a few HSIL-like cells (the so-called ASC-H). The latter points at a fast progression to HSIL from the very beginning (**Figure 8**). Nevertheless, the ASC-H category was challenged by other investigators as being problematic, since the morphologic difference in the appearance of such single metaplastic and/or neoplastic cell is not always clear and opens the way to false positive as well as false negative diagnoses [80–82]. Nuclear hyperchromasia and irregular nuclear membrane contours count as the most reliable diagnostic feature in the so-called pre-neoplastic atypia. The presence of such cells in the smears possibly corresponds to the dysplastic cells in histological sections [83], which, however, cannot be present in the smear in the case of CIN I/LSIL (compare **Figure 1**), but may be already easily collected in the cases of CIN II/HSIL (compare **Figure 4**). For better orientation in the most uncertain cases, a short course of local estrogen cream therapy followed by repeated PA tests has been suggested for helpful [84].

According to our experience, the relatively high percentage of p16 reactive atypical cells causing false positive LSIL grading appears in smears if handled by the conventional PA method. Therefore, the LBC should be preferred at repeated examination of patients which had been selected according to the classical Papanicolaou diagnostic system (when scored PA IIIa/IIIb roughly corresponding ASCUS and/or LSIL, as well as PA IV corresponding to HSIL and/or even PA V). The advantage of recently introduced improved diagnostic approach can be demonstrated on the results from the Cytology Laboratory (CL) of the St. Elisabeth Cancer

Institute (SECI) in Bratislava obtained within the years 2012–2013. The total number of gynecological cytology samples enrolled during the given period was 17,272, which corresponds to approximately 46% of samples examined at the Cytology Laboratories of Alpha medical presented in **Table 4**. While **Table 4** shows the positive rate of the screening from samples enrolled by practitioners, the Cancer Institute samples were submitted by specialists to whom the positively screened patients were sent for further care and/or treatment. Therefore, the diagnostic approach met was more complex combining the LBC technic (for example the Liqui-PREP kit) along with the detection of HPV DNA in nearly 53% of samples reflects the advantage of the interpretation of results, which have been achieved by comparison of both methods (**Table 9**). When considering the fact there was no logical need to request the HPV DNA test in the majority of negative cases enrolled (out of these 28% were tested for HPV only) then in becomes clear that the number of smears complete by DNA testing prevailed not only in essentially inevitable cases, such as ASCUS and LSIL, but by average the HPV DNA was tested by a proportion of 52.3% of all smears examined. When considering all the important facts mentioned, then the complete (dual examination) approach has been applied in 68% of ASCUS/LSIL cases (shown in bold type). It comes from **Table 10** that the HPV positive rates ranged from 27 to 33% in ASCUS and/or LSIL cases, respectively, indicating that the probability of further progression in these mild lesions could be quite low. In contrast, by the HSIL-graded smears the HPV DNA test was positive in 92% of cases. The same was true for the combination of positive HPV test with the p16 antigen staining. This was seen by 32% of LSIL-graded cases, in which the probability of progression was then relatively high, since as has been such transition really may occur by up to 15% of LSIL cases (citácia), which means that each second such double positive LSIL case might progress into HSIL. As our statistics concerns, this was really the case, but by a lower probability equal to one out of three LSIL cases. Byun et al. [85] conducted a comparative study including the p16 INK4a/Ki-67 double staining as well as the L1 capsid protein immunostaining along with human papillomavirus (HPV) DNA detection and typing in 56 ASC-H or LSIL-H cases diagnosed by LBC stained smears came to the conclusion that their approach was sufficient to predict CIN II+ and/or CIN III+ later on diagnoses at histological examinations of biopsies obtained from patients who underwent conization. Further interesting considerations are coming from the comparison of the biopsies subsequently resulting from the previous cytological diagnostic based their histological grading made at different Pathology Departments. While in the Diagnostic Center at the Pathology in Martin revealed that the probability of negative and/or non-neoplastic findings (including false positive and/or reactive staining) was approximately 22%. In contrast, the probability of the occurrence of the same result at the Pathology Department of the Oncology Institute in Bratislava was slightly below 10% only (**Table 11**). This difference in the negative scoring of CIN lesions in cervical biopsies reflects a better forecast based on a complex diagnostic approach, involving the use of LBC technology at repeated examination of cervical smears with contemporary HPV DNA tests along with the staining of smears for at least two serological markers. We believe that the cytological diagnosis should reflect a better degree of cooperation between clinicians and laboratory workers. As seen according to the presented algorithm, the suggested minimum of 2 or 3 colposcopic sessions may be satisfactory for precise diagnosis (**Figure 9**). In positive cases (i.e., those graded over PA III according to

conventional Papanicolaou system), the repeated smear taken should be handled by the LBC-based technic and stained for at least two markers (p16 and Ki-67 and tested HPV DNA presence.

