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Prox 1, VEGF-C and VEGFR3 expression during cervical neoplasia progression as evidence of an early lymphangiogenic switch

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Summary. Prox1 is a key regulator of lymphatic endothelial cell commitment during embryonic development. No correlations between Prox1 and VEGF-C/VEGFR3 expression in cervical cancer has been done until now. The aim of the present study was to evaluate the peculiarities of Prox1, VEGF-C and VEGFR3 expression during uterine cervix neoplasia progression. *Material and methods*. One hundred and four specimens taken from women with macroscopically detectable lesions were classified by histopathology and analyzed by immunohistochemistry for Prox1, VEGFR3 and VEGF-C expression. *Results*. The presence of Prox1 nuclear expression was detected starting from CIN2 and CIN3 lesions to microinvasive carcinoma, in the nuclei of lymphatic and venous endothelial cells and scattered stromal cells. All Prox1 positive lymphatic vessels were positive for VEGFR3. A significant correlation was found between expression of VEGF-C in tumor cells and nuclear density of Prox1 positive lymphatic cells (p=0.044). *Conclusion*. The commitment of Prox1 positive cells through a lymphatic lineage is an early event for cervical neoplastic progression, being present starting with intraepithelial cervical lesions, and is strongly associated with VEGFR3 and VEGF-C expression. These findings suggest an early active lymphangiogenesis during cervical neoplasia progression and explain, in part, the early presence of lymph node metastasis in cervical cancer. By the detection of Prox1 expression in lymphatic and venous endothelial cells, and also in stromal cells, it has been suggested that there are at least three different mechanism of lymph vessel development during cervical neoplasia progression.

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Introduction

The development of lymphatic vessels is a complex, multistep, highly-organized biological event (Oliver and Harvey, 2002). A huge amount of information regarding the mechanisms (intrinsic and extrinsic) involved in this process has been learned in the last two decades (Oliver and Detmar, 2002). The characterization of lymphatic endothelial cells markers as podoplanin (Breiteneder-Gellef et al., 1999), VEGFR3 (Kaipainen et al., 1995), LYVE-1 (Banerji et al., 1999), and Prox1 (Wigle and Oliver, 1999) represented a real breakthrough in the field of lymphangiogenesis study. The discovery and characterization of several lymphangiogenic markers led to the development and study of an increased number of drugs that are able to modulate lymphatic vessels growing at different stages of lymphangiogenesis. Despite the efforts to design new modulators of lymphangiogenesis, most of them demonstrated their low efficiency in clinical practice. This fact strongly supports a highly organ-dependent specificity of lymphatic vessels (Sabin, 1902) and suggests the need for more preclinical studies.

Lymphatic vessels develop from the cardinal veins during embryonic life (Wigle and Oliver, 1999).

Endothelial cells that line the veins are morphologically identical and express the same markers. The fate of venous endothelial cells towards the lymphatic phenotype involve a specific and mandatory molecular factor -Prox1- able to determine the future lymphatic phenotype.

Prox1 plays a crucial role in the embryonic development of a variety of organs such as eye (Duncan et al., 2002), liver (Burke and Oliver, 2002) or pancreas development (Wang et al., 2005) and also, in the development of the lymphatic system (Wigle and Oliver, 1999).

Prox1 homedomain is highly conserved between species, indicating a similar role of Prox1 in these species. Sequence comparison of human Prox1 homedomain revealed 65% homology with Drosophila Prox1, and 94% homology with mouse and chicken Prox1 (Zinovieva et al., 1996).

An increased number of papers concerning Prox1 expression in different tumors have been published in the last years. Prox1 low expression enhances pancreatic cancer development (Schneider et al., 2006) and, also, contributes to the development of hepatic carcinoma, biliary system neoplasia and breast cancer (Shimoda et al., 2006; Laerm et al., 2007; Versmold et al., 2007). Also, Prox1 overexpression has been strongly associated with colon cancer progression (Petrova et al., 2008).

VEGF-C is one of the most powerful growth factors involved in the proliferation, migration and survival of lymphatic endothelial cells. Secreted by both tumor and stromal cells, VEGF C expression was highly associated with an increased lymphatic microvascular density and regional lymph nodes metastases (Mandriota et al., 2001).

