
Shadow Keyplayers of the Uterine Cervix Lesions Progression and Metastasis

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1. Introduction

Cervical neoplasia remains one of the most controversial issues for clinicians, pathologists and researchers. Screening programs have reduced the incidence of invasive neoplastic lesions but have not changed the rate of precursor lesions. Human papilloma virus (HPV) is confirmed to be involved in the etiology of uterine cervix lesions. Recently, Coutlée *et al.* reported that samples from women with low grade squamous intraepithelial lesions (LSIL) contained higher HPV-6 loads than women without this lesion. Despite the existence of an anti-HPV vaccine, HPV gene expression is dramatically altered during cervical carcinogenesis and novel biomarkers are needed for a better characterization of neoplastic cervical progression[2]. Incomplete characterization of the uterine cervix cancer from molecular point of view represents the main problem for the use of a proper therapy in this disease. Angiogenesis and lymphangiogenesis remain a paradigm of cervical lesions progression and metastasis. Most of the papers concerning angiogenesis were based on description of growth factors and/or microvessel density assessed in cervical lesions, especially in invasive carcinomas. The cellular and molecular mechanisms of the angiogenic switch in preneoplastic lesions of the uterine cervix are still less studied. The assessment of angiogenesis in uterine cervix lesions was performed using pan-endothelial markers such as CD31, CD34 and von Willebrand factor, which are not specific enough to differentiate between endothelial cells from normal, activated or tumor vessels[3-5]. All these antibodies stain both normal and tumor vessels. In 2008 Mazibrada *et al.* reported a correlation between CD105 microvascular density (MVD) and CD31 MVD and found an increase in both MVD as a continuum from benign lesions to invasive squamous cell carcinoma⁶. Endothelial cell activation and proliferation might be the clue for an early angiogenic switch and must be study more deeply.

Uterine cervical lesions and the involvement of LV in the tumor development, progression and metastasis still remains a field of debate. Few articles describe lymphatic vessel density and the potential role of lymphangiogenesis in the progression of cervical neoplasia[7-8]. The relationship between lymphangiogenesis and the invasion of cervical cancer was suggested by Hashimoto *et al.*, who considered that vascular endothelial growth factor C (VEGF)-C could be a promoter of pelvic lymph node metastasis in invasive cervical cancer [9]. Usually, malignant lesions of the uterine cervix are considered more important than precursor lesions. This fact may explain the lack of data about the promotion of lymphangiogenesis in pathological conditions of the uterine cervix. No data about the origin of lymph vessels, the prognostic impact of lymphangiogenesis in precursor lesions of the uterine cervix, or their involvement in nodal pelvic metastasis development have been confirmed. Available data about lymphatic vessels (LV) in tumors of the uterine cervix are scattered and controversial, and moreover, there are almost no references regarding lymphangiogenesis in precursor lesions and also for the normal cervix [10, 11].

Based on the controversial data concerning expression of CD105 and Ki-67 in endothelial cells from uterine cervix lesions, the aim of the present study was to investigate the distribution and co-localization of mentioned markers in benign and malignant conditions of the uterine cervix. Concerning early steps of lymphangiogenesis, the purpose was to evaluate morphology, distribution, lymphatic microvessel density and lymphatic proliferation in different stages of cervical lesions. Comparison of type-specific features of lymphangiogenesis in premalignant and malignant lesions, correlated with proliferative status of lymphatic endothelial cells, could help detect the early lymphangiogenesis in pathological conditions of the uterine cervix. A complete characterization of the morphological and immunohistochemical data of LV could have a prognostic and therapeutic impact and confirm diagnosis in cervical lesions.

