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SIGNIFICANCE OF SURVIVIN IMMUNOREACTIVITY AND MORPHOMETRIC ANALYSIS OF HPV-INDUCED CERVICAL DYSPLASIA

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Abstract - Genomic integration of high-risk human papilloma virus in the nucleus of cervical epithelial mucosal cells leads to epithelial dysplasia. The aim of this study was to determine the relevance of correlation between epithelial survivin expression and the degree of human papilloma virus (HPV)-induced cervical epithelial dysplasia, and to establish the significance of morphometric analysis of the nuclear area in the assessment of the degree of cervical dysplasia. This retrospective study included 99 women with primary, previously untreated lesions, and colposcopic findings indicating dysplasia, in whom a cytological test by Papanicolaou method was interpreted according to the Bethesda criteria as low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), and atypical squamous cells of undetermined significance (ASCUS). We performed human papilloma virus (HPV) typing by PCR for evidence of viruse types 16, 18, 31, 33. After biopsy of the cervical mucosa, we performed hematoxylin-eosin (H-E) and Periodic Acid Schiff (PAS) staining, and immunohistochemical and morphometric analysis of tissue samples. The control group consisted of 12 women without dysplasia and without a verified infection of cervical high-risk HPV. A high statistical correlation between the degree of dysplasia and expression of survivin was found in patients with different types of cervical dysplasia ($p = 0.003$). We observed a high statistical difference between the area of nuclei at different degrees of cervical dysplasias ($p = 0.000$). The high-grade cervical dysplasia had a more than 2-fold higher level of ranking in comparison to low-grade dysplasia, and a more than 10-fold higher ranking than the control group without cervical dysplasia.

Key words: Cervical dysplasia, human papilloma viruses (HPV), morphometric analysis, survivin

INTRODUCTION

Cervical epithelial dysplasia (minor and severe) describes a group of interconnected morphological changes in the histological architecture of the cervical mucosa. The degree of dysplasia is determined by the presence of dysplastic cells and their proliferation, with a loss of maturation in relation to the total thickness of the epithelium (Wentzen et al., 2010).

Etiologic risk factors for dysplasia are infection with the human papilloma virus (HPV), hereditary factors and a history of sexually transmitted diseases associated with the early start of sexual activity and promiscuity, as well as cigarette smoking, obesity, use of oral contraceptives, etc. (Wentzen et al., 2010; Jović et al., 2008).

However, infection by so-called high-risk human papilloma viruses is the major etiologic factor in the

development of cervical epithelial dysplasia. According to many authors, the DNA of a high-risk human papilloma virus (HR-HPV) can be detected in over 90% of cases of cervical dysplasia and cancer (Hlaing et al., 2010).

According to its oncogenic potential, human papilloma viruses are classified into low-risk viruses (LR-HPV; types 6, 11, 42, 43, 44), medium-risk viruses (IR- HPV; types 33, 35, 39, 51, 52), and high-risk viruses (HR-HPV; types 16, 18, 31, 45, 56) (Hlaing et al., 2010; DeYoung and Ellisen, 2007; Regauer and Reich, 2007; Lesnikova et al., 2009; Lopes et al., 2007).

In spatial terms, HPV infection initiates damage in the basal layer of the epithelium, where the virus integrates into the cell nucleus. After this, the virus expresses the E6 and E7 genes that lead to the deregulation and progression of the cell cycle and inhibition of apoptosis (Brenan et al., 2008; Barbosa et al., 2011; Frost et al., 2002).

