

Expression of VEGF, VEGF-C and VEGFR-2 in *in situ* and invasive SCC of cervix

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1. ABSTRACT

Cervical squamous cell carcinoma (SCC) arises from the metaplastic epithelium and develops slowly through dysplastic changes (i.e., cervical intraepithelial neoplasia - CIN) to carcinoma *in situ* and invasive cancer. There is little data concerning the quantitation of vascular endothelial growth factor (VEGF) and its correlation to the clinical or pathologic characteristics of SCC. This study assessed the expression of VEGF, VEGF-C and their receptor VEGFR-2 in 35 samples of normal cervical tissue, 35 - CIN1, 35 - CIN2 (25 non-pregnant, 15 pregnant women), 35 - CIN3 and 30- SCC. VEGF, VEGF-C and VEGFR-2 were analyzed using RT-PCR, RQ-PCR, immunohistochemical staining and Western blot. VEGF, VEGF-C and VEGFR-2 were not detected in normal cervical epithelium. In CIN and SCC, both forms of VEGF and its receptor were identified, indicating a correlation between the increasing expression and staging of carcinoma. Results show the important role of VEGF in cervical progression and that the switch to the lymphangiogenesis phenotype occurs prior to the stage of invasion likely at CIN2/3.

2. INTRODUCTION

Cervical squamous cell carcinoma (SCC) is the second most common cancer in women and the leading cause of cancer-related death in females from underdeveloped countries. Each year, approximately 500,000 cases of cervical cancer are diagnosed worldwide. Routine screening has decreased the incidence of invasive cervical cancer in the United States, where approximately 13,000 cases of invasive cervical cancer and 50,000 cases of cervical carcinoma *in situ* (i.e. localized cancer) are diagnosed yearly. Cervical SCC arises from the metaplastic epithelium of the transformation zone (TZ) (squamocolumnar junction) and develops slowly through progressive dysplastic changes to carcinoma *in situ* (CIS) and invasive cancer. Cervical intraepithelial neoplasia (CIN) is divided into three stages according to degree of epithelial dysplasia and differentiation. Lesions are accessible to colposcopic evaluation and biopsy, which makes monitoring disease progression relatively easy. Low grade lesions (i.e., CIN1 and, in some cases, CIN2) may spontaneously regress or not progress further, while the malignant potential of CIN 3 is 36% over 20 years (1)

There is considerable controversy regarding the possible over-treatment of patients with mild cervical abnormalities, with lesions being often excised or ablated. Thusly, identifying the markers of potentially malignant lesions would be of a great prognostic value (2). Adenocarcinoma accounts for approximately 20% of invasive cervical cancers and its incidence is increasing notably among women aged less than 35 years, with no evidence of a reduction in the number of diagnoses since the introduction of mass screening programs (3-5)

Over the past few decades, significant progress has been made in understanding the molecular genetics underlying the development of cervical cancer. There are many associated risk factors, including number of sexual partners, parity, oral contraceptive use, smoking, immune system alterations and lack of antioxidants (6-7), whereas persistent type 16 and 18 human papillomavirus (HPV) infection is the most significant factor in the etiology of this disease (8-9). The development of tumors has conventionally been considered to follow a pre-vascular phase, where the growth of the primary tumor is restricted to a few millimeters in diameter due to the diffusion limit of oxygen (10). Folkman's (11) findings, that growth of solid tumors and their metastasis is dependent on angiogenesis, have been confirmed by several experimental and clinical studies providing indirect (12) and direct (13) evidence. Of all the factors discovered thus far, Vascular Endothelial Growth Factor (VEGF) is one of the most specific and strongest epithelial growth factors. VEGF, a multifunctional cytokine, stimulates angiogenic activity by increasing vascular permeability and acting as an endothelial cell mitogen. This protein family includes VEGF-A (14), -B (15), -C (16), -D (17), -E (18) and placenta growth factor (PlGF) (19), each with different VEGF receptor affinity, various expression levels and different localization. The number of amino acids, their ability to bind heparin and their presence in specific types of cancer has become the basis for differentiating the different VEGF isoforms. Alternative splicing of VEGF-A pre-mRNA leads to secretion of six variants of this homodimeric molecule consisting of 121, 145, 165, 183, 189 and 206 amino acid residues, respectively (20). VEGF-C and VEGF-D regulate lymphatic angiogenesis (21), emphasizing the unique role of this gene family in controlling growth and differentiation of multiple anatomic components of the vascular system. It is controlled dynamically by the local balance of pro- and anti-angiogenic factors in favor of blood vessel formation, where increased pro-angiogenic factors are thought to constitute "angiogenic switches" (10). Recent data has clearly shown that inhibiting VEGF by using bevacizumab (a monoclonal antibody against VEGF) with fluorouracil-based combination chemotherapy resulted in clinical benefit, including increased survival of patients with advanced malignancies. This trial provided the first clinical validation of the hypothesis that blocking angiogenesis may serve as a strategy for treating cancer (22). Thus far, Toi (23) extracted cancer tissue and quantitated VEGF concentrations in breast carcinoma and Obermair (24) evaluated the relationship between VEGF concentration and microvessel density and their correlation with the

prognosis of breast cancer patients. In several types of human tumors, such as breast, colorectal and cervical, high VEGF-C and/or VEGF-D expression has been linked to a poor prognosis (25). However, the quantitation of VEGF and its correlation to their clinical or pathologic characteristics of cervical carcinoma remain unknown.

This study evaluated the correlation between VEGF-C, VEGFR-2 and clinicopathologic parameters in cervical cancer. Normal cervical epithelium did not express VEGF-C or VEGFR-2 in the samples analyzed. Significant differences were found between CIN1-2 and CIN3, but not between CIN3 and cervical SCC. VEGFR-2 expression was strongest in the CIN3 samples, while the CIN1 group remained comparable to control group specimens.

These observations confirm that VEGF is an important angiogenic factor in the progression of cervical carcinoma, further highlighting the role of molecular growth stimulation via VEGFR-2 in cervical carcinogenesis. Results show that the switch to the lymphangiogenic phenotype occurs prior to the stage of invasion, probably between CIN2 and CIN3. Blocking VEGFR-2 signaling may represent a novel therapeutic approach to the treatment of a subset of cervix cancers and cervical intraepithelial neoplasia.