Cytology	Completed	Not completed	Total examined
Non-suspicious	63 (28%)	225	288
ASCUS	**139 (59%)**	96 (41%)	235
ASC-H	**5 (63%)**	3	8
LSIL	**304 (68%)**	143 (32%)	447
HSIL	124 (53%)	108 (47%)	232
SCa	0	4	4
Total	**635 (52.3%)**	**579**	**1214**

Abbreviations: LBC—liquid-based cytology; HPV—human papilloma virus; ASCUS—atypical squamous cells of undetermined significance; ASC-H—Atypical squamous cells-cannot exclude high-grade squamous intraepithelial lesion; LGSIL—Low-grade squamous intraepithelial lesion; HGSIL—High-grade squamous intraepithelial lesion; SCa—squamous cell carcinoma.
* The CL is a part of the Pathology Department of the St. Elisabeth Cancer Institute, which also is a teaching center of Slovak Postgraduate University in Bratislava
** Negative.
*** HPV DNA not tested.

Table 9. LBC examinations of cervical smears during the period of 2012–2013 at the CL of the St. Elisabeth Cancer Institute, Bratislava.

Cytological diagnosis	Number		Antigenic markers			HPV DNA test	
		Per cent*	p16 positive	Ki-67 positive	p16+/ Ki-67+	Positive alone	p16+/HPV+
Negative**	63	9.9	0	0	0	3	0
ASCUS	139	21.9	87 (63%)	7	4	38 (27%)	21 (15%)
ASC-H	5	0.8	2	2	2	2	2
LSIL	304	47.9	268 (88%)	66	114	99 (33%)	97 (32%)
HSIL	124	19.5	120 (96%)	106	103	114 (92%)	112 (90%)
SCa	0	0	0	0	0	0	0
Total	635	100%	477	181	223	256 (40%)	232 (37%)

LBC—liquid-based cytology; HPV—human papilloma virus; ASCUS—atypical squamous cells of undetermined significance; ASC-H—Atypical squamous cells-cannot exclude high-grade squamous intraepithelial lesion; LSIL—Low-grade squamous intraepithelial lesion; HGSIL—High-grade squamous intraepithelial lesion; SCa—squamous cell carcinoma.
* Calculated from the total completed examinations.
** Scored as non-suspicious, see also in **Table 9**.

Table 10. The completed LBC-based cervical smears according to staining procedures and correlated with the HPV DNA test result.

Cytological diagnosis	Diagnosis at biopsy						Examination
	Non-neoplastic**	CIN I	CIN II	CIN III	SCa	Number total	
ASCUS	20	12	2	4	0	38	Histology
ASC-H	3	0	2	0	0	5	Histology
LSIL	0	56	0	0	0	56	Histology
HSIL	0	4	60	76	0	140	Histology
SCa	0	0	0	0	4	4	Histology
Total (Pathology 1)	**23 (9.4%)**	**72 (30%)**	**64 (26%)**	**80 (33%)**	**4 (1.6%)**	**243 (100%)**	Histology
Example shown at	Figure 2**, Figure 3	Figure 1	Figure 4	Figure 5	Figure 6		
Pathology 2	**55 (22%)**	**90 (36%)**	**46 (18%)**	**52 (21%)**	**7 (3%)**	**250 (100%)**	Histology
		3/11 (27%)		**7/8 (88%)**			HPV DNA

LBC—liquid-based cytology; HPV—human papilloma virus; ASCUS—atypical squamous cells of undetermined significance; ASC-H—Atypical squamous cells-cannot exclude high-grade squamous intraepithelial lesion; LGSIL—Low-grade squamous intraepithelial lesion; HGSIL—High-grade squamous intraepithelial lesion; SCa—squamous cell carcinoma; CIN—cervical intraepithelial neoplasia and its grading.
* Concerns the biopsies examined at CL of St. Elisabeth Cancer Institute.
** Includes the reactive not-dysplastic lesions, such as false positive p16 staining as well as the p16 negative (normal) squamous epithelium.
*** The data concern the Diagnostic Center of Pathology Ltd, Alpha medical, Martin.
# Important notice: even though the proportion of patients in which the HPV DNA was tested at The Pathology center was relatively low (12 and 15%, respectively), the application of a complex approach Figure 9 proven the prevalence of HPV DNA presence by CIN III/HSIL.

