

## VEGF and CD105 immunoexpression in squamous cervical carcinomas and associated precancerous lesions

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### Abstract

In this study, we analyzed the VEGF and CD105 immunoexpression in 24 cervical squamous cell carcinomas and CIN associated lesions with different degrees. For both lesions, MVD values were higher in patients who had associated risk factors. VEGF and MVD expression increased in both categories for high-grade lesions, respectively CIN III lesions compared with CIN I/II and poorly differentiated carcinomas compared with well-differentiated ones. Also, there was a statistically significant association between VEGF and MVD in poorly differentiated carcinoma and CIN III. The study indicated that analyzed markers were specific for both early and advanced stages of cervical angiogenesis. Maximum values of VEGF and MVD in CIN III designate this lesion as critical to the progression of neoplasia.

**Keywords:** CIN, cervical squamous carcinoma, VEGF, CD105, angiogenesis.

### ✉ Introduction

Squamous cell carcinoma of the cervix is a health issue, worldwide being diagnosed each year about 529 000 new cases [1]. Most cervical cancers are preceded by a long preclinical period and detection of the precursor lesions is essential to prevent and reduce the incidence and mortality [2, 3]. Currently, cervical intraepithelial dysplastic lesions (CIN) are diagnosed more frequently and at younger ages [2]. From this perspective, it has always tried to identify molecular markers of prognosis for these lesions. In the last years, one of the mechanisms involved in cervical carcinogenesis which has been extensively investigated was the angiogenesis [4, 5].

First described by Folkman J, angiogenesis provides a microenvironment conducive to tumor development, being the result of the imbalance between the anti-angiogenic and proangiogenic factors of the tissue [6, 7]. Among proangiogenic factors, VEGF was identified as the most important promoter of neovascularisation by increasing vascular permeability and the mitogen effect on the endothelial cells [8]. VEGF has proven its efficiency as biomarker prognosis for oral, breast, stomach carcinomas and is also a potential serum marker of angiogenesis and tumor progression [9–11].

Tumoral angiogenesis in cervical cancer has been intensively studied and quantified in recent years but data are unclear regarding the prognostic value of VEGF [4, 12, 13]. Also, there is no consensus about which

marker should be used to quantify the newly formed blood vessels [4, 14]. Recent studies indicated CD105 as a specific marker for cervical neoformed vessels and the decrease of CD105 positive vessels appears to be associated with tumor invasion [15]. It is also possible that cervical tumor angiogenesis may not be an indispensable feature of invasive tumors [16].

The aim of this study was the morphometric and immunohistochemical investigation of tumor angiogenesis in cervical squamous carcinomas and adjacent CIN lesions using CD105 and VEGF.

### ✉ Materials and Methods

We accomplished a retrospective study, which has included a total of 24 cervical squamous carcinomas with associated cervical intraepithelial neoplasia (CIN). Cases were selected and diagnosed during 2009–2011, in the Pathology Laboratory of Emergency County Hospital of Craiova. The biological material was represented by biopsies pieces from patients hospitalized in the Gynecology Clinics of the same hospital, which were processed by common histopathological technique using 10% formalin fixation, paraffin embedding and Hematoxylin–Eosin stain. We analyzed epidemiological data and histopathological classification of invasive malignant lesions was done in conformity with criteria established in 2003 by IARC nominated work group for female genital tract tumors within World Health Organization [17].

Immunohistochemical analysis was performed in accordance with instructions of LSAB2 System–HRP kit (Dako, code K0675) for VEGF, respectively Biotin-free Tyramide Signal CSAII Amplification System (Dako, code K1479) for CD105 (endoglin). It were used monoclonal mouse anti-human VEGF (clone C1, dilution 1/100, DAKO) and mouse anti-human CD105 (clone SN6h, dilution 1/1000, Santa Cruz Biotechnology) antibodies. For the visualization of the reaction, the diaminobenzidine tetrahydrochloride (DAB, Dako) was used, followed by counterstained with Hematoxylin. Negative external control staining was done by omitting primary antibodies.