2. Morphologic and immunohistochemical methods for blood and lymphatic vessels assesment in cervical neoplasia

One of our research included 128 retrospective targeted biopsies of the uterine cervix and specimens taken from conization in patients with macroscopically detectable lesions. Specimens were fixed in buffer formalin and embedded in paraffin, based on the conventional histological technique. Step sections, 5 mm thick, were prepared for each case. Initial sections were stained with HE, for the pathological diagnosis and grade of the tumor. Lesions were stratified as follows: squamous cell metaplasia ($n = 27$); cervical intraepithelial lesions (LSIL, $n = 25$) and (high grade intraepithelial squamous lesions (HSIL), $n = 23$); carcinoma *in situ* ($n = 10$); microinvasive carcinoma ($n = 15$); and invasive carcinoma ($n = 20$). Normal uterine cervix specimens (biopsies collected from patients with inconclusive results of colposcopy when conization was recommended by gynecologist) were used as control ($n=8$). We applied a double stain method for colocalization of CD105 and Ki-67 markers on the same section. On dewaxed and rehydrated slides we performed endogenous peroxidase

blocking with 3% hydrogen peroxide for 5 min followed by pretreatment with proteinase K for 15 min at room temperature. Incubation with primary CD105 antibody, clone SN6h from DakoCytomation (Glostrup, Denmark) for 1 h (dilution 1:10) preceded the first application of avidin–biotin system LSAB+HRP (Dako, Carpinteria, CA, USA), and visualization was performed with 3,3 diaminobenzidine as chromogen. Automated method with PT Link module of heat-induced epitope retrieval in citrate buffer pH6 (DakoCytomation) was used for 30 min to unmask the Ki-67 epitope. After 30 min incubation with Ki-67 ready-to-use antibody (clone MIB1, Dako, Carpinteria, CA, USA), the LSAB+HRP system was used, followed by visualization with amino-ethylcarbazol as chromogen for 10 min. Colocalization of the Ki67 proliferation marker (monoclonal antibody, clone MIB1 from Dako, (Carpinteria, CA, USA), with D2-40 in lymphatic endothelium was obtained by applying the double immunostaining method followed by the use of two different chromogens (3,3'diaminobenzidine for nuclear brown staining of Ki67 and amino ethyl carbazole for cytoplasmic red staining of D2-40 epitope). Basal cells of stratified squamous epithelium of the cervix and lymphatic endothelial cells were considered positive control. Also, PROX1, VEGF C and VEGFR3 were immunohistochemically assessed by using the same labeled streptavidin biotin methods. Counterstain was performed with Lillie's modified hematoxylin. The entire immunohistochemistry procedure was performed with DakoCytomation Autostainer.

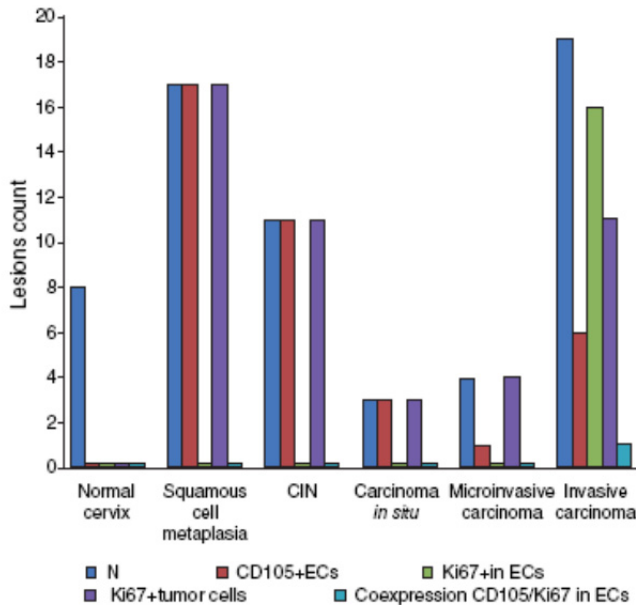


Figure 1. Statistic analysis of uterine cervix lesions based on CD105 and Ki-67 expression. Distribution of CD105-positive endothelial cells, Ki-67 immunoreactivity in endothelial cells and tumor cells according to histopathological type of uterine cervix lesions. Note the presence of Ki-67+ endothelial cells only in invasive carcinoma. Coexpression of CD105 and Ki-67 was also restricted to the invasive type carcinoma. CIN, cervical intra-epithelial neoplasia; EC, endothelial cells.