3. MATERIALS AND METHODS

3.1. Patients and sampling

A total of 175 patients, aged 18-72 years, diagnosed with pre-invasive or invasive cervical lesions were included in this study, subsequent to EBB approval by the Local Research Ethic Committee of Jagiellonian University in Krakow, Poland. Between 2004-2006, the Department of Gynecology, Obstetrics and Oncology, Jagiellonian University Medical College, Krakow, Poland obtained consecutive punch or cone biopsy specimens of pre-invasive CIN1 (n=35), CIN2 (n=40) and CIN3 (n=35) lesions. Additionally, specimens of primary cervical SCC (n=30) were included into the study from patients who underwent radical hysterectomy with pelvic lymph node dissection. Another group of patients included pregnant women diagnosed with CIN2/3 (n=15). Normal cervical tissue was obtained from 35 patients who underwent total hysterectomy for various benign conditions.

Patients were screened with Pap smear and recruited to the colposcopy unit at the Department of Gynecology, Obstetrics and Oncology Jagiellonian University Medical College. Colposcopically guided biopsy of the ectocervix and endocervical curettage were performed in all cases with the exception of pregnant women who were not curettaged. The sampling of tissue for this study was especially difficult in early stages of CIN and in cases where the colposcopically diagnosed lesion was of small volume. Samples were divided into two parts: one part was sent for histopathological evaluation and the remainder for analysis as part of this study. Samples were included into this study in cases of histologically confirmed CIN and cervical cancer. The sampling procedure included punch biopsy, LEEP, LLETZ, cold knife conisation, simple

Table 1. Mode of surgical procedure used for obtaining tissue for the study

Surgical procedure	Normal	CIN1	CIN 2	CIN 3	SCC	Pregnancy	Total
Biopsy	0	13	5	0	0	7	25 (14,29%)
LEEP	0	7	3	0	0	0	10 (5,70%)
LLETZ	0	3	14	25	0	0	42 (24,80%)
Cold knife conisation	0	0	0	10	16	4	30 (17,14%)
Simple hysterectomy	35	12	3	0	7	0	57 (32,58%)
Radical hysterectomy	0	0	0	0	7	4	11 (6,29%)
Total	35	35	25	35	30	15	175 (100%)



Figure 1. Colposcopic pictures of the cervix from normal, CIN1 - 3, SCC and pregnancy tissues. Colpophotography 1. Control - Normal cervix. Squamo-columnar junction visible around anatomic external orifice of the cervical canal (n=35). Colpophotography 2. CIN1 - Fine mosaic and fine punctuation of geographic borders. Subclinical HPV infection (n=35). Colpophotography 3. CIN2 - Secondary punctuation and mosaic with slightly elevated borders - (n=25). Colpophotography 4. CIN3 - Secondary coarse mosaic and punctuation. Sharply demarcated elongated borders (n=35). Colpophotography 5. SCC - Secondary coarse mosaic and punctuation. Atypical vessel (neoangiogenesis - endothelial tube) – invasive cervical cancer (n=30). Colpophotography 6. CIN3 in pregnant women (n=15). A total of 175 patients ranging from 18 to 72 years of age diagnosed with pre-invasive or invasive cervical lesion were included in the study. Punch or cone biopsy specimens were obtained from consecutive pre-invasive tissues (Department of Gynecology, Obstetrics and Oncology, Jagiellonian University, Medical College, Krakow). Ethical approval was obtained from the Local Research Ethic Committee of Jagiellonian University in Krakow.

hysterectomy and radical hysterectomy (Table 1). All specimens were formalin fixed and paraffin-embedded for storage. Precise pTNM analysis was performed.

3.2. Tissues

Each tissue specimen was collected during surgery and immediately frozen in RNase later solution (Qiagen, Hilden, Germany): one part was reserved for mRNA analysis and the second for protein analysis. Both parts were stored at -80°C until in this study. Cancer tissue preparation was done as previously reported (26).

3.3. Immunohistochemistry

Formalin-fixed, paraffin embedded, 4 µm sections were air-dried, deparaffinized in xylene, rehydrated with graded ethanol-water mixtures, and then washed with distilled water.

For VEGFR-2 and lymphatic vessels - LV(D2-40) - staining slides were incubated for 15 minutes in 0,3% hydrogen peroxide in methanol at room temperature. Antigen retrieval was achieved by placing the slides in

citrate buffer (pH 6) and then microwave treatment at 100°C for 18 minutes. Sections were washed in distilled water, soaked in a mixture of Tris-buffered saline (TBS) and polyoxyethylene-sorbitan monolaurate for 3 minutes and incubated in 5% normal horse serum at room temperature for 3 minutes. Primary antibodies for VEGFR-2 and LV(D2-40) were applied as 1:200 and 1:10 buffer dilution respectively and slides were incubated for 40 minutes at room temperature. After subsequent incubation for 20 minutes with a biotinylated secondary antibody, VEGFR-2 and LV(D2-40) were detected, according to manufacturer's instructions, using a Vectastain Elite ABC kit (Vector Laboratories, USA). Counterstaining was performed with hematoxylin. Negative controls were performed by omitting the primary antibody or using rabbit IgG. To confirm the specificity of staining against VEGFR-2 and LV(D2-40) both antibodies were pre-incubated with exogenous human VEGFR-2 and LV(D2-40) as