For the quantification of VEGF expression, we have made a histoscore (HScore), which reflected the heterogeneity of the stain regarding the percentage of labeled cells and intensity, using the formula:  $[HScore = \%positive \times (intensity + 1)]$ ; positive cell counts was done at 40× microscope field [4, 18]. In this formula, the intensity of reaction was measured as a score between 1–3, which defined the reactions with low, moderate and high intensity.

For the quantification of CD105, we assessed the microvascular density (MVD) by “hot spot” method, which was the average number of stained blood vessels in three microscopic fields (MF) with the richest vasculature [19]. Areas were identified at 10× MF, and quantification was performed at 20× MF.

Statistical analysis of the results was performed in SPSS 10 software using the *chi-square* test for dependence assessment. The acquisition of the images was done with Nikon Eclipse E600 and software program Lucia 5.

## Results

The analyzed squamous cervical carcinomas were mainly diagnosed in patients aged between 40–60 years, which represented 62.5% of all cases. In the antecedents of patients were identified risk factors for developing cervical carcinoma as HPV confirmed infection, hormone therapy, and associations in 20.8% of cases (Table 1).

**Table 1 – Epidemiological and histopathological parameters**

Epidemiological and histopathological parameters		No. of cases	%
Age [years]	<40	7	29.2
	40–60	15	62.5
	>60	2	8.3
Risk factors	HPV	6	25
	Hormonal therapy	4	16.6
	Associations	5	20.8
Degree of differentiation	Well-differentiated	5	20.8
	Moderately differentiated	10	41.6
	Poorly differentiated	9	37.6
Associated lesions	CIN I + CIN II + CIN III	3	12.5
	CIN II + CIN III	12	50
Immunoexpression	CD105	24	100
	VEGF	24	100

Histopathological analysis of the selected carcinomas indicated in 20.8% of cases well-differentiated tumors, moderately differentiated in 41.6% of cases and poorly

differentiated in 37.6% of cases. All carcinomas were frankly invasive. In 62.5% of cases, were identified adjacent CIN lesions, most commonly CIN II and CIN III in association (Table 1).

VEGF immunoreaction was identified at cytoplasmic level in all cases. VEGF expression was heterogeneous, with focal or diffuse positive areas (Figure 1). For carcinomas, immunoexpression was diffuse, mostly in the periphery of tumor islands, as identified in the stromal elements, such as plasma cells, fibroblasts and lymphocytes. Medium histological score (HScore) was 130.5 in the cases of well-differentiated carcinomas, 280 in case of moderately differentiated, and 300.5 in poorly differentiated ones. In case of associated lesions, the highest medium HScore values were recorded in cases of CIN III, which was 310.8.

