

ORIGINAL PAPER

Neoangiogenesis in cervical cancer: focus on CD34 assessment

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Abstract

Despite recent advances in understanding the immune mechanisms of cervical cancer (CC), relapse remains still an actual issue and recognition of new predictive biomarkers is essential. **Aim:** The purpose of this retrospective study was to investigate neo-angiogenesis in CC and its possible utility as prognostic biomarker. **Material and Methods:** Paraffin-embedded tissue samples from 61 consecutive women with CC were immunostained for CD34 and E-cadherin. Statistical analysis was performed in SPSS-12 software, $p < 0.05$. **Results:** Statistically significant differences between CD34 distribution among three interest tumor regions: micro-vessels density increase from central to peripheral area (χ^2 , $p < 0.05$); statistically significant correlation between CD34 expression, particularly in stromal and peripheral sites, E-cadherin (Spearman $r_1 = -0.321$) and lymphatic invasion (Spearman $r_2 = 0.455$) ($p < 0.05$) were reported. Overall five-year survival is clearly dependent on level and distribution of tumor angiogenesis among defined area of interest as suggested by Kaplan–Meier analysis. **Conclusions:** Angiogenesis is essential for guiding CC evolution and prognosis, particularly in squamous invasive types.

Keywords: cervical cancer, angiogenesis, CD34 antigen.

Introduction

Cervical cancer (CC) represents an important cause of morbidity and mortality in women worldwide. Despite complex therapeutic opportunities, ranging from classical surgery to newly approved anti-*Human Papilloma Virus* (HPV) vaccines, relapse still occurs in about 40% of women with CC [1].

At least three key factors are involved in tumor aggressiveness including *uncontrolled proliferation activity, adhesion, migration and tumor cell invasiveness*, and *tumor neo-angiogenesis*, resulting in a complex vicious circle [2–4].

Neo-angiogenesis or new micro-vessels formation advances through a multifaceted interaction between pro- and anti-angiogenic signals released by endothelial and stromal tumor cells and is critical for tumor growth, progression and metastases. The angiogenic activity, revealed by the development of novel micro-vessels in tumor tissue, can be quantified by intra-tumoral micro-vessel density, which, in turn, can be assessed by tissue expression of several representative molecules involved in angiogenesis such as VEGF (*Vascular Endothelial Growth Factor*), factor VIII-related antigen/von Willebrand's factor, CD31 (*Platelet Endothelial Cell Adhesion Molecule*, *PECAM-1*), TSP-1 (*Thrombospondin-1*), UEA-1 (*Ulex Europaeus Lectin I*) and CD34 [1–4].

The CD34 antigen, a member of a sialomucin family, is a single heavily chain transmembrane 67 kDa glycoprotein, expressed mainly on human hematopoietic

stem and progenitor cells, vascular endothelial cells, but absent on fully differentiated hematopoietic cells; while the main function of CD34 is intercellular adhesion, anti-CD34 antibody is a highly sensitive biomarker for endothelial cell differentiation, that has been extensively studied in tumor angiogenesis [1–5].

Both classical (including tumor size, depth of stromal invasion, lymphatic metastasis, positive resection margins, histological type, tumor grading) and modern prognostic factors (tumor and immune biomarkers) have already been described, but not yet validated in cervical cancer [1, 2]. However, new predictive biomarkers are still necessary to identify and stratify patients according to their risk of relapse and to optimize disease management, especially in early cervical cancer.

Despite increased knowledge on tumor angiogenesis, research in cervical cancer have suggested rather conflicting data regarding the potential prognostic value of (neo)angiogenesis [6–9].

The aim of this work was to investigate cervical cancer angiogenesis by assessing tissue expression of CD34 and to evaluate the association between micro-vessel density and classical prognostic factors in cervical cancer.

Material and Methods

We performed a retrospective observational study on sixty-one consecutive women diagnosed with invasive cervical cancer undergoing radical hysterectomy with or

without bilateral pelvic lymphadenectomy; all patients have attended Gynecology Department of “Cuza-Vodă” Hospital in Iassy between 2000 and 2003 and data regarding five-years overall free survival were retrieved from regional oncology files.