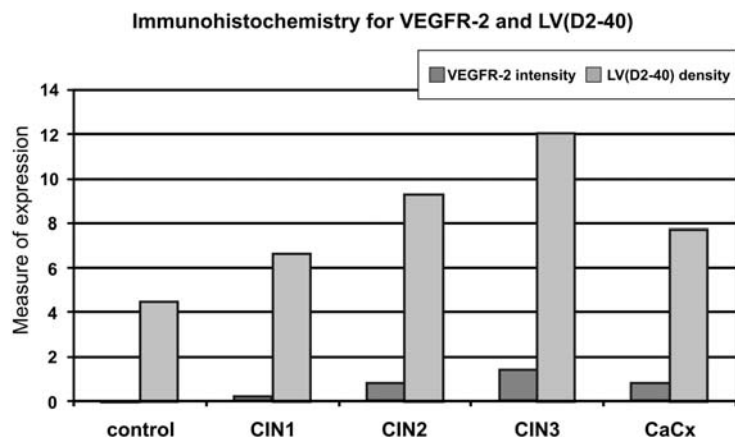


Figure 2. Immunohistochemistry of VEGFR2 expression and lymphatic vessels LV(D2-40) concentration (SCC-pT1a, pCIN2-CIN2 in pregnant women) The presence of VEGFR-2 and LV(D2-40) in squamous epithelium was determined by a semiquantitative method using the following scale: 0 - no expression reaction, 1 - weak expression (positive in less than 10% of cells); 2 - moderate expression (positive in 10-50% of cells); 3 - strong expression (positive in more than 50% of cells). Positive was defined as a moderate and strong expression comparable with control group samples (normal human placenta sections). LV(D2-40) positivity was determined using the “hot spots” method within the gray zone 2 mm under the paraepidermoid epithelium and, in cases of cancer, also within the tumor. After selecting a section, vessels were counted in three big areas of observation (40x, 0.196 mm²). Slides were examined independently by two pathologists without any knowledge of the patients’ clinicopathological features. Control - Normal cervix. Squamo-columnar junction visible around anatomic external orifice of the cervical canal (n=35). CIN1 - Fine mosaic and fine punctuation of geographic borders. Subclinical HPV infection (n=35). CIN2 - Secondary punctuation and mosaic with slightly elevated borders - (n=40). CIN3 - Secondary coarse mosaic and punctuation. Sharply demarcated elongated borders (n=35). SCC - Secondary coarse mosaic and punctuation. Atypical vessel (neovascularization - endothelial tube) – invasive cervical cancer (n=30).

competition controls. Cervical cancer specimens with a high level of LV(D2-40) were used as positive controls for LV(D2-40). Normal human placenta sections were used as positive controls for VEGFR-2.

The presence of VEGFR-2 and LV(D2-40) in squamous epithelium was calculated with semiquantitative methodology, using the following scale: 0 - no expression reaction, 1 - weak expression (positive in less than 10% of cells), 2 - moderate expression (positive in 10-50% of cells), 3 - strong expression (positive in more than 50% of cells). Positive was defined as moderate and strong expression comparable with control (normal human placenta sections). Positive Lymphatic vessels (D2-40) were calculated using “hot spots” methodology within the grey zone, 2 mm under paraepidermoid epithelium, and, in cases of cancer, also within the tumor. After selecting, section vessels were calculated in three big areas of observation (40 x 0,196 mm²). Slides were examined independently by two pathologists without knowledge of clinicopathological features (Figure 2).

3.4. RNA extraction:

Tissues in RNA^{later} RNA stabilization reagent were stored at -80°C until their use for RNA extraction. Each tissue sample was homogenized in lysis buffer (RLT) using a conventional rotor-stator homogenizer until the sample was uniformly homogeneous (usually 20-40s). Total RNA was extracted, according to manufacturer’s instructions, using the RNeasy Protect Mini Kit (Qiagen, Germany). The yield and purity of isolated RNA was

checked by UV spectrophotometric measurement and by electrophoresis in 1.2% agarose gel, followed by routine ethidium bromide staining. RNA preparations characterized by A260/A280 ≥ 1.8 and showing only 18S (1/3) and 28S (2/3) rRNA bands were used for further analysis. These isolated RNA samples were stored in -80°C until applied in RT-PCR analysis.

3.5. cDNA synthesis for RT-PCR analysis

Preparation of cDNA and RT-PCR reaction were carried out as previously described elsewhere (27). The temperature profile of RT-PCR amplification consisted of activation of Taq polymerase at 95°C for 4 min., denaturation of cDNA at 95°C for 30-45 s, primer annealing temperature for 30-60s, elongation for 40-60s at 72°C for the following 35 cycles and finished by a 10 minutes extension step. Identification of expressed mRNA specific for selected proteins was performed with primers for VEGF-isoforms, VEGF-C and VEGFR-2, respectively (Oligo Company, Poland - Table 2). PCR reaction products were separated electrophoretically in a 2% agarose gel and visualized with ethidium bromide.

3.6. cDNA synthesis for real-time PCR

In Parallel, cDNA was synthesized using 1000ng of aliquots of total RNA primed with Superscript II reverse transcriptase (Invitrogen) and oligo d(T) primers (Sigma) in 20µl reactions volume in thermocycler DYAD (MJ Research, Inc., Waltham, USA) After reverse transcription, cDNA was dissolved in water (2:3). Next, 5µl of cDNA solution was taken to RQ-PCR reaction.

Table 2. Primers used for RT-PCR

Gene	Size ¹	Forward 5' - 3'	Reverse 5' - 3'	Conditions
GAPDH	510	CACCGCCTCGGCTTGTCACAT	CTGCTGTCTTGGGTGCATTGG	95°C, 30"; 59°C, 30"; 72°C, 60"; 35 cycles
VEGF-C	727	GATCTGGAGGAGCAGTTACGG	CTCTGCAGACACTGACAGG 3'	94°C, 30"; 68°C, 40"; 72°C, 40"; 35 cycles
VEGFR-2	660	GTGACCAACATGGAGTCGTG	CCAGAGATTCCATGCCACTT	94°C, 30"; 68°C, 40"; 72°C, 40"; 35 cycles
VEGF ²	431	GCCAAGCAGCAGTACATTCTACACG	GTCGTTCTTCACGTGCTCAAAATCC	94°C, 30"; 68°C, 40"; 72°C, 40"; 35 cycles