VEGF-C has a high affinity for VEGFR3, a tyrosin-kinase transmembrane receptor (Joukov et al., 1996; Makinen et al., 2001) with specificity for both VEGF C and VEGF D. In the early embryonic life, VEGFR3 is expressed by endothelial cells of blood and lymph vasculature. Later, during fetal development, and, than in adult life VEGFR3 expression becomes restricted to lymphatic vessels and sinusoid capillaries endothelial cells (Kaipainen et al., 1995) and, in pathologic conditions promotes tumor invasion and metastases (Su et al., 2007).

Cervical cancer is the second most widespread cancer among women throughout the world (http://www.who.int/reproductivehealth/topics/cancers/en/). Lymphatic vessels from cervical neoplasia play a crucial role in cancer cells spreading but the mechanisms of tumor lymphatics development and pathways of lymphatic metastasis are not fully characterized for cervical lesions. Early proliferation of lymphatic vessels found in the preneoplastic lesions of the uterine cervix (Cimpean et al., 2011), together with a strong correlation between high expression of VEGF, VEGF-C, VEGFR2 and CIN or cervical invasive carcinoma, suggested the involvement of these factors in the pathogenesis of cervical neoplasia (Jach et al., 2010).

The mandatory lymphangiogenesis inducing factor as VEGFR3 and Prox1 are not fully characterized in cervical neoplasia, especially in preneoplastic lesions of the uterine cervix.

Based on previously described evidence, we proposed, as the aim of the present work, the study of cervical cancer lymphangiogenesis by assessement of Prox1 and its correlation with VEGF-C and VEGFR3 in preneoplastic and neoplastic lesions of the uterine cervix.

Material and methods

We collected one hundred and four cervical biopsies from patients with macroscopically detected lesions of the uterine cervix. The specimens were fixed in 10% buffered formalin and paraffin embedded. Three micrometer serial step sections were performed from each case. One section was stained with haematoxylin and eosin method for histopathologic evaluation of the following lesions of the uterine cervix: squamous metaplasia - 12 cases (n=12), CIN1 (n=8), CIN2 (n=6), CIN3 (n=24), microinvasive carcinoma (n=16), invasive carcinoma (n=26). Uterine cervix normal tissues from twelve specimens obtained by autopsy were used as a control group. Lymphatic endothelial cells and committed cells through lymphatic phenotype were highlighted by immunohistochemistry using anti-Prox1 antibodies (polyclonal, Acris Antibodies, dilution 1:200), and anti-VEGFR3 antibodies (ready to use, Labvision/Neomarkers, USA). Assessement of VEGF C expression in tumor and stromal cells was done by using anti VEGF-C antibodies (polyclonal, dilution 1:200, Santa Cruz Biotechnology, USA). Incubation with primary antibodies was followed by the use of avidin-biotin/HRP working system (LSAB+ kit, code 0690, Dako Cytomation, Golstrup, Denmark), 3,3'diamino-benzidine as chromogen and nuclear counterstain with modified Lillie's Haematoxylin. All immunohisto-chemical steps were performed in an automatic manner by using PT Link system for automated antigen retrieval and DakoAutostainer for the next immunohistochemical procedures.

VEGFR3 positive vessels were quantified with the method proposed by van der Auwera et al. (2006).

Prox1 positive nuclei were also counted in an automated manner using Lucia G software. Assessment of VEGF-C expression in tumor cells was done by a previously described method proposed by Raica et al. (2007). This method quantified VEGF expression by using a score from 0 to 3 where 0 means the absence of expression, 1 - weak expression, 2 - moderate expression and 3 - strong expression.

Statistical analysis was performed with SPSS 13.0 software system, using bivariate correlation and Pearson correlation test. A p-value less than 0.05 was considered statistically significant.

Results

Assessement of the epithelium, lymphatic and venous blood vessels of the normal uterine cervix specimens revealed the lack of Prox1 positive reaction for control group. The same pattern was observed in squamous metaplasia lesions associated or not with other cervical lesions. In contrast, VEGF-C and VEGFR3 were present in the epithelial layer of the normal cervix and the epithelial cells from cervical squamous metaplasia. Interesting and divergent expressions of VEGF-C and VEGFR3 were observed in the normal cervix epithelium. If VEGF-C had a weak but homogeneous expression restricted to the basal cell layer (and scattered parabasal cells) of the normal cervix epithelium, VEGFR3 lacked the positive reaction in the basal and parabasal epithelial layers, but showed an intensely positive staining in the intermediate and superficial normal epithelial cells (Fig. 1)

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 (Fig. 2a), whereas VEGF-C had a wide expression in the tumor, lymphatic endothelial and scaterred stromal cells (Fig. 2b). VEGFR3 had a strong expression in lymphatic endothelial cells from peritumoral or intratumoral lymphatic vessels (Fig. 3a,b), and also in the intravascular tumor emboli from invasive carcinoma cases (Fig. 3c,d).