3. Tumor blood vessels activation and proliferation is different for each histopathologic type of cervical neoplasia

CD105 expression was absent in all cases of normal cervix. CD105 immunoreaction was restricted to the vascular activated endothelium from dysplasia, carcinoma *in situ* and microinvasive carcinoma. Intense activation of blood vessels was observed in both squamous cell metaplasia and dysplasia, with a CD105-MVD that varied between 0 and 25 vessels/x400 field with a range of 6.5 vessels/x400 field. Also, *in situ* carcinoma had positive vessels for CD105 with no endothelial cell proliferation. The activated vessels were agglomerated close to the epithelial lesions and had a lower density far from them, similar to the dysplasia findings. In microinvasive carcinoma positive reaction for CD105 was found in only one case, and the other three cases were negative. Almost 68% of invasive carcinomas were negative for CD105 and most of them had a tumor cells proliferative index <5%. CD105-negative endothelial cells from invasive carcinoma had a higher proliferation rate compared with CD105-negative vascular endothelium from normal cervix blood vessels. We found a significant correlation between the lack of endoglin expression in proliferative endothelial cells and invasive type of cervix carcinoma ($P = 0.004$). Also, low proliferative index of tumor cells was correlated with lack of CD105 expression in tumor vessels ($P = 0.003$). Lack of coexpression for CD105 and Ki-67 in endothelial cells was a constant phenomenon. In only one case of invasive carcinoma with high CD105 MVD (13 vessels/x400 field) was a positive signal for Ki-67 observed in the nuclei of CD105-positive endothelial cells in the activated vessels located only in the tumor periphery.

4. Lymphangiogenesis and lymphatic vessels in cervical neoplasia- early development with a potential prognostic impact

Basal cells of the epithelium from normal exocervix and cervical squamous metaplasia were positive for D2-40 as a continuous layer over the lamina propria of the cervix. This distribution was also observed in LSIL. A lower intensity of immunostaining was detected for basal cells of HSIL. A discontinuous pattern of the basal layer or clusters of positive cells also characterized HSIL. There were differences concerning the distribution and density of LV between the histopathological types of cervical lesions included in our study. In the normal cervix we found large LV, round or oval in shape without branches, found deep in the lamina propria, far from the stratified squamous epithelium. In the normal cervix, D2-40-positive lymphatic vessels had no positive reaction for Ki67 in endothelial cells. The same pattern was found in squamous metaplasia of the uterine cervix. Lymphatic microvascular density (LMVD) ranged between 4.8 and 6.6 vessels/200 magnification, with an average of 5.8 in the normal cervix. In squamous cell metaplasia the number of LV was not significantly different from results found in the normal cervix. A significant increase in the number of LV was found in cases with HSIL and *in situ* carcinoma. All cases with LSIL, HSIL and carcinoma *in situ* showed D2/40+/Ki67+ proliferative lymphatic endothelial cells. Proliferative vessels were distributed close to the lesions. A significant correlation was

found between the proliferative status of lymphatic endothelial cells in both cervical intraepithelial neoplasia types. In LSIL a higher correlation was found compared with HSIL ($P = 0.009$ in LSIL compared with $P = 0.044$ for HSIL). This finding supports early onset of lymphangiogenesis in cervical lesions. High proliferative activity of lymphatic endothelial cells found in LSIL, HSIL and *in situ* carcinoma was also present in microinvasive carcinoma. A significant correlation of lymphatic proliferative activity and the microinvasive type of cervical cancer was also found ($P = 0.002$). In invasive carcinoma, LV were observed in the tumor and peritumoral areas. A few intratumoral LV were found as small D2-40-positive vessels with narrow or collapsed lumen, irregular walls and no tumor cells inside. Peritumoral LV were significantly more numerous, larger, sinuous, with a discontinuous wall and often containing proliferative lymphatic endothelial cells and tumor cells. Density of lymphatics varied between 1 and 15.2 with an average of 7.25. A significant correlation was also evident for invasive carcinoma ($P = 0.002$).

Expression of Prox1, VEGF-C and VEGFR3 was different concerning the histopathology of cervical lesions. Prox1 was restricted to the lymphatic and venous endothelial cell nuclei, whereas VEGF-C had a wide expression in the tumor, lymphatic endothelial and scattered stromal cells. VEGFR3 had a strong expression in lymphatic endothelial cells from peritumoral or intratumoral lymphatic vessels, and also in the intravascular tumor emboli from invasive carcinoma cases.