¹base pair, ², isoforms, "": seconds

Table 3. Primers used for real time PCR

Gene	Name	sequence 5' - 3'
ABL	Forward primer ENF1003	TGG AGA TAA CAC TCT AAG CAT AAC TAA AGG T
	Reverse primer ENR1063	GAT GTA GTT CTT GGG ACC CA
	Molecular probe ENP1043	Fam - CCA TTT TTG GTT TGG GCT TCA CAC CAT T-Tamra
VEGF	Forward primer VEGF	TTG CTG CTC TAC CTC CAC CA
	Reverse primer VEGF	TGA TTC TGC CCT CCT CCT TCT
	Molecular probe VEGF	6Fam- TGG TCC CAG GCT GCA CCC ATG -Tamra
VEGF-C	Forward primer VEGF	TTG CTG CTC TAC CTC CAC CA
	Reverse primer VEGF	TGA TTC TGC CCT CCT CCT TCT
	Molecular probe VEGF	6Fam- TGG TCC CAG GCT GCA CCC ATG -Tamra
VEGF-R2	Forward primer VEGF	TTG CTG CTC TAC CTC CAC CA
	Reverse primer VEGF	TgA TTC TGC CCT CCT CCT TCT
	Molecular probe VEGF	6Fam- TGG TCC CAG GCT GCA CCC ATG -Tamra

Real-time PCR (RQ-PCR) was performed using the 7500 Real-Time PCR System (Applied Biosystems, foster City, USA) and TaqMan probes. Specific primers and probes for *VEGF*, *VEGF-C*, *VEGFR-2* genes and the *ABL* control gene were purchased from TIB MOLBIOL (Poznań, Poland) and Applied Biosystems (Applied Biosystems, Cheshire, UK), respectively. Primer and probe sequences are shown in Table 3.

The standardized protocol for the comparative threshold cycle (Ct) method was used as described in the *Europe Against Cancer* program (28). Amplification was done using recommended standard conditions (28). RQ-PCR results were expressed using the Δ CT method and relative RNA expression was calculated according to Macabeo-Ong (29). All experiments were performed in triplicate, and mean values were calculated. Negative controls lacking template RNA were always included in each experiment.

3.7. Protein detection

Protein concentrations were determined using the Peterson protein assay kit (Sigma) according to manufacture's instructions (30). Total cell extracts amounting to 15 pg – 50 pg of protein/lane were separated by 10% (β-actin) or 8% (VEGFR-2) SDS-PAGE according to Laemmli (31). Total protein detection was analyzed by the Coomassie Blue Binding method described by Sedmak and Grossberg (32)

3.8. Western blot analysis

Tissue samples stored in -80°C were homogenized (usually 20-40s three times) in lysis buffer using a conventional rotor-stator homogenizer until the sample was uniformly homogeneous. Total cellular protein was isolated according to the procedure described previously (27). Western blot analysis was performed on equal amounts of each protein sample. Protein identification was performed with monoclonal anti-beta-actin antibody 1:10000 (Sigma) as a control protein and monoclonal anti-VEGFR-2 antibody in 1:2000 dilution (Pharmingen), respectively. Goat anti-mouse or anti-rabbit

immunoglobulins conjugated with alkaline phosphatase (Sigma) were used as the second antibody. The reaction was examined by the reduction of 4-nitroblue tetrazolium salt in the presence of 5-bromo-4-chloro-3-indolyl-phosphate (Roche) in Tris/HCl (pH 9.5) buffer containing 0.05 M MgCl₂ and 0.1 M NaCl.

3.9. Statistical analysis

Sperman's rank correlation was used to assess whether the immunostaining scoring results given by the two pathologists were concordant and to determine any significant correlations between VEGF-C and VEGFR-2 expression. Chi-square testing was used to compare and determine whether there was a significant difference for VEGF-C and VEGFR-2 expression amongst the different groups of cervical lesions and to determine whether there was a correlation between any of the above variables with clinicopathological prognostic features (i.e., tumor grade, LVSI and lymph node involvement).

Each experimental point for Western Blot analysis was performed in triplicate with the objective to calculate the mean value and standard deviation. T-student testing for small samples was used for data analysis. Statistically significant values were set at the level of $p \leq 0.05$.

4. RESULTS

The presence of VEGF-C and VEGF isoform in 175 cervical lesions was studied by RT-PCR and real time PCR methods, while VEGFR-2 in uterine cervical cancers was determined both at the mRNA (RT-PCR and RQ-PCR) and protein level (Western blot) in CIN1 (n=35), CIN2 (n=25), CIN3 (n=35), cervical SCC (n=30), CIN2/3 specimens originating from pregnant women (n=15) and normal cervical tissue samples (n=35) (Figure 1).

4.1. Immunostaining results for VEGFR-2 and LV(D2-40) in pre-invasive and invasive cervical lesions

Using Spearman's rank correlation testing, significant concordance ($p < 0.0001$) was found between

immunostaining scoring results given independently by two pathologists. Figure 2 illustrates the staining results in both pre-invasive and invasive cervical cancer lesions. Significant differences were found in protein expression as well as LV(D2-40) concentrations between CIN1-2 and CIN3 ($p < 0.001$ in both cases).

Immunostaining results for VEGFR-2 and LV (D2-40) in pre-invasive and invasive cervical lesion samples revealed significant differences in VEGFR-2 staining as well as LV(D2-40) concentrations between CIN1-2 and CIN3 ($p < 0.001$ in both cases) and in LV(D2-40) concentrations between CIN3 and cervical SCC. As far as CIN is concerned, VEGFR-2 expression was strongest in CIN3 samples. In cases of invasive cervical SCC, most times, no expression of VEGFR2 was observed. However, in four cases it could be observed in the majority of cells. In dysplastic lesions, a higher grade of CIN correlated with a higher number of LV(D2-40). In CIN1 samples, LVD2-40 was 6.6 (4-13), in CIN2 - 8.6 (3-16) and the highest value was seen in CIN3 - 11.7 (5-22). In the cervical SCC group, the number of LV(D2-40) was lower than in the CIN2 group, namely - 7.3 (2-13) (Figure 2).

No significant differences were found between CIN3 and cervical cancer. Positive expression of VEGFR-2 was observed in all examined groups, but only in two cases from the control samples. Expression was highest in CIN3 samples, while in the CIN1 group it was comparable to that of the control group. With respect to invasive cervical SCC, no expression of VEGFR2 was observed in the majority of cases. Only in four cases was it noted in the majority of cells. A strong positive VEGFR-2 reaction was found in endothelial cells of some vessels. The number of lymphatic vessels LV(D2-40) in control group samples reached the median value of 5.5 (0-12). In dysplastic lesions, a higher grade of CIN correlated with a higher number of LV(D2-40) (Figure 2).