Prox1 expression was found in 38 cases (36,5%) and had a nuclear pattern only. Its distribution was found on lymphatic endothelial cells, scattered cells from tumor stroma and venous 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 microscopic field was 2/x200 microscopic field in CIN2, 4.6/x200 microscopic field in CIN3 and 4/x200 microscopic field in microinvasive carcinoma (Fig. 4). 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

Table 1. Significant 2 tailed Pearson correlation of Prox 1expression and VEGF C in the uterine cervical lesions.

		VEGF C IE	Prox1 ND
VAR00001	Pearson Correlation	1	0.333*
	Sig. (2-tailed)		0.044
	N	38	38
VAR00002	Pearson Correlation	0.333*	1
	Sig. (2-tailed)	0.044	
	N	38	38

* Correlation is significant at the 0.05 level (2-tailed).

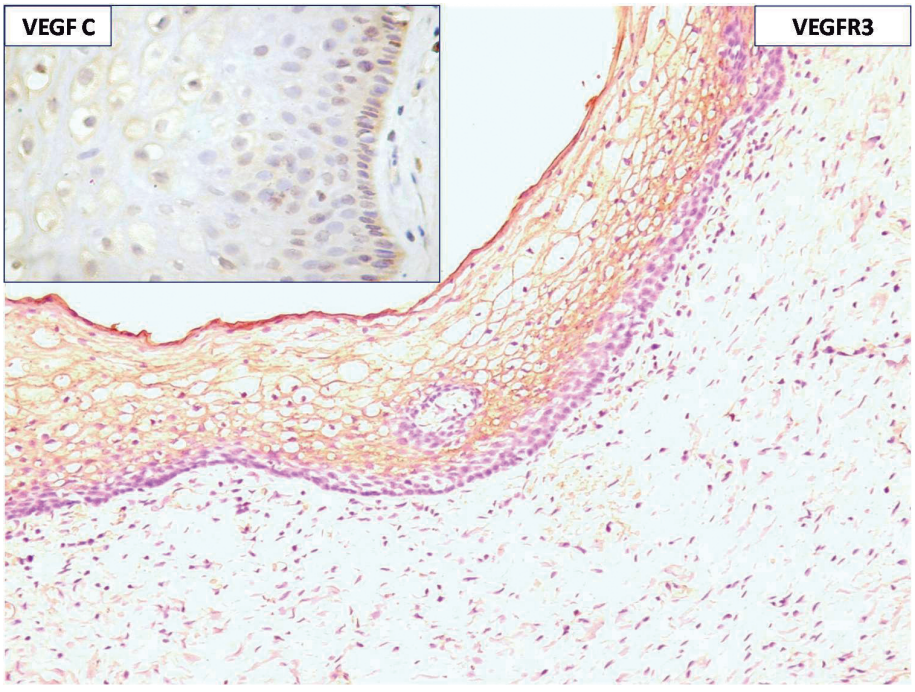


Fig. 1. Expression of VEGFR3 and VEGF C (inset) in the normal epithelium of the uterine cervix. Note restricted expression on basal layer for VEGF C (inset) and VEGFR3 expression on intermediate and superficial cell layers. x 100; inset x 400

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 (Fig. 5). VEGF-C was highly expressed in tumor cells, less and inconstant in stromal cells and lymphatic vessel endothelial cells. From 38 cases with Prox-1 positive specimens, 3 were associated with weak expression of VEGF-C, 29 with intermediate and 6 with strong VEGF-C expression (Fig. 6). Based on these findings, we obtained a significant correlation between intermediate grade of VEGF-C expression and Prox1 nuclear density (p=0.044). However, we need to mention that we compared the Prox-1 positive nuclear density with tumor cell VEGF-C expression, neglecting the stromal cell VEGF-C expression (Table 1).

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.