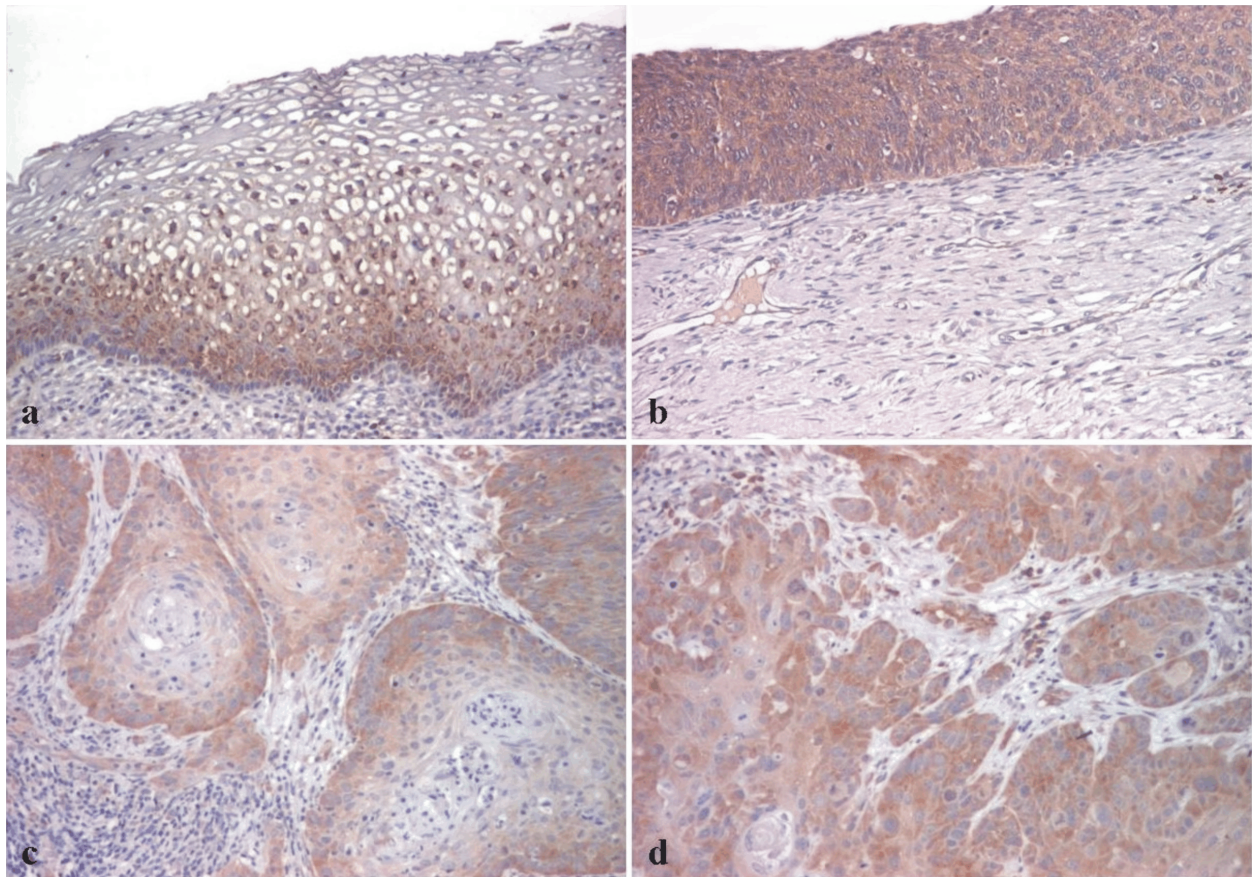
Immunohistochemical investigation indicated CD105 positivity in all cases, the immunoreaction occurring in the cytoplasm of endothelial cells. The immunostain was present in blood vessels with small diameter, irregular, sometimes with branches or collapsed lumen and was absent in large blood vessels. The endoglin-marked vessels were located mainly in the periphery of tumor islands and under dysplastic epithelium in CIN lesions. In CIN lesions, the shortest distance from the epithelial basal membrane of stained vessels was 135.28 µm and the maximum distance was 52 090.8 µm (20× MF). Note that some of the larger vessels distant from the epithelium or stroma tumor showed focal stain that were interpreted as negative.

The mean MVD value for carcinomas varied depending on the degree of tumor differentiation, the highest values being found in poorly ( $12.6 \pm 2.29/MF$ ) and moderately ( $10.8 \pm 1.03/MF$ ) differentiated carcinomas, compared with the well-differentiated ( $9.6 \pm 1.29/MF$ ) ones (Figure 2). Morphometric analysis indicated a maximum microvascular density (MVD) for poorly differentiated carcinomas, which are associated with CIN III lesions. Thus, in dysplastic associated lesions, MVD value varied with the lesion degree, highest values being recorded for CIN III ( $15.5 \pm 2.62/MF$ ), compared with CIN II and CIN I ( $10 \pm 3.32$ , respectively  $8.6 \pm 3.21/MF$ ).