4.2. Proteins determination – mRNA and protein level

Simultaneously with immunochemical determinations, analyses were carried out at the mRNA and protein level. Expression of the VEGF-C genes was studied in all tissues (Figure 3A, 4A and 5B). There was a direct relationship between increasing stage vs. grade of the lesion and intensity of expression. Using four categories of staining (negative, weak, moderate and intense), moderate and intense expression of VEGF-C was associated with CIN3 and SCC. In CIN1 lesions, negative and weak expression was seen in 75% and 25%, respectively. The expression of VEGFC was meaningfully higher in CIN2: 13% negative, 73% weak and 14% moderate. In the control group, negative expression was 100%, but in cervical SCC lesions we observed an opposite effect, notably 20% moderate and 80% intense (Figure 3A). The relationship between VEGFC tumor growth in cervical squamous cells was studied by mRNA (RT-PCR and RQ-PCR) (Figure 4A and 5B). Positive expression of VEGFC was observed with various degrees of intensity in all the examined CIN1-3 groups and in SCC and was not detected in the control group. Expression was highest in CIN3 samples, while in the CIN2 pregnancy group it was comparable to that of the

CIN3 group. No significant differences were found between CIN3 and SCC. In RQ-PCR analysis, overexpression of mRNA ranging from 14- to 18-fold in the CIN2 pregnancy group and about 50-fold in cervical SCC group was observed. No significant differences in VEGF-C expression in CIN3 and SCC tissues were noticed.

The presence of VEGF isoforms was determined by RT-PCR and RQ-PCR. Among the four subtypes of VEGF, the populations of VEGF₁₆₅ and VEGF₁₂₁ were predominant in normal uterine cervixes and uterine cervical cancers. Compared to other cases, including normal cervixes, levels of VEGF and VEGF₁₆₅ and VEGF₁₂₁ mRNAs were remarkable in some CIN2 samples and the majority of CIN3 adenocarcinomas of the cervix. Also, the expression of VEGF₂₀₆ was higher in some CIN2- and CIN3-stage cancers and in two pregnancy samples. Therefore, the elevation of VEGF₁₆₅ and VEGF₁₂₁ might contribute to the relatively late advancing, via angiogenic activity, of some cervical adenocarcinomas (Figure 4A). The very high expression of mRNA levels was identified in the CIN3 and cervical SCC groups (Figure 4 and 5).

In a parallel analysis, we measured the expression of VEGFR-2 at the mRNA level. Figure 3B shows that the expression of VEGFR-2 in CIN1 was 6% moderate, while CIN2 was positive in 33% of weak and 40% of moderate cases. VEGFR-2 overexpression was observed in CIN3-SCC, identified as intense in 48% of CIN3 and 80% of cervical SCC, respectively. Of those diagnosed with CIN3 ($n=35$), only one woman showed negative staining for VEGFR-2 expression. A similar effect (i.e., increased expression of VEGFC or VEGFR2) was observed in the case of CIN3, cervical SCC and pregnancy sample groups. However, these tissues did not show any pronounced differences between CIN3 and SCC (Figure 4A-C). Positive expression of VEGFR-2 (i.e., on mRNA and protein levels) was observed in all the examined groups and only in one control group participants (Figures 3B, 4A-C and 5C). VEGFR-2 expression was strongest in CIN3 samples, while in the CIN1 group it was comparable to the control group (Figures 3B, 4A-C and 5A-C). In the first group (i.e., normal cervical tissue) the analyzed samples showed no expression of VEGF-C or VEGFR-2.

These findings provide evidence that VEGF is involved in the promotion of angiogenesis in cervical cancer and may play an important role in early invasion.

5. DISCUSSION

Several issues are important in relation to the prediction of cervical SCC and CIN (31). According to the task force on prognostic factors in cervical SCC, there is an urgent need for more specific markers predicting the disease outcome in individual patients (32). One of the key mechanisms leading to the invasive phenotype in progressing CIN lesions is angiogenesis, which is induced by several angiogenic factors, such as those of the VEGF family. The identification of VEGF transcripts in cultured vascular smooth muscle cells (33) and the response of