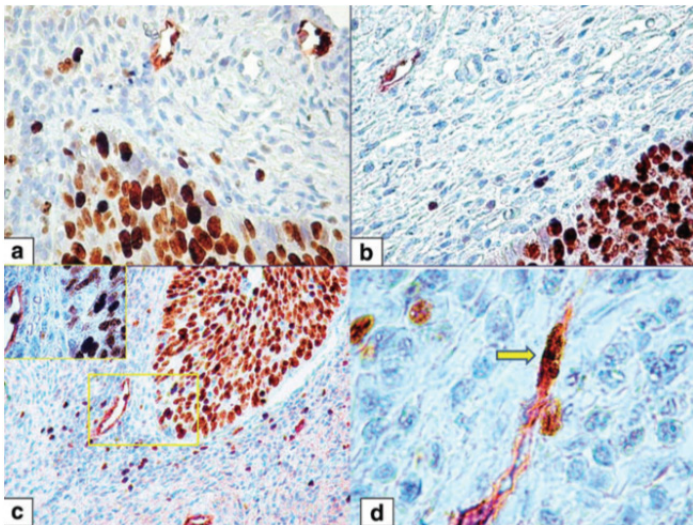


Figure 2. Proliferative status of lymphatic vessels in various cervical lesion types (D2-40 in red and Ki 67 brown immunostain). (a). High grade intraepithelial squamous lesions and (b) *in situ* carcinoma with a high number of Ki 67 proliferative endothelial cells (nuclear, brown) lining D2-40-positive lymphatic vessels (red). (c) Microinvasive carcinoma also presented proliferative lymphatic endothelial cells. (d) Intratumoral lymphatic vessel from invasive carcinoma with proliferating lymphatic endothelial cells at the tip of the lymphatic sprout (arrow) and collapsed lumen lined by D2-40-positive lymphatic endothelial cells.

Prox1 analysis showed the presence of positive reaction from CIN2 lesions (33,3% from total cases) to CIN3- 91,6% and microinvasive carcinoma – 14 cases (87,5%). Prox1 positive endothelial cells circumscribed the lumen of the lymphatic vessels in close vicinity with the epithelial proliferation of uterine cervix “*in situ*” preneoplastic lesions. The average number of Prox1 positive nuclei/x200 increased from CIN2 and CIN3 lesions to microinvasive carcinoma. All Prox1 positive lymphatic and venous blood vessels were also positive for VEGFR3. A significant correlation was found between density of Prox1 positive nuclei of lymphatic endothelial cells and LMVD assessed for VEGFR3 positive lymphatic vessels ($p=0.001$).

VEGF-C expression was found to be positive in all types of cervical lesions. Intensity of VEGF-C expression and number of positive cases increased from squamous metaplasia to invasive carcinoma. VEGF-C was highly expressed in tumor cells, less and inconstant in stromal cells and lymphatic vessel endothelial cells. , We obtained a significant correlation between intermediate grade of VEGF-C expression and Prox1 nuclear density ($p=0.044$). Tumor cells were negative for Prox-1 in 100% of cases. No positive reaction was found in normal specimens, squamous metaplasia, low grade intraepithelial neoplasia or invasive carcinoma.

5. Angiogenesis and lymphangiogenesis controversies in cervical neoplasia-review of the literature

Benign lesions has not been studied as much with regard to CD105 expression. We observed a high CD105 expression in vessels from cervical metaplasia and dysplasia, and absence of endothelial proliferation was quantified using Ki-67 marker. During the early stages of tumor development TGF-b acts as an inhibitor of neoplastic proliferation. When tumor cells escape from the anti-mitotic signal exerted by TGF-b, they secrete large amount of TGF-b, promote cell invasion and metastasis and create an optimal microenvironment for promoting neo-angiogenesis[2,13]. Also, it is known that as cells progress toward fully malignant tumor cells, they undergo changes that result in reduced expression of TGF-b receptors, increased expression of TGF-b ligands, and resistance to inhibition of growth by TGF-b. The present findings suggest the same changes in the endothelial cells of activated vessels from premalignant lesions and invasive carcinoma of the uterine cervix on expression of CD105 (co-receptor for TGF-receptors I and III) without Ki-67—in cervical metaplasia and dysplasia activated vessels— and lack of CD105 staining in Ki-67-positive endothelial cells from tumor vessels of cervical invasive carcinoma. This is sustained by significant correlation found by our team between low tumor cell proliferative index (<5%) and absence of CD105 expression in the vascular bed from cervical invasive carcinoma. Many studies associated CD105 immuno-expression with proliferating endothelial cells[14-16]. The present data showed that in CD105-positive endothelial cells from premalignant and malignant lesions of the uterine cervix, activation and proliferation are two distinct processes in most of the cases. In conclusion, activation