Table 11. Correlations between cytology and biopsy during the same time period of 2013–2014 in two different Pathology Departments.

Diagnosis	Sample number	HPV (all types)		HR HPV genotypes			HR total
		Negative	Positive	HPV16	HPV 18	Not frequent**	
ASCUS/LSIL	520	329	191	59	18	114	191 (34%)
HSIL	45	19	26	2	0	24	26 (57%)
Not determined	932	608	302	93	21	120	(25%)

* Department of Clinical Microbiology, Alpha medical Ltd, Ružomberok, suitable to recognize the genotypes.
** HPV types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68.

Table 12. Results of HPV DNA testing by means of the Cobas 4800 equipment*.

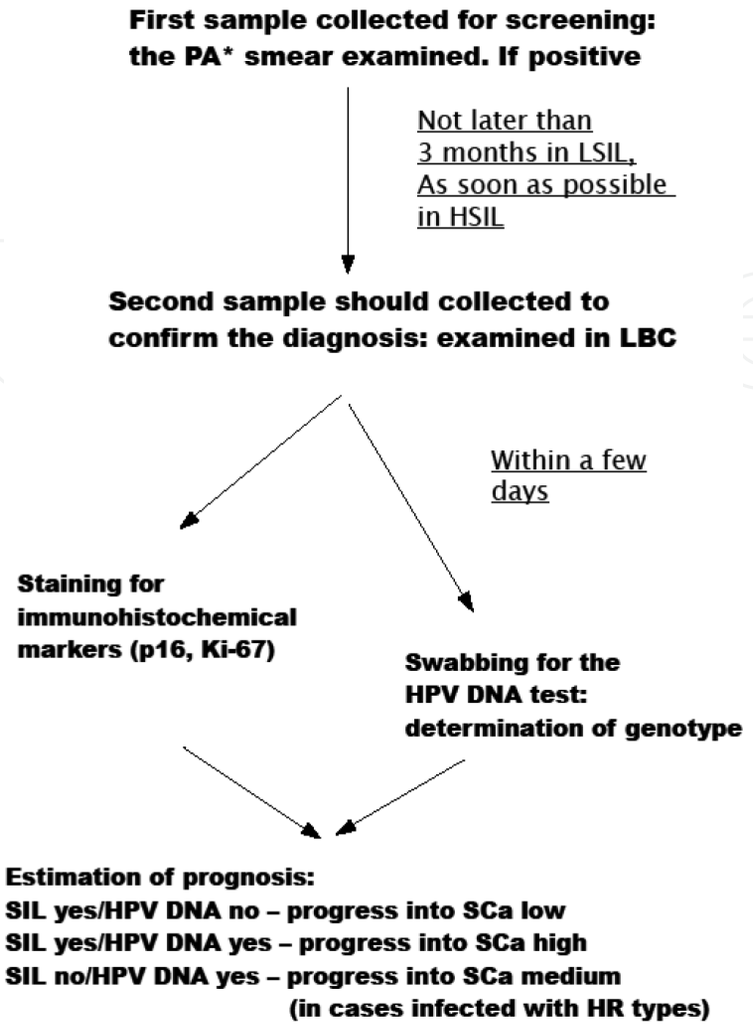

Figure 9. A simple algorithm allowing to assess the precise diagnosis of dysplasia and to estimate its prognosis (all the basic information shown should be at disposal for exact decision making).

5. HPV DNA detection in association with cytological and histological examinations

In countries where cytological diagnostic is widely used for screening, the most useful option is to use HPV DNA testing especially for getting more reliable information allowing to lengthen safely the next smear examination interval [86]. In general, the DNA tests are based either on DNA/DNA hybridization (using labeled complementary DNA probes), or on amplification of vDNA by polymerase chain reaction (PCR) as well as on DNA/RNA hybridization (using complementary RNA probes) followed by visualization of the labeled hybrid signal. Early approaches utilized various modifications of the vDNA to DNA probe hybridization tests, such as in situ hybridization and/or various blotting techniques [87]. To improve the PCR method, multiple primers for L1 and/or E1 gene amplifications have been introduced, aiming to identify the most frequent genotypes (i.e., HPV 6, 11, 16, and 18) in a single-tube