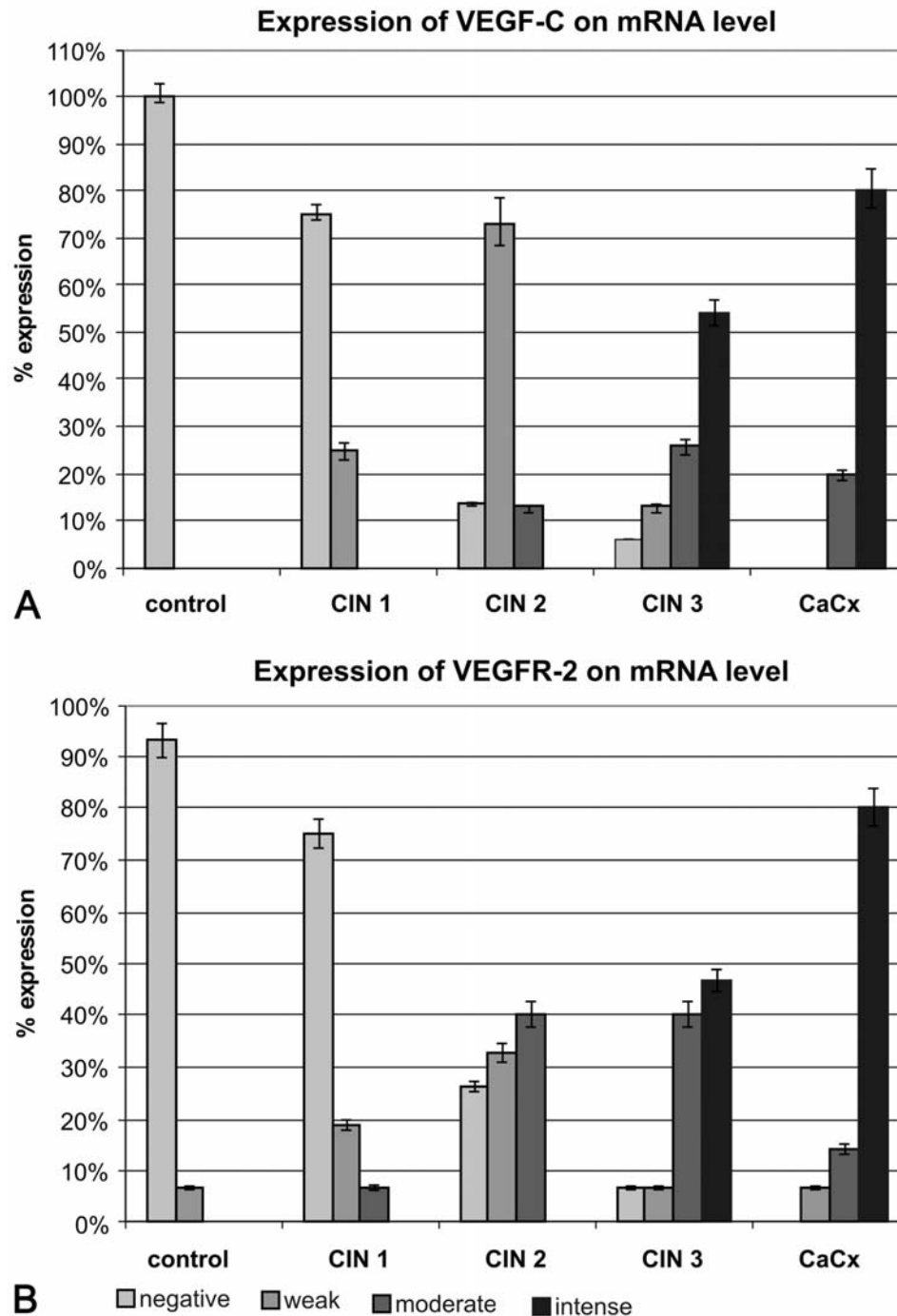


Figure 3. Expression of VEGF-C (A) and VEGFR-2 (B) on the mRNA level vs. stage of cancer progression. A total of 175 patients ranging from 18 to 72 years of age diagnosed with pre-invasive or invasive cervical lesions were included in the study. Total RNA was prepared from various human cervix tissues. Normal cervix of non-pregnant women (controls=35). CIN 1 (n=35). CIN 2 (n=25). CIN 3 (n=35). Cervical SCC (n=30). Cervix of pregnant women (n=15). The identification of the expression of mRNA specific for selected proteins was performed with primers for VEGF-C and VEGFR-2, respectively (Oligo Company, Poland - Table 2). PCR reaction products were separated electrophoretically in a 2% agarose gel and visualized with ethidium bromide. The results present the percent of changes observed in each group, as a mean calculated result. Negative - no expression of gene. Weak - positive expression in less than 10% of mRNA. Moderate - positive expression in 10-50% of mRNA. Intense - positive expression in more than 55% of mRNA.