Inhibition and deregulation of apoptosis is one of the major events of the HPV-induced process of dysplasia (Frost et al., 2002; Branca et al., 2005; Nayar and Solomon, 2004). There are two major categories of inhibitors of apoptosis: the bcl-2 protein family and the family of inhibitors of apoptosis (IAP). Survivin belongs to the family of inhibitors of apoptosis, with eight members: NAIP, Apollon, c-IAP1, c-IAP2, XIAP, ML-IAP/vivin, ILP-2 and survivin. From 1997, when survivin, which is normally present only during fetal development, was discovered, it has been studied in epithelial and mesenchymal tumors. Survivin inhibits apoptosis indirectly through intermediate proteins, or directly by inhibiting caspases-3 -7 and -8 (Brenan et al., 2008; Barbosa et al., 2011; Frost et al., 2002). It is believed that survivin is a multifunctional protein, present in cytoplasmic and nuclear areas and controlling cell survival and cell division (Nayar and Solomon, 2004). The results of previous studies suggest that the nuclear translocation of survivin may be important for the proliferation of cancerous cells, whereas cytoplasmic survivin activity may be more important for cell survival. High levels of inhibitors of apoptosis are a predictor for a poor clinical prognosis of cervical dysplasias. Survivin has a role in the progression of cervical dysplasia, specifically, by binding to the division spindle through microtubules (Barbosa et al., 2011; Frost et al., 2002; Branca et al., 2005). The molecular mechanism of deregulation of survivin in the cervical epithelium in dysplasia is mediated by HPV proteins E6, E7 or E2. Cytoplasmic survivin reactivity may be present in individual epithelial cells during normal cervical cell maturation. During HPV-mediated cell destruction, nuclear and/or cytoplasmic reactivity is present in a large number of cells (Nayar and Solomon, 2004).

Immunohistochemical analysis represents an important approach in the analysis of cervical dysplastic cells. The light microscope renders the cervical epithelium accessible to analysis. Previous results showed a positive correlation between the degree of dysplasia and the expression of immunohistochemical reactivity for survivin (DeYoung and Ellisen, 2007; Lesnikova et al., 2009; Brenan et al., 2008; Nayar and Solomon, 2004).

According to the Bethesda System criteria for histological classification of dysplasias, low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL) are defined by the thickness of the dysplastic cells layer in the cervical epithelium (Cheah and Looi, 2008). Squamous intraepithelial lesions (SIL) are characterized by cytological atypia, which manifests as nuclear pleomorphism, enlargement and hyperchromasia, with irregular shapes and abnormal chromatin distribution, and an increased nucleocytoplasmic ratio.

The morphometric technique is a method of computer analysis of histological and cytological preparations using appropriate morphometric software (Terlikowski et al., 2004). Numerical parameters express the characteristics of pleomorphic, enlarged and hyperchromatic nuclei (Terlikowski et al., 2004). Mathematically objective morphometric analysis of

nuclei diameters is an important step in determining the cytomorphological substrate in premalignant lesions of the cervix (Terlikowski et al., 2004; Perić et al., 2011).

The most accurate method for determining the presence of high-risk types of HPV in the cells is by PCR (14). According to previous results, there is a positive correlation between the detection of high-risk HPV types with the histological finding of dysplasia (Deligdish et al., 2003). Some authors also used and correlated various methods in the diagnosis of squamous intraepithelial lesions considering that dysplasias are a set of morphologic genetic and functional changes (DeYoung and Ellisen, 2007; Lesnikova et al., 2009).

The objectives of this study were to determine the diagnostic value of immunohistochemical analysis of survivin and morphometric analysis of dysplastic cervical epithelium in patients with a verified presence of high-risk HPV infection by polymerase chain reaction (PCR), after the standard method of analysis of biopsy specimens stained by hematoxylin and eosin (H & E) and Periodic Acid Schiff (PAS).

MATERIALS AND METHODS

Patients

This retrospective study included 99 patients examined at the Department of Gynecology of the Military Medical Academy, Belgrade, in the period from 2009 to 2012. This study was realized according to the Declaration of Helsinki, and performed in accordance with Ethics Committee of the Military Medical Academy. The criteria for inclusion into the study were primary, previously untreated lesions, colposcopic findings that suggest the presence of premalignant lesions of the cervix, findings from a Papanicolaou cytological test, interpreted by the Bethesda criteria with the findings of LSIL, HSIL and ASCUS. HPV typization was done by PCR with evidence of virus types 16, 18, 31 and 33 in all women. After a biopsy of the cervical mucosa, we performed pathohistological analysis by hematoxylin-eosin and PAS staining, immunohistochemical analysis for survivin and morphometric analysis of the cervical epithelium.

The control group consisted of 12 women without dysplasia and without verified infection of cervical high-risk HPV, with a pathological diagnosis of leiomyoma in material obtained after hysterectomy.

Criteria for exclusion from the study were previously diagnosed and treated dysplasia or squamous cell carcinoma.