reaction [88]. The principle of multiple genotype amplification was further modified using general primers (GPs), which flank the strongly conserved regions located either on L1 and/or E1 ORFs, enabling detection of a wide spectrum of genotypes [89]. The GPs annealed not only to the ORFs of genotypes, which they had been designed for, but also to some another which sequence was not known at that time (later on these were identified as HPV 13, 30, 31, 45, and 51). The consensus or general primer GP5+/GP6+ based procedure became widely used, since it enabled the differentiation between several HR and LR HPV genotypes in a single assay [90, 91]. The GP-PCR technique became further improved in order to detect more genotypes (at least 14 HR HPV along with the 6 frequent LR HPV genotypes). In addition, it was modified in order to visualize the reaction product by enzyme linked immunosorbent assay, that is ELISA [92]. The high-risk types being assessed this way were, as a rule, HPV 16, 18, 31, 33, 39, 45, 51, 52, 56, 58, 59, 66, and 68, and the low-risk ones were at least HPV 6, 11, 40, 42, 43, and 44. Further improvements of GPs allowed their annealing to the DNA of additional genotypes, such as HPV 26, 30, 53, 70, 73, 82, and 83, in order to increase the number of routinely detectable genotypes to 27 out of the 40 possible mucosal human papillomavirus types [93]. Additional consensus primers, having been introduced for L1 ORF amplification, were further modified to avoid synthesis of irreproducible fragments [94]. The latter primer set (PGMY07/11) increased the number of multiple HPV genotype infections detected by adding the rare genotypes such as HPV 26, 35, 42, 45, 52, 54, 59, 66, and 73. Any routine HR versus LR HPV testing may be influenced by the DNA extraction technic, namely depending whether a recommended manual extraction procedure or an automated extraction protocol supplied by the manufacturer of given equipment was used [95]. To avoid the methodic variations, automated vDNA extraction was recommended for HPV genotyping by both, the classical PCR (GP5/GP6 primers) as well as for the real-time PCR-based quantitative TaqMan assay. Further modification of HPV detection in the direction of immunochemistry has resulted into an assay omitting vDNA amplification, while introducing the labeled signal amplification instead. In a latter assay, the denatured vDNA was hybridized under high stringency conditions to single-stranded RNA probes either for LR genotypes (at least 6, 11, 42, 43, and 44) and/or for HR genotypes (at least 16, 18, 31, 33, 35, 45, 51, 52, and 56). The RNA/DNA hybrid complex was then bound to microplates (or tubes) coated with an alkaline phosphatase conjugated monoclonal antibody, able to capture the specific RNA/DNA hybrid [96]. The reaction is then visualized by addition of the chemilumiscent substrate, in which emission light is amplified and measured in a luminometer; the results are expressed in relative light units (RLU). This method is referred to as hybrid capture (HC). At its beginning HC showed lower sensitivity, as compared to as few as 10–100 vDNA copies (about 100 fg HPV DNA) were detectable per 1 ml sample when using the classical PCR. The recent HC2-based high-risk HPV DNA test (Qiagen), which was previously used in the Alpha medical laboratory as well, detects 13 HR genotypes (HPV 16, 18, 31, 33, 35, 35, 39, 45, 51, 52, 56, 58, and 68) at a sensitivity of 1–2 pg/ml (i.e., about 100,000 HPV DNA copies/ml). Another variation of this assay can be also used for detection of at least 5 LR genotypes (HPV 6, 11, 42, 43, and 44). Recio et al. [97] used the first generation HC test for investigating the HPV DNA presence in patients with ASCUS, LSIL, HSIL, and carcinoma *in situ* smears, the latter being used as a relevant standard. Altogether 44% of patients were tested positive, mainly for HR HPV genotypes. The authors

concluded that testing of HPV DNA by the HC method is helpful for clinical diagnostic. Monsenogo et al. [90] reported that the HC2 test as compared in 470 patients with the PCR-based Roche Amplicor HPV test, reached an agreement of 96.2%; only 18 cases were found discordant. It should be mentioned that the Amplicor HPV test identifies the PCR-amplified vDNA by means of 13 HR-HPV genotype probes and that the vDNA is being obtained from cervical cells collected into a transport medium [98]. In patients revealing ASCUS smears, both tests for HPV DNA showed a positive rate of 42.3%, while in patients showing LSIL smears, the HPV DNA positive rate was 66.3 and/or 66.8%, respectively, depending on the test performed (the PCR-based test seemed in this case even less sensitive). In patients with HSIL smears, the DNA was positive in both tests at the highest rate of 92.8%.