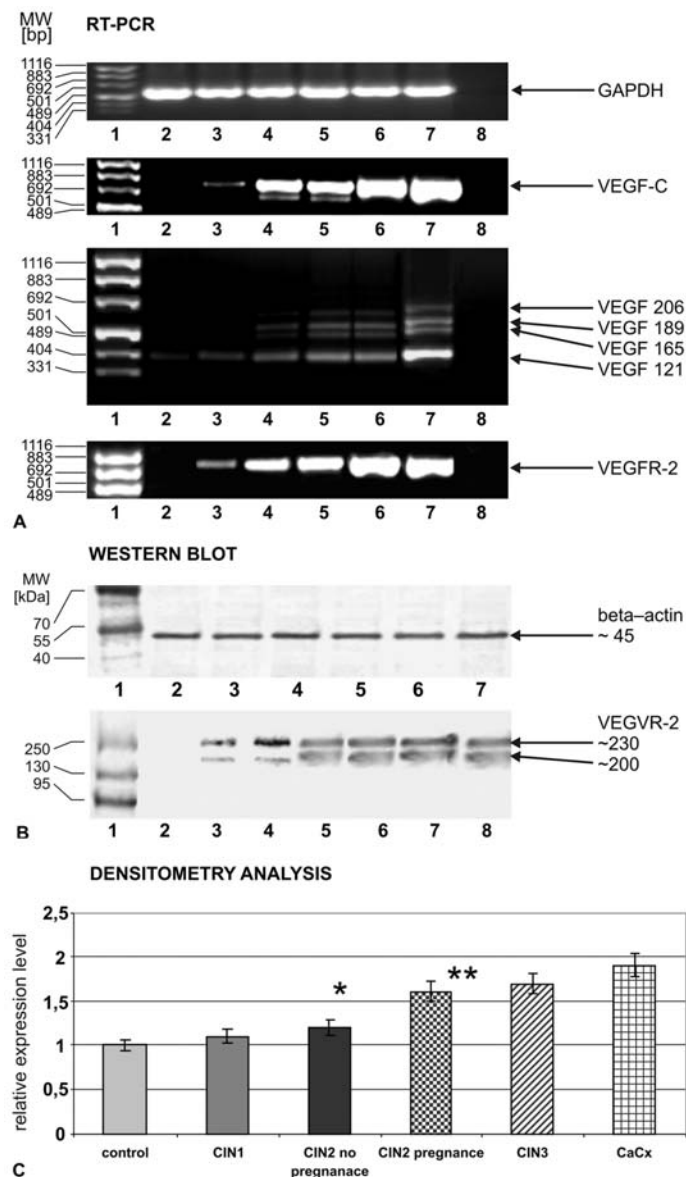


Figure 4. Expression of VEGF-C, VEGFR-2 and VEGF-isoforms transcripts in various human cervix tissues. A. Ethidium bromide stained 1.5% agarose gel of expression of mRNA of VEGF and GAPDH in cervix from patients (CIN1 - 3, SCC, pregnancy) and controls. The upper line shows the expression of the house-keeping gene GAPDH under the same conditions (from the same tissue). Details are described under Materials and methods. 1. DNA ladder. 2. Control - Normal cervix tissue. Squamo-columnar junction visible around the anatomic external orifice of the cervical canal (n=35). 3. CIN1 - Fine mosaic and fine punctuation of geographic borders. Subclinical HPV infection (n=35). 4. CIN2 - Secondary punctuation and mosaic with slightly elevated borders - (n=25). 5. CIN2 in pregnant women (n=40). 6. CIN3 - Secondary coarse mosaic and punctuation. Sharply demarcated elongated borders (n=35). 7. SCC - Secondary coarse mosaic and punctuation. Atypical vessel (neoangiogenesis - endothelial tube) - invasive cervical cancer (n=30). 8. Negative control. B. The expression level of VEGF-R2 protein in whole cell protein extracts of cervix tissue: Equivalent amount of protein cell lysates were separated by electrophoresis (PAGE-SDS, 8% gel) and probed by Western blotting with individual antibodies. The upper line shows the expression of the house-keeping gene beta-actin under the same conditions (from the same tissue). Details are described under Materials and Methods. 1. DNA ladder. 2. Control - Normal cervix tissue. Squamo-columnar junction visible around the anatomic external orifice of the cervical canal (n=35). 3. CIN1 - Fine mosaic and fine punctuation of geographic borders. Subclinical HPV infection (n=35). 4. CIN2 - Secondary punctuation and mosaic with slightly elevated borders - (n=25). 5. CIN2 in pregnant women (n=15). 6. CIN3 - Secondary coarse mosaic and punctuation. Sharply demarcated elongated borders (n=35). 7. SCC - Secondary coarse mosaic and punctuation. Atypical vessel (neoangiogenesis - endothelial tube) - invasive cervical cancer (n=30). 8. Protein standard - VEGFR-2. C. Relative expression of protein from different cervix tissues to beta-actin levels (densitometry analysis). The ratio for control (untreated cell) to β -actin was set at 1. The values are mean \pm SD from three independent experiments. Data represent the mean of four independent experiments, each conducted in triplicate, expressed as the percentage of activity of the control. Significantly different from the corresponding control * $P < 0.05$, ** $P < 0.001$.

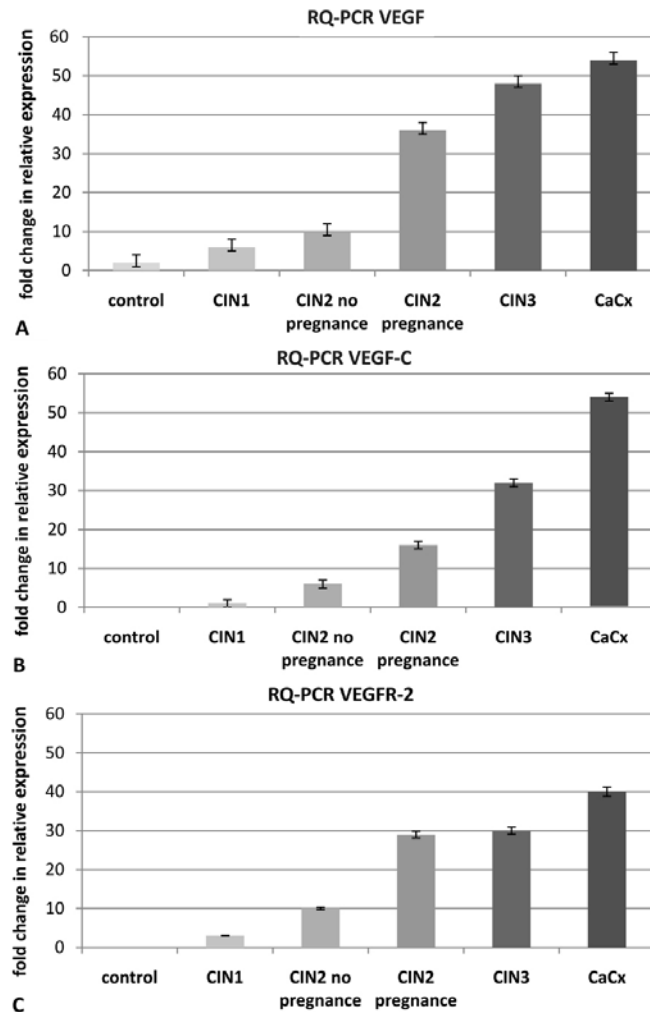


Figure 5. The quantitative real-time PCR (RQ-PCR) illustrating the differential expression of VEGF (A), VEGF-C (B) and VEGFR-2 (C) transcripts in various human cervix tissues. The amplification for VEGF, VEGF-C, VEGFR-2 and ABL was carried out as described under Materials and Methods.

endothelial cells suggests that VEGF was a typical paracrine factor involved in endothelial proliferation and/or survival.

VEGF and VEGF-C can induce blood vessel and lymphatic growth due to their ability to activate VEGFR-2. A large number of studies have shown that metastasis to regional lymph nodes is stimulated when these factors induce lymphangiogenesis in the vicinity of tumors (21).

This study has shown that, in cervical carcinogenesis, a potential switch to the lymphangiogenic phenotype may occur at stage CIN2 and, in pregnant women, CIN3 (Figures 3 - 5). Trappen (36) found a significant difference in the expression of VEGF-C and VEGF-D between CIN1-2 and CIN3 ($p < 0.001$ in both cases), but no significant difference between CIN3 and cervical cancer. Up to 78% of CIN3 lesions showed moderate/strong staining for VEGF-C or VEGF-D, whereas only one case of CIN1 and CIN2 showed moderate/strong

staining for these factors. He concluded that this switch to the expression of VEGF-C may determine the further progression of CIN3 to an invasive disease, inducing metastatic spread to lymph nodes.