Tissue preparation, immunohistochemical and morphometric analysis

After cervical biopsy, specimens were fixed in 5% buffered neutral formaldehyde and routinely processed in a Leica ASP300 and embedded in paraffin. Tissue samples were cut on an automated rotary microtome (LKB Historange) on 4 micrometers (μm) thick slices, and then stained with H&E and PAS.

Tissue sections for immunohistochemical detection of survivin were transferred to Superfrost + glass slides. Survivin epitope unmasking was done in a water bath at 95°C in Dako Target Retrieval solution S1699. As a primary antibody, monoclonal Mouse anti-human survivin antibody Dako (code M3624; clone 12C4) was used at a 1:100 dilution, and as a system for visualizing the CSA II sytem code K1497 Dako was used.

The presence of nuclear and/or cytoplasmic reactivity for survivin in more than 10% of cells in dysplastic areas of the cervical mucosa specimens is considered as positive immunoreactivity for survivin. The absence of survivin reactivity and the presence of reactivity in less than 10% of cells are considered as negative immunoreactivity for survivin.

Morphometric analysis was performed on H&E and PAS stained slides at 400 x magnification. We analyzed four representative fields of dysplasia; 70 nuclei were photographed, using a digital optical microscope (Nikon Coolscope, Japan) and the Image

Table 1. Survivin immunoreactivity in LSIL, HSIL and control group

		Survivin expression		Sum
		Negative	Positive	
Dysplasia degree	Without dysplasia	10 (9.01%)	2 (1.80%)	12 (10.81%)
	LSIL	20 (18.02%)	14 (12.61%)	34 (30.63%)
	HSIL	23 (20.72%)	42 (37.84%)	65 (58.56%)
Sum		53 (47.75%)	58 (52.25%)	111 (100%)

LEGEND: Negative-without nuclear and/or cytoplasmic immunoreactivity; positive-with nuclear and/or cytoplasmic immunoreactivity

Table 2. Descriptive analysis of nuclear areas according to degrees of cervical dysplasia

Dysplasia degree	N	Mean (μm²)	Min. (μm²)	Max. (μm²)	Ranking (μm²)	Std. Dev.
Without dysplasia	12	18.409	14.20	24.87	10.67	2,941
LSIL	34	31.921	22.11	44.45	22.34	5.073
HSIL	65	96.223	11.40	134.43	123.03	18.753

N – number of patients; mean (μm²) – mean nuclear area expressed in squared micrometers; min – minimal nuclear area expressed in μm²; max – maximum nuclear area expressed in μm²; std. dev – standard deviation

Table 3. The results of Kruskal-Wallis test for ranking

	Diagnosis	Number	Arithmetic mean of the rank
Nuclear area	Without dysplasia	12	7.67
	LSIL	34	30.44
	HSIL	65	78.29
	Sum	111	

J program (Java-based image processing program), as previously described (Perić et al., 2011; Perić et al., 2012). The nuclear areas were measured and expressed numerically in squared micrometers.

Statistical analysis

Survivin immunoreactivity in biopsy material was compared among the patient groups with LSIL, HSIL and the control group. We analyzed the data with descriptive and analytical statistical methods. For comparison of the degree of dysplasia and survivin expression, we used the chi-square test. A p value of 0.05 and less was considered statistically significant. For comparing the degree of dysplasia and nuclear area, we used the Kruskal-Wallis test.

RESULTS

Survivin immunoreactivity of 1.80% was observed in the control group, 12.61% in the group of patients with LSIL, and 37.84% in the group of patients with HSIL (Fig. 1, Table 1). The chi-square value was 11.74 for df = 2, p = 0.003. Survivin immunoexpression was significantly higher in patients with HSIL than in patients with LSIL and in the control group. Fig. 2 shows survivin nuclear and cytoplasmic immunoreactivity in the cervical mucosa of the patients with HSIL.