of endothelial cells is an early event that predominates in benign and premalignant conditions of the uterine cervix, while endothelial cell proliferation is observed in tumor vessel endothelial cells from invasive carcinoma of the uterine cervix.

Using the method of step sections, Roche and Norris[17] and later Leman *et al.*[18] demonstrated lymphatic invasion in microinvasive carcinoma without association of lymph node metastasis. Later, Zivaljević *et al.* reported similar results and recommended a personalized treatment based on an exhaustive pathological evaluation of an adequate cone biopsy specimen[19]. These findings support the hypothesis that in microinvasive carcinoma the newly formed LV (based on a higher LMVD found in the present study compared with normal cervix) are already functional. The increase of LMVD in the early stages of LSIL and the similar LMVD value found in the present study in HSIL, LSIL, and carcinoma *in situ* could partially explain the early development of a lymphatic network and may promote early nodal metastases in cervical cancer. Scattered data characterizes lymphangiogenesis in cervical intraepithelial neoplasia lesions. Lymphangiogenic growth factors VEGF-C and VEGF-D, and their corresponding receptor VEGFR3 mRNA levels, significantly increase in HSIL lesions compared with LSIL. Correlation of these data with our findings concerning a doubled LMVD value in LSIL and HSIL compared to the normal cervix suggests an early lymphangiogenic switch in cervical carcinogenesis. Present findings concerning lymphangiogenesis correlated with our previous published data about proliferation of endothelial cells from blood vessels in cervical lesions[20] show an early lymphatic endothelial proliferation in preneoplastic stages of cervical lesions even before the development of the angiogenic response.

Prox1 has been extensively studied, mainly in the context of embryonic development of lymphatic vasculature, liver, pancreas, lens fibers cells and progenitor cells of retinal photoreceptor neurons.). In our study, no Prox1 positive immunostaining was found for normal cervix epithelium or dysplastic one. Up to now, there are no data about Prox1 expression in tumors derived from stratified squamous epithelia. Based on our results, we assume that Prox-1 is not involved in the uterine cervix epithelial carcinogenesis as previously described for other types of neoplasia. Commitment of endothelial cells through a lymphatic phenotype by its Prox1 expression appear early in the development of cervical cancer. A high number of Prox1 positive cells was observed starting from the intraepithelial neoplastic stages (CIN2 and CIN3) to microinvasive carcinoma. These findings can be partially explained by previously published data concerning lymphangiogenesis. all Prox-1 positive lymphatic vessels were positive for VEGFR-3. Similar results were previously reported in lymphangiomas by Wilting *et al.* Furthermore, similar Prox1 and VEGFR-3 positivity of venous endothelial cells might be evidence for the origin of lymphatic endothelial cells from venous endothelium as a main lymphangiogenic mechanism during cervical neoplasia progression. No similar data has been previously reported in the uterine cervix cancer. Thus, we consider that in cervical

neoplasia, venous endothelial cells could specify through a lymphatic phenotype, by activation of Prox1 expression during tumor progression, being able to give rise to lymphatic endothelial cells. The presence of stromal Prox1 positive cells found in the present study suggests the existence of a stromal pool of cells capable of acquiring the lymphatic phenotype.

In summary, the present findings suggests by microscopy, immunohistochemistry and statistical analysis that angiogenesis and lymphangiogenesis are early events in the lesions of the uterine cervix before an overt invasion certified by morphological and immunohistochemical tests. This explains the early development of metastasis from microinvasive carcinoma stage, aggressiveness and poor response to the therapy of cervical invasive carcinoma. Further studies are necessary to evaluate angiogenesis and lymphangiogenesis in preneoplastic lesions of the uterine cervix to show their value for prognosis and therapeutic management.

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