Discussion

VEGF-C and VEGFR3 are well known crucial players of the lymphangiogenic process (Onimaru and Yonemitsu, 2011). Few and scattered data are available concerning expression of these lymphangiogenic factors in structures other than tumor cells and/or lymphatic endothelial cells. There was reported the presence of VEGF C and VEGFR3 in normal breast epithelial cells (Longatto Filho et al., 2005) and cornea (Cursiefen et al., 2006), but no data about expression of VEGFR3 and its

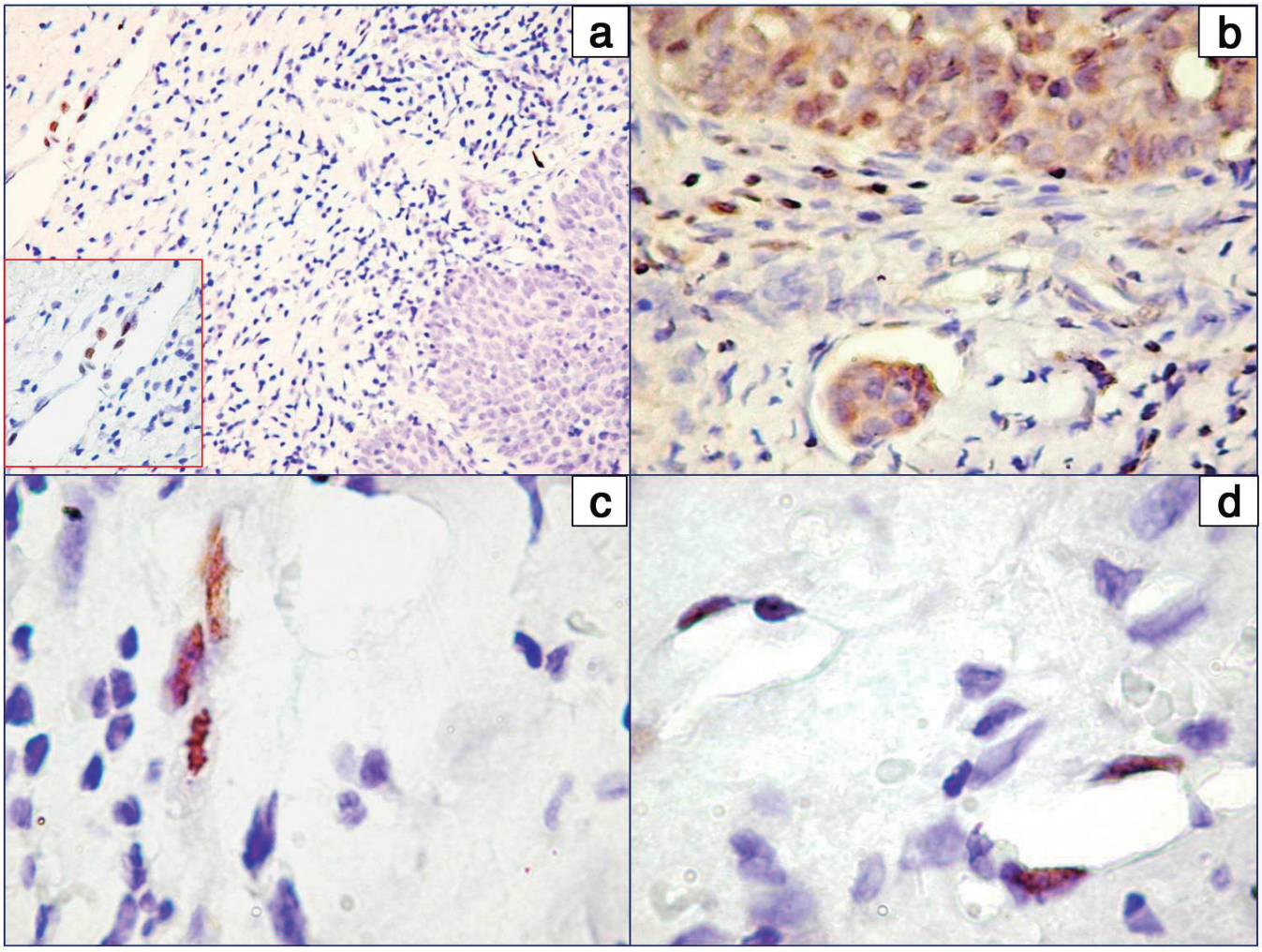


Fig. 2. Nuclear expression of Prox1 in lymphatic endothelial cells from CIN3 lesions **(a)** and VEGF C expression in tumor cells, intravascular emboli and stromal cells of invasive carcinoma **(b)**. Stromal Prox1 positive cells were organized as cords **(c)** or lining newly formed lymphatic vessels **(d)**. a, x 100; b, x 200; c, d, inset in a, x 400

ligand VEGF-C in the uterine cervix normal epithelium have been found. In the present study, we observed by immunohistochemistry the presence of VEGF-C and VEGFR3 in the normal cervix epithelium with a particular immunohistochemical and morphologic pattern. VEGF-C was found in the basal layer, whereas VEGFR3 was restricted to the intermediate and superficial layer of the normal cervix epithelium.