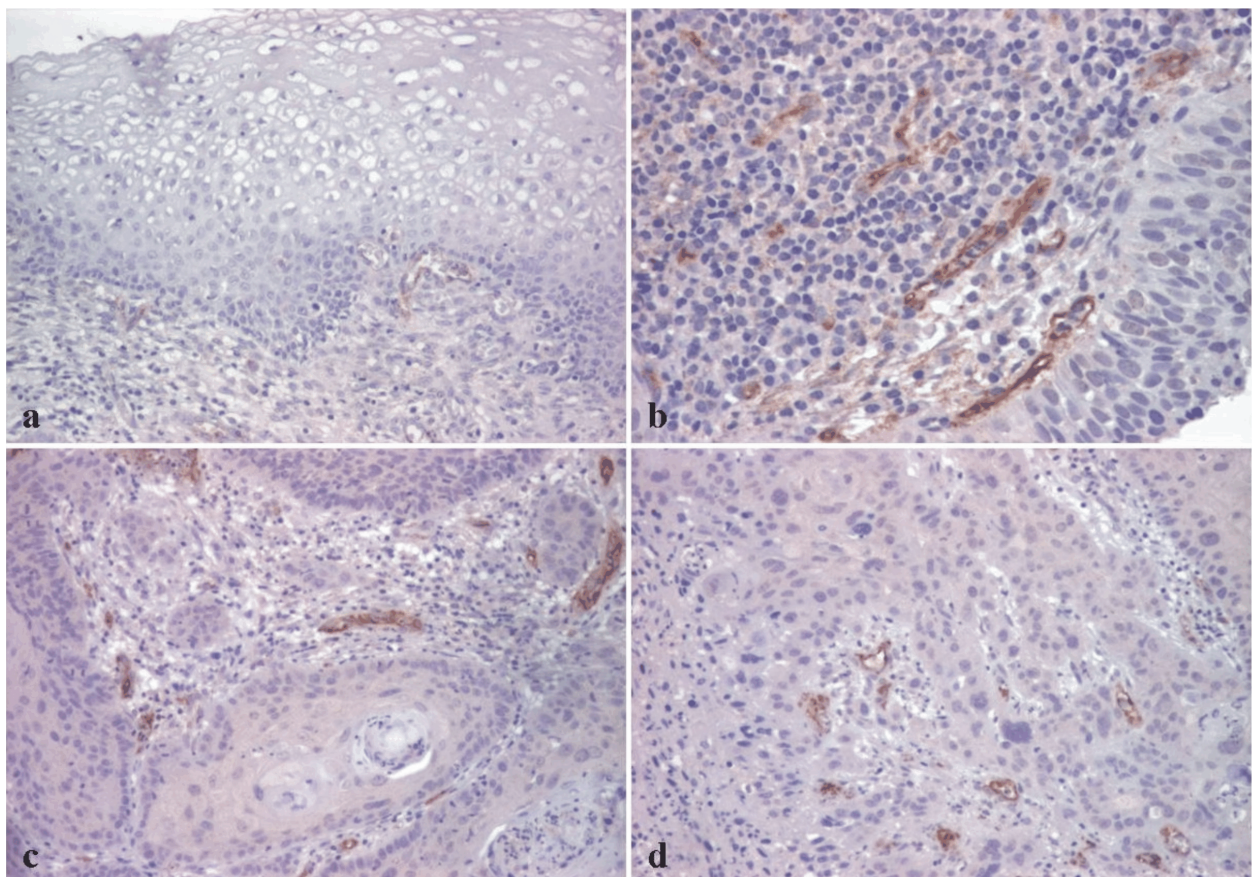
There were statistically significant differences between HScore values of well-differentiated carcinomas and moderately/poorly differentiated, and also between CIN III and CIN I/CIN II lesions ( $p < 0.01$ , *chi-square* test).

*Chi-square* test indicated significant differences between the degree of tumor differentiation and MVD, moderately and poorly differentiated carcinomas having high values ( $p < 0.01$ ). Also, were significant differences of MVD according to degree of carcinomas associated lesions, with highest values for CIN III lesions ( $p < 0.01$ , *chi-square* test). MVD values were higher in cases of patients who have risk factors without statistical significance ( $p > 0.05$ , *chi-square* test). Between VEGF and CD105 we found a close association for CIN III and poorly differentiated carcinomas ( $p > 0.05$ , *chi-square* test). There were no statistical differences between MVD and depth of the surface with CD105-positive vessels.





**Figure 1 – VEGF immunostain, ob.  $\times 10$ :** (a) CIN I; (b) CIN III; (c) Well-differentiated carcinoma; (d) Poorly differentiated carcinoma.



**Figure 2 – CD105 positive vessels, ob.  $\times 10$ :** (a) CIN I; (b) CIN III; (c) Well-differentiated carcinoma; (d) Poorly differentiated carcinoma.