Table 2 shows the results of descriptive analysis of nuclear areas in comparison with the degree of cervical dysplasia. The mean nuclear area was higher in patients with a high degree of cervical epithelial

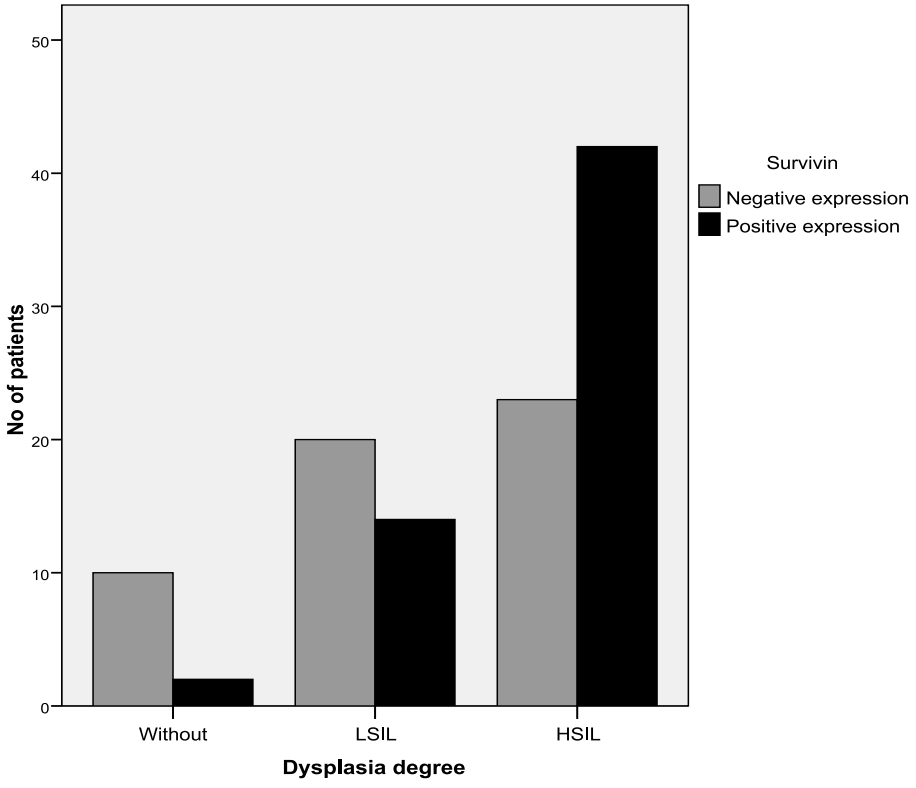

Fig. 1. Survivin immunoreactivity in LSIL, HSIL and control group

dysplasia. Fig. 3 shows the irregular nuclear characteristics of dysplastic cells in the cervical mucosa of patients with HSIL.

Using the Kruskal-Wallis test, we found a statistically significant difference in the nuclear areas in different types of cervical dysplasia (chi square = 79.69, p = 0.000, df = 2) (Table 3). These findings showed that HSILs have more than two times higher ranking of the nuclear area than those with LSIL, and more than ten times higher ranking than the control group without dysplasia.

DISCUSSION

Cervical cancer has been one of leading causes of death in women in the 20th century (Wentzen et al., 2010; Jović et al., 2008). Dysplasia, as a precursor lesion, is a morphologic substratum group of pathogenic events at the cellular level. As benign lesions with potential for malignant transformation, dysplasias are reversible, and potentially self-limiting or progressive types of lesions. Cervical cancer and dysplasias are usually associated with high risk-HPV (Jović et al., 2008). Risk monitoring in patients with an HPV-induced dysplastic epithelium using immunohistochemical markers is an important practical and theoretical task for the prevention of carcinoma formation. Survivin is a potential biomarker of dysplasia caused by HPV oncoproteins (DeYoung and Ellisen, 2007; Regauer and Reich, 2007). Inhibition of apoptosis by HR-HPV oncoprotein is an important step in the pathogenesis of cervical dysplasia. The results of previous investigations showed colocalization of HPV DNA and survivin, suggesting that survivin plays a role in HPV-caused cervical dysplasia (Lesnikova et al., 2009; Lopes et al., 2007; Brenan et al., 2008; Barbosa et al., 2011; Frost et al., 2002).