Prox1 is a transcription factor involved in cellular fate, proliferative activity and differentiation of a large variety of the cells during embryogenesis. It plays a crucial role in the early stages of lymphangiogenesis.

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. Some years ago, the first data regarding the potential implication of Prox1 in colon carcinogenesis was reported (Parr and Jiang, 2003). It was found that Prox1 is strongly expressed by tumor cells of advanced colon cancer. Moreover, by genetic experiments on transgenic mice it was proved that Prox1 enchances the transition from normal colon epithelium to a dysplastic phenotype by remodeling of actin cytoskeleton and by disturbing the intercellular junctions and cellular polarity, whereas loss of Prox-1 produces the full restoration and quiescence of epithelium (Petrova et al., 2008). On the other hand, low expression of Prox1 achieved tumor progression in hepatic cancer (Shimoda et al., 2006). 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

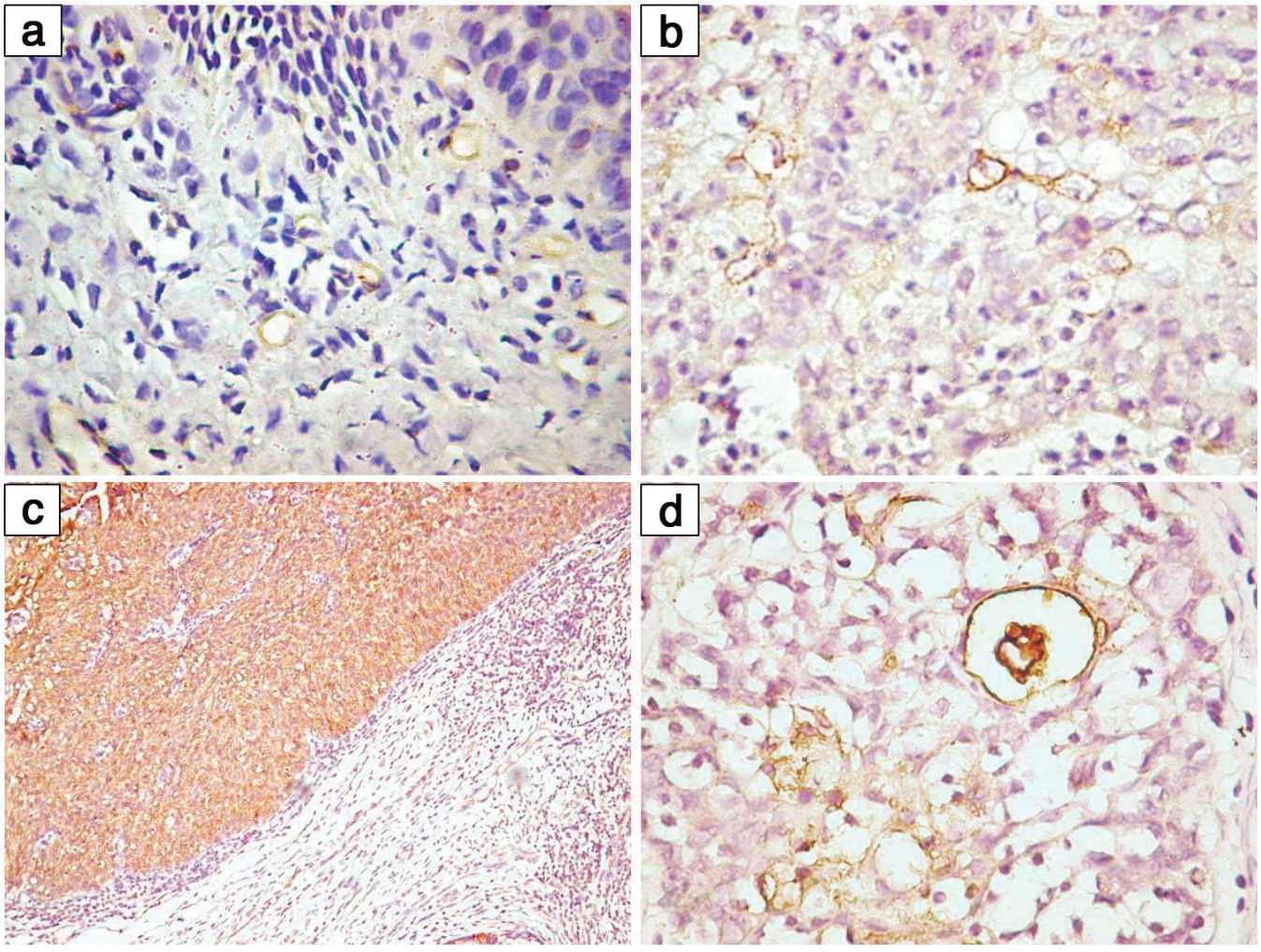


Fig. 3. VEGFR3 expression in peritumoral lymphatic vessels from CIN3 lesion **(a)**, intratumoral lymphatic vessels from invasive carcinoma **(b)**, invasive carcinoma tumor cells **(c)** and lymphatic tumor emboli **(d)**. Note VEGFR3 positive reaction of lymphatic endothelium, tumor emboli and tumor cells with different intensity in **d**. a, b, d, x 400; c, x 200

uterine cervix epithelial carcinogenesis as was previously described for other types of neoplasia.

If Prox1 involvement in cervical carcinogenesis is not evident, its role in tumor lymphangiogenesis seems to be crucial in cervical lesions. Committment 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. Recently, Cimpean et al. (2011) demonstrated an intense and particular