## Discussion

In this study, there was a progressive increase of VEGF immunoexpression to the lesions with low differentiation, the highest values being present in CIN III and carcinomas. The VEGF–VEGFR2 system is the most important stimulator of tumor angiogenesis and also an ideal target for angiogenic therapy [7, 20]. This system determines tumor angiogenesis through autocrine and paracrine mechanisms, sources of VEGF being both tumor and fibroblast cells, plasma cells, lymphocytes, endothelial cells, macrophages [21].

The VEGF immunoexpression is controversial in cervical carcinomas. In 2003, Lee JS *et al.* support the involvement of VEGF in cervical angiogenesis and indicate that protein expression increased from normal cervical epithelium, the precursor and invasive lesions [22]. Gaffney DK *et al.*, on a group of 55 cervical squamous carcinomas treated with radiotherapy, notes that increased VEGF expression correlates with reduced survival interval and poor prognosis [23]. Also, Lee IJ *et al.*, considering in 2002 a group of 117 cervical squamous carcinomas, found that increased VEGF expression is associated with tumor size, depth of invasion and lymph node metastasis [13].

On the contrary, Tjalma W *et al.* indicates the absence of any correlation with clinicopathological parameters of VEGF expression and supports the lack of VEGF as a prognostic marker and No JH *et al.* indicate that between VEGF scores of CIN II, III and carcinoma there is no significant differences [12, 24]. These differences in results can be attributed to heterogeneity of the studied groups and different methods of quantification.

Some studies have indicated that HPV may induce angiogenesis by HIF1 alpha dependent mechanism, with stimulation of VEGF production by infected cells [5, 24]. In this study there was no a higher VEGF expression for patients with proven HPV infection.

The MVD quantification with CD105 revealed correlation with preinvasive and invasive degree, the highest values being present for CIN III and poorly differentiated carcinomas.

Previous studies that have quantified the cervical tumor angiogenesis with panendothelial markers indicated unclear results on the marker that should be optimally used. Some authors consider that CD34 is an excellent marker of tumor vessels and invasiveness, while others prefer CD31 or von Willebrand factor [4, 13, 14].

In 2009, Randall LM *et al.* has investigated VEGF expression and quantified tumoral angiogenesis by CD31 and CD105 in a study witch included 139 cases of squamous cervical carcinomas [4]. The authors found that high MVD/CD31 is associated to a favorable evolution and a higher survival period, being an independent prognostic factor, while the number of increased CD105 vessels is found in patients with decreased survival, with no statistical differences [4].

MVD value for studying tumor progression is also controversial. Thus, a large study in 2006, that studied 474 cases, which included normal cervix, CIN of different degrees, microcarcinoma and invasive carcinoma,

Triratanachat S *et al.* showed that microvascular density of CIN III, microinvasive and invasive carcinoma is higher than normal, CIN I and CIN II and did not observe correlation of MVD with deep stromal invasion, vascular invasion or presence of metastases [25]. In 2003, Ozalp S *et al.* found on a group of 27 CIN, 27 cervical squamous carcinomas and 12 normal specimens that MVD values for CIN II and III are higher than in CIN I and those of carcinomas are higher than CIN [26]. The MVD value is invalidated in 2002 by Graflund M *et al.*, which on a group of 172 patients with early cervical squamous cell carcinomas found no correlation with the presence of metastases, tumor grade, surgical margins status and survival [27].

The prognostic value of CD105 is proven in several studies of tumors involving other sites, such as head and neck carcinoma, breast carcinoma, colorectal carcinoma [28–30]. In studies in which were used panendothelial markers, MVD values are over 15 vessels/MF, values that are greater than in this study (3–20 vessels/MF), which indicates that CD105 is a specific marker for newly vessels unlike CD34, CD31, von Willebrand factor that marks both mature and immature vessels.

## Conclusions

The immunohistochemical study indicated that the VEGF and CD105 are characteristic for cervical tumor angiogenesis in both early and advanced stages. VEGF expression and MVD increased with the degree of preinvasive and invasive lesions. Achieving a maximum rate of MVD and VEGF in CIN III lesions indicates that in the cervical tumor angiogenesis exists a critical angiogenic-dependent step, later probably involving other growth mechanisms.

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