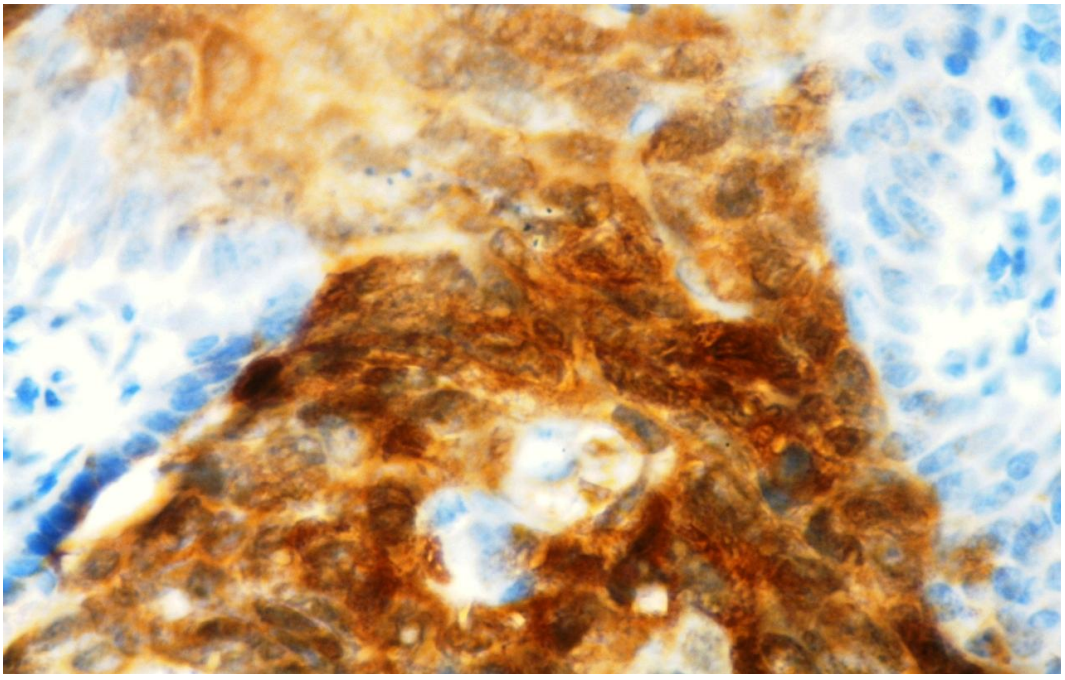

Fig. 2. Survivin nuclear and cytoplasmic immunoreactivity (400 x magnification)