lymphangiogenic response found in low and high grade squamous intraepithelial lesions, and microinvasive carcinoma by lymphatic proliferation occurred early in cervical lesions. From the present study data, one more step was added to the previous findings concerning cervical lymphangiogenesis, because a strong correlation between Prox1 expression and the main lymphagiogenic markers VEGF-C and VEGFR3 has been highlighted. This correlation strongly supports the mechanism of a more active lymphangiogenesis in premalignant lesions and microinvasive carcinomas compared to invasive lesions. In this research, all Prox-1 positive lymphatic vessels were positive for VEGFR-3. Similar results were previously reported in lymphangiomas (Wilting et al., 2002). 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. Their distribution in cord-like structures is strong evidence for their active involvement in the development of new lymphatic vessels starting from stromal precursors at an early stage of cervical neoplasia.

VEGF-C induces LEC proliferation via interaction with VEGFR3. There is a lot of experimental and clinical evidence concerning the correlation between LMVD and a high level of VEGF-C expression. It has been shown that expression of Prox1 in primary endothelial cells leads to upregulation of VEGFR3, a receptor tyrosine kinase that is specific for the lymphatic endothelium after mid-gestation and essential for proper lymphatic growth and function (Johnson et al., 2008), whereas inactivating mutations of VEGFR3 in humans

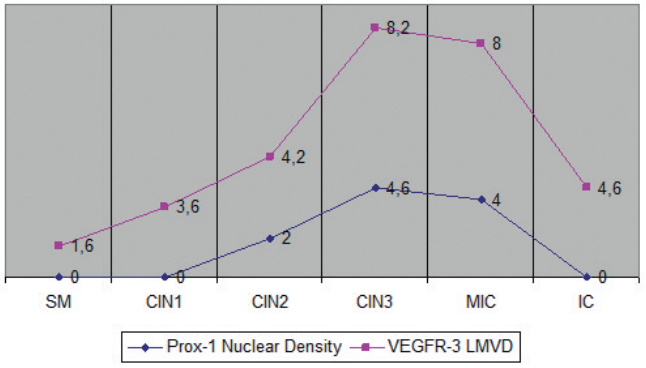
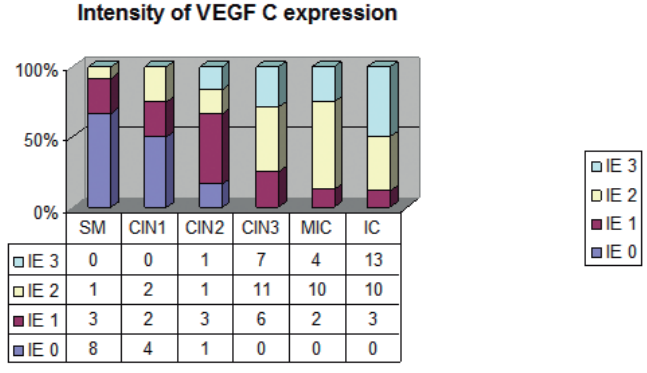


Fig. 4. Graphic expression of VEGFR3 and Prox1 correlation in cervical lesions. A high number of both Prox1 and VEGFR3 positive vessels were detected for intraepithelial lesions and microinvasive carcinoma.