The HC2 high risk HPV DNA Test takes advantage of the Hybrid Capture 2 (HC2) technology; it is a nucleic acid hybridization assay with signal amplification that utilizes microplate chemiluminescent detection for the qualitative detection of 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) in cervical specimens. Specimens containing the target DNA hybridize with a specific HPV RNA probe. The resultant RNA:DNA hybrids are captured onto the surface of a microplate well coated with antibodies specific for RNA:DNA hybrids. Immobilized hybrids are then reacted with alkaline phosphatase conjugated antibodies specific for the RNA:DNA hybrids and detected with chemiluminescent substrate. Several alkaline phosphatase molecules are conjugated to each antibody. Multiple conjugated antibodies bind to each captured hybrid resulting in substantial signal amplification. In contrast to the HC technology, the Cobas® 4800 Human Papillomavirus (HPV) Test is a qualitative *in vitro* test for the detection of human papillomavirus in cervical specimens. The test utilizes amplification of target DNA by the polymerase chain reaction (PCR) and nucleic acid hybridization for the detection of 14 high-risk HPV types in a single analysis. The test specifically identifies HPV16 and HPV18 while concurrently detecting the other high-risk types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) at clinically relevant infection levels. The Cobas® 4800 HPV Test primers define a sequence of approximately 200 nucleotides within the polymorphic L1 region of the HPV genome. An additional primer pair would target the human β-globin gene (330 bp amplicon) to provide a process control. A pool of HPV primers present in the Master Mix is designed to amplify HPV DNA from 14 high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). The detection of amplified DNA is performed during thermal cycling using oligonucleotide probes labeled with four different fluorescent dyes. The amplified signal from twelve high-risk HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), is detected using the same fluorescent dye, while HPV16, HPV18, and β-globin signals are each detected with their own dedicated fluorescent dye. Fluorescent oligonucleotide probes bind to polymorphic regions HPV and human β-globin gene within the sequence defined by primers.

Summing up, the DNAs can be distinguished according to (1) the ability to identify a pool of high-risk HPV types, with or without genotypization of the most common high-risk viruses (i.e., HPV16 and 18) or (2) to detect a broad spectrum of oncogenic and non-oncogenic HPVs along with individual genotyping. While the assays of the first group are mainly used in screening programs, where there is no clinical benefit from the knowledge of specific HPV

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types, the assays of the second group are primarily used in HPV surveillance studies and to monitor the eventual spreading of particular viral types in vaccinated women [99]. The HPV16 and HPV18 genotyping, for its high specificity, have been included in the US guidelines for the triage of HPV positive and cytology negative women [100]. In general, the probability of developing precancerous (HSIL) lesions was high in women who were LSIL as well as DNA positive; a lower, but still medium probability for developing cancer was found in HPV DNA positive, but by cytology negative women. The lowest probability was noted in HPV negative but ASCUS/LSIL positive cases. Similar findings were observed in the Microbiology Department of Alpha medical (**Table 12**) showing that the positive rate of HR genotypes was the highest in HSIL cases, the closet in cases with not precisely determined diagnosis. As stressed by Mandelblatt et al. [101], screening of HPV plus patients with LSIL tests within 2 years appears to save lives and is more reasonable than performing the cytology test alone. Another large cohort study in US (performed from 2003 to 2005) found that women aged less than 30 who have ASCUS-grade smears showed HPV positive rate at 53% [102]. On the other hand, women older than 30 years with NIL PAP test were HPV (HC2) positive at a rate of 9%. Since ASCUS smear diagnosis is a clinical and prognostic challenge, it should be combined with HPV testing and repeatedly investigated to show whether or not the transition to HSIL occurs [103]. Also in this follow up, a relatively low proportion (6.7%) of ASCUS positive patients developed HSIL or cancer. Among the patients with HSIL smears, up to 98% was found HPV 565 DNA positive; theoretically such women harbor the integrated incomplete genome in the cervical tissue. It was concluded that the residual specimens collected from routine cervical cytology in ASCUS cases could provide additional information about the HPV DNA status that is of substantial help by identifying those patients, who are likely to develop HSIL, especially if they test positive for the HPV DNA presence.

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