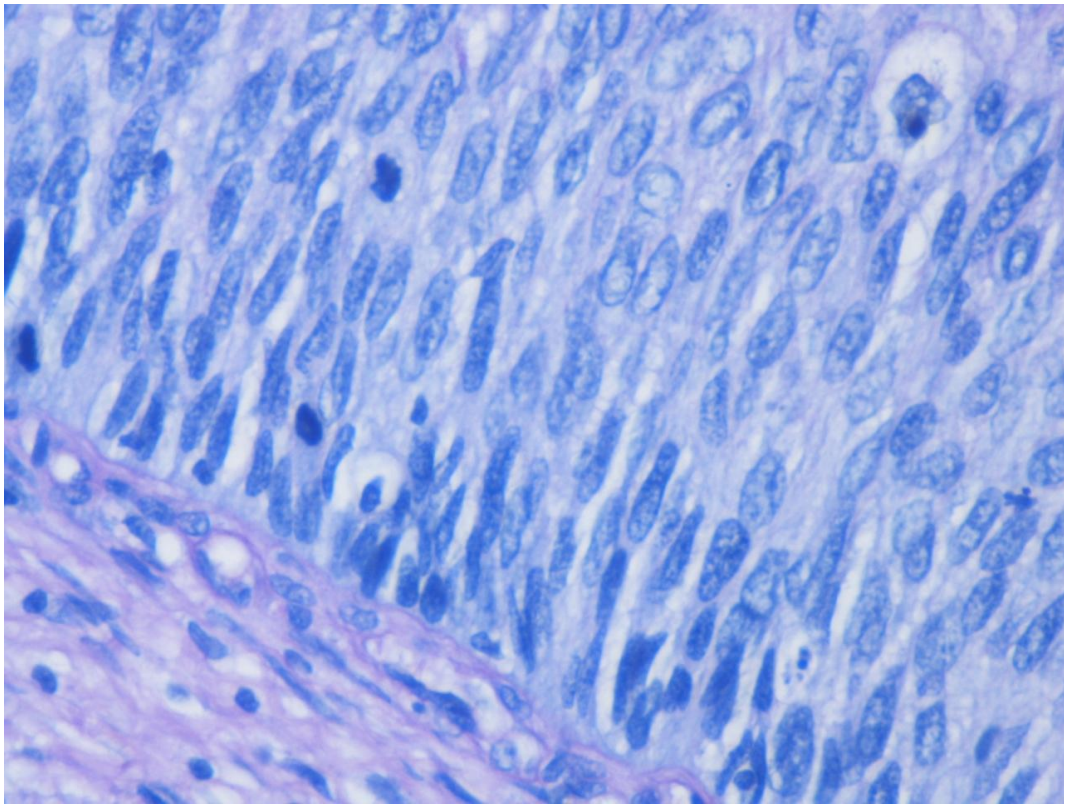

Fig. 3. Irregular nuclear characteristics of dysplastic cells in the cervical mucosa (Periodic Acid Schiff staining; 400 x magnification)

In our study, in the control group we found survivin immunoreactivity in individual cells in the basal and parabasal layers. There are studies in which survivin immunoreactivity in the epithelium without dysplasia was absent (Branca et al., 2005), and studies showing survivin immunoreactivity in the basal and parabasal cells in normal epithelium (Frost et al., 2002). We could explain a positive immunoreactivity in areas without dysplasia by unrecognized infection with HPV types that were not identified in our study in combination with other risk factors. We found a correlation between the degree of cervical dysplasia and expression of survivin in patients with different degrees of cervical dysplasia ($p = 0.003$). Immunoreactivity was cytoplasmic and/or nuclear. This localization of immunoreactivity may be explained by the intracellular distribution of survivin that could be nuclear or cytoplasmic. The increase in immunoreactivity with the degree of dysplasia is caused by the inhibition of apoptosis by high risk-HPV oncoproteins. Survivin immunoreactivity is considered an indicator of genomic instability caused by the E6 and E7 oncoprotein (Cheah and Looi, 2008). Therefore, some authors suggest that survivin is a specific marker for HR-HPV-induced cervical dysplasia (Barbosa et al., 2011). In our study, we had cases of dysplasia that did not show immunoreactivity for survivin. We explain this with the fact that some HPV-induced dysplasias spontaneously regress through the mechanisms of cellular repair, helped by factors of local and general immunity.

Morphometric analysis showed a statistically significant association between the nuclear area and the degree of dysplasia. We found a high statistical difference between the area of nuclei in cervical dysplasias of different degrees ($P = 0.000$). The high-grade dysplasia had more than a 2-fold ranking than those with LSIL, and a more than a 10-fold higher value than the control group without cervical dysplasia. These results can be explained by the fact that the initial infection of basal cells does not always create a clear morphological lesion. After a longer period of productive infection with an integration of viruses and viral DNA replication, the intermediate and superficial layers of the epithelium are occupied and damaged. The expression of E6 and E7 HR-HPV oncoproteins leads to defects in cell cycle regulation with activation of cell cycle, the appearance of mutations and violation of the genomic balance, with abnormal mitotic spindles and polyploid cells. Therefore, cellular morphological and phenotypic changes are lower in latent HPV infection with a small number of copies of the virus, where compatibility with the chromosomal DNA of the host persists. In productive HPV infection, viral DNA replication is independent of host chromosomal DNA. In addition, epithelial cells produce transcription factors that cause an increase in morphological nuclear changes. All nuclear changes are summarized during progress of the dysplasia, which is the key element of morphological diagnosis. Morphometric analysis of dysplastic nuclei offer the possibility of categorization and comparison of dysplasias based on physical quantities in the latent and productive infection (Nayar and Solomon, 2004; Cheah and Looi, 2008; Deligdish et al., 2003). Such analysis gives objectivity to the assessment of nuclear characteristics and determination of cytomorphological substrate (Deligdish et al., 2003; Terlikowski et al., 2004).

Our results suggest that the use of the immunohistochemical marker survivin and morphometric analysis are useful in the assessment of cervical epithelial dysplasias.

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