	SM	CIN1	CIN2	CIN3	MIC	IC
IE 3	0	0	1	7	4	13
IE 2	1	2	1	11	10	10
IE 1	3	2	3	6	2	3
IE 0	8	4	1	0	0	0

Fig. 5. Graphic distribution of VEGF-C expression patterns and number of cervical lesions. Note that +2 (moderate) and +3 (intense) expression patterns predominate from CIN3 to invasive carcinoma, with the highest intensity (+3) in most cases of invasive carcinoma. These findings suggest VEGF-C involvement not only in lymphangiogenesis but also could have a role in tumor invasion.

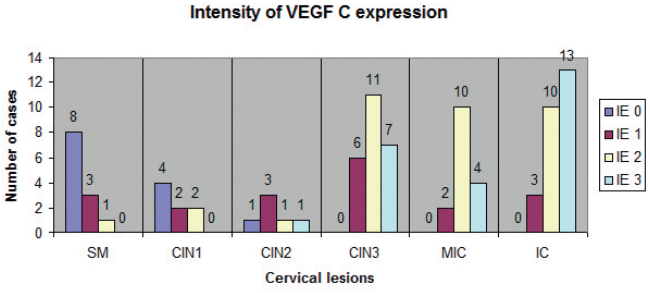


Fig. 6. Intensity of VEGF-C expression in tumor cells from premalignant to malignant lesions of the uterine cervix. Compared with benign lesions (squamous metaplasia) where most of the cases were VEGF-C negative, for invasive carcinoma 50% of cases had intense expression of VEGF-C. An increased expression with moderate pattern (+2) was found starting from CIN3 lesions but this expression was constant from CIN3 to invasive carcinoma.

and mice leads to lymphatic hypoplasia and lymphedema (Jeltsch et al., 1997; Irrthum et al., 2000; Karkkainen et al., 2000). Mishima et al. (2007) demonstrated that Prox1 increased the LEC chemotaxis as a response to VEGF-C via integrin α9 pathway. Prox1 expression inhibits chemotaxis of BECs to VEGF-A, and promotes it to VEGF-C via modulation of their receptors.

The implication of the VEGF-C, VEGFR3 and Prox1 triad in the development of embryonic lymphatic system was demonstrated by Karkkainen et al. in 2004. They showed that VEGF-C signaling is essential for lymphatic vessels sprouting from the embryonic veins but is not mandatory for the venous endothelial cell commitment to a Prox1 phenotype. This latter step required VEGFR3 involvement and only those endothelial cells which co-express VEGFR3 and Prox1 are capable of the sprouting process and consecutive upregulation of other lymphatic markers. In cervical lesions similar processes seem to be developed. In the present study, the active lymphangiogenic process was demonstrated, as starting early in the development of intraepithelial lesions by the presence of VEGFR3 and Prox 1 in lymphatic vessels. We hypothesized that proliferating cervical epithelial cells of intraepithelial and microinvasive carcinomas overexpressed VEGF-C and VEGFR3 and induced an early lymphangiogenic switch by lymphatic endothelial cell commitment and lymphatic sprouting followed by an early tumor lymphatic network development.

In conclusion, Prox1, VEGF-C and VEGFR3 represent the key players of early lymphangiogenesis in cervical cancer, being more active in high grade intraepithelial neoplasia and microinvasive carcinoma than in invasive lesions. The present study reported for the first time the correlation between VEGF-C expression and Prox1 nuclear density in early stage of cervical neoplasia. Our results revealed the presence of three main lymphatic endothelial cells sources, supporting early lymphangiogenesis in cervical neoplasia: activation of previously existing lymphatic vessels, lymphatic phenotype acquisition by the venous endothelial cells and induction of stromal cell differentiation into lymphatic endothelial cells by the presence of VEGF C ,VEGFR3 and a stromal pool of cells able to aquire lymphatic phenotype through Prox1 activation.

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