The findings of this study indicate that VEGF-C expression may serve as the indicator of the switch from LSIL (CIN1) to HSIL (CIN2/3) phenotype. To the best knowledge of the authors, this is the first study to present the results of VEGF, VEGF-C and VEGFR-2 expression at mRNA, protein, and cellular levels in CIN tissue of pregnant women. Interestingly, our data show that the lymphatic vessels concentration in cervical SCC is lower than in CIN3 and even CIN2 specimens. These findings may be the result of the CIN2 of pregnancy group, where high LV(D2-40) may be related to physiological changes of the cervix during pregnancy. This result supports previous observations (37) that pregnancy does not accelerate cervical carcinogenesis. The highest expression of VEGF, VEGF-C and VEGFR-2 measured on mRNA and protein

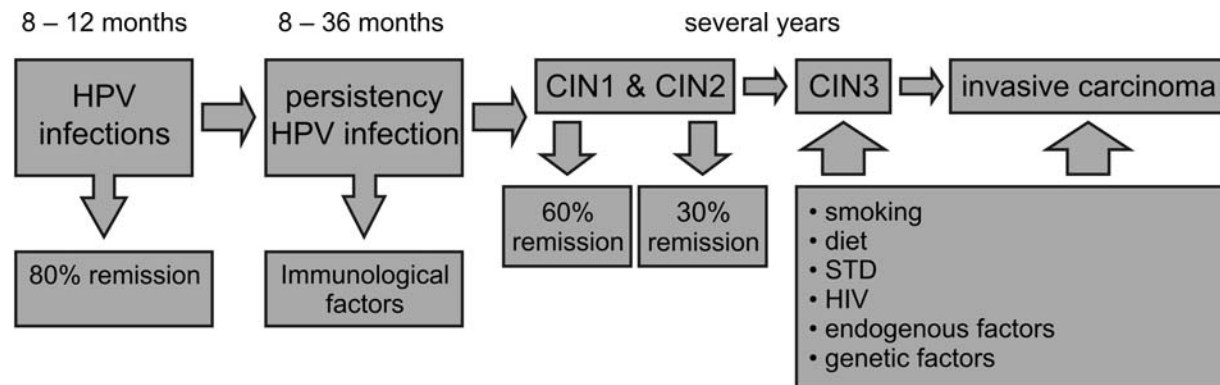


Figure 6. Model of carcinogenesis (Basta A., The Cervix, 2nd edition, Ed. J. Jordan, A Singer, 2006, Blackwell Publishing Ltd, 122).

levels (VEGFR-2) as well as by immunostaining confirm that these cytokines and their receptor expression in cervical carcinogenesis are acquired at pre-invasive stages (CIN3), before invasion. Obviously, other factors (e.g., chemokines, adhesion molecules, persistency of HR HPV infection) play an important role in establishing a background for lymphangiogenesis and lymph nodes metastases (Figure 6). Recent evidence suggests that high risk HPV is able to induce expression of the angiogenic factor VEGF-A (38). It is possible that the high expression of VEGF-C and VEGFR-2 in our CIN3 specimens is associated with HPV infection.

VEGF-C was cloned in 1996 and found to have a region 30% homologous to VEGF-A and 27% homologous to VEGF-B (36, 39, 40). The 23 kDa VEGF-C molecule is a ligand for both VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4) tyrosine kinases (41, 42). During the past few years, VEGF and its tissue manifestation (i.e., microvessel density-MVD), measured in this study using with LV(D2-40), have attracted increasing attention in studies examining different human malignancies, as the major regulators of physiological and pathological angiogenesis and lymphangiogenesis in human tissue (41). This subject has become increasingly important since the recent discovery of the link between HPV and VEGF, suggesting that the HR-HPV E6 oncoprotein upregulates VEGF expression via a promoter region that contains four Sp-1 sites and that this is done in a p53-independent manner (41, 43-46).

VEGF-C was absent in 35 control group tissue samples. This absence of VEGF-C in normal epithelium is consistent with data established in several previous CIN lesions and cervical cancer studies (25, 47-52). In the majority of these studies, VEGF expression was usually associated with minimal MVD, increasing in parallel with increasing CIN grade and being noticed first in low grade CIN lesions. In our specimens, we found an almost linear relationship between the CIN-grading and the intensity of VEGF-C expression and breakdown of the cervical SCC phenotype. This fact suggests that VEGF expression is an early phenomenon in cervical carcinogenesis. VEGF-C over expression proved to be a 100% specific marker of CIN, because it was never found in biopsies without CIN.

Undoubtedly, angiogenesis is one of the key mechanisms leading to the invasive phenotype found in progressing CIN lesions and is induced by several angiogenic factors, such as those of the VEGF family (43-45). Based on our results and taking into consideration current literature, as anticipated by the participation of the same molecules (e.g., p53 and high-risk HPV type E6) in several of these pathways, multiple interactions exist between the different molecular pathways in cervical carcinogenesis.

In conclusion, our results suggest that VEGF-C expression may be a diagnostic marker of the early stages of cervical carcinogenesis. There is a linear increase in VEGF-C and VEGFR-2 expression from a low to high grade of CIN and a reversal of this trend in cervical cancer (SCC). Interestingly, VEGF-C expression is related to its tissue manifestation in the form of MVD, with the exception of pregnancy. The CIN2 pregnancy group, although small (n=5), indicates that the relation may not be specific to MVD.

On the level of VEGF receptors, our results are consistent with findings supporting the contention that the angiogenic switch that occurs prior to invasion may be a good indicator for CIN progression.

6. ACKNOWLEDGMENTS

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Abbreviation: SCC: squamous cell carcinoma; CIN: cervical intraepithelial neoplasia; CIS: carcinoma *in situ*; HPV: human papillomavirus; HSIL: high grade squamous intraepithelial lesion; LEEP: loop electrosurgical excision procedure; LLETZ: Large Loop Excision of the Transformation Zone; LSIL: low grade squamous intraepithelial lesion; LV(D2-40): lymphatic vessels; LVSI: lymphatic vascular space involvement; MVD: microvessel density; pCIN: pregnancy cervical intraepithelial neoplasia; PDGF: platelet-derived growth factor; PIGF: placenta growth factor; pTNM: pathologic Tumor Node Metastases; pT1a: subgroup of pTNM; VEGF: vascular endothelial growth factor; TZ: transformation zone.

Key Words: Cervical cancer, Pathology, Vascular Endothelial Growth Factor, VEGF-C, VEGF-R2

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