Paraffin-embedded cervical tissues were processed at the time of diagnosis at the Pathology Department of “Cuza-Vodă” Hospital and immunohistochemistry (IHC) was done at the Immunopathology and Genetics Laboratory of “Sf. Spiridon” Hospital in Iassy. The study was approved by the local Ethics Committee.

Immunohistochemistry

To identify neo-angiogenesis, tissue sections were immunostained for CD34 biomarker (CD34 class II mouse monoclonal antibody, Clone QBEnd, Code M7165, DAKO, in dilution of 1:25) and streptavidin–biotin method was used [10]. CD34 was assessed in tumor gradient on five representative microscopic fields, three area of interest being selected (central, median, stromal and peripheral tissue); distinction between low, mild and high micro-vessel density was based on micro-vessel count: 0–33, 33–66, and 66–99 vascular elements per examined field. Micro-vessels were counted in the area with the highest density (“hot spot”), after the identification with a smaller magnification; a brown-staining endothelial cell obviously separated from adjacent micro-vessels, tumor cells and other connective tissue elements was considered a single quantifiable micro-vessel.

Tissues have also been stained also for E-cadherin antibodies (DAKO); assessed as brown color of the cell membrane, E-cadherin was classified either *negative* (loss of expression), meaning loss of intercellular adhesion and increased tumor invasiveness, or *positive*, non-homogenous reaction.

Statistical analysis

Descriptive statistics, non-parametrical tests (Spearman’s correlation, Mann–Whitney and chi-squared tests) and Kaplan–Meier survival analysis were performed in SPSS–12 software, $p < 0.05$.

Results

Baseline characteristics of selected cases with cervical cancer related to classical prognostic factors are shown in Table 1.

Table 1 – Patients’ distribution based on classical prognostic factors

Variable	Percentage of cases [%]
<i>Tumor size</i>	
<4 cm	50.8
>4 cm	49.2
<i>Tumor grading</i>	
G1 (well-differentiated)	24.6
G2 (moderate differentiated)	49.2
G3 (undifferentiated)	9.8
<i>Histological type</i>	
Invasive squamous cervical cancer (SICC)	68.9
Micro-invasive cervical cancer (MICC)	16.4

Variable	Percentage of cases [%]
<i>FIGO classification</i>	
IA	19.7
IB	31.1
IIA	4.9
IIB	39.3
<i>Lymph node invasion</i>	
With invasion	31.1
Without invasion	68.9
<i>Five-years overall survival</i>	
Death	42.6
Survival	57.4
<i>Relapse</i>	
With	55.7
Without	44.3

Figure 1 presents different aspects of CD34 expression in cervical cancer, as defined by IHC.

Several critical differences regarding the distribution of CD34 expression between three-tumor area of interest and their relation with classical prognostic factors have been suggested in our study.

CD34 expression in central tumor area – association between immunostaining and clinico-pathological variables

CD34 expression in central tumor area – association between immunostaining and clinico-pathological variables: (i) no difference between levels of angiogenesis in CC in young, while patients aged more than 55-year-old mainly presented with low micro-vessel counts (72%); (ii) low CD34 expression in the up to 90% of micro-invasive CC, but also in more than half of squamous invasive type (55%); (iii) low micro-vessels count in both G1 (67%) and G2 (60%) grading; (iv) low angiogenesis in the majority (62%) of CC without lymph node metastasis (moreover, no lymphatic invasion has been reported in highly vascularized central tumor areas); (v) moderate neo-angiogenesis in 56% of recurrences, while (vi) increased global survival rate in CC with low CD34 expression (60%) have been noted in our study.

CD34 expression in median tumor area – association between immunostaining and clinico-pathological variables

CD34 expression in median tumor area – association between immunostaining and clinico-pathological variables: (i) no significant differences between young and elder CC based on angiogenesis in above mentioned area; moderate CD34 expression in the more than half of cases (67% under 35-year-old, 54% between 35–55-year-old, 61% above the age of 55 years); (ii) moderate neo-angiogenesis in about 50% MICC and 60% of SICC; (iii) moderate count of micro-vessels per field in G1 (73%) and G2 (47%) tumors; (iv) moderate angiogenesis in CC with lymphatic invasion (53%); (v) moderate angiogenesis in 65% of relapsed CC and (vi) worse five-years overall survival rate for women with moderate CD34 expression (65%) have been showed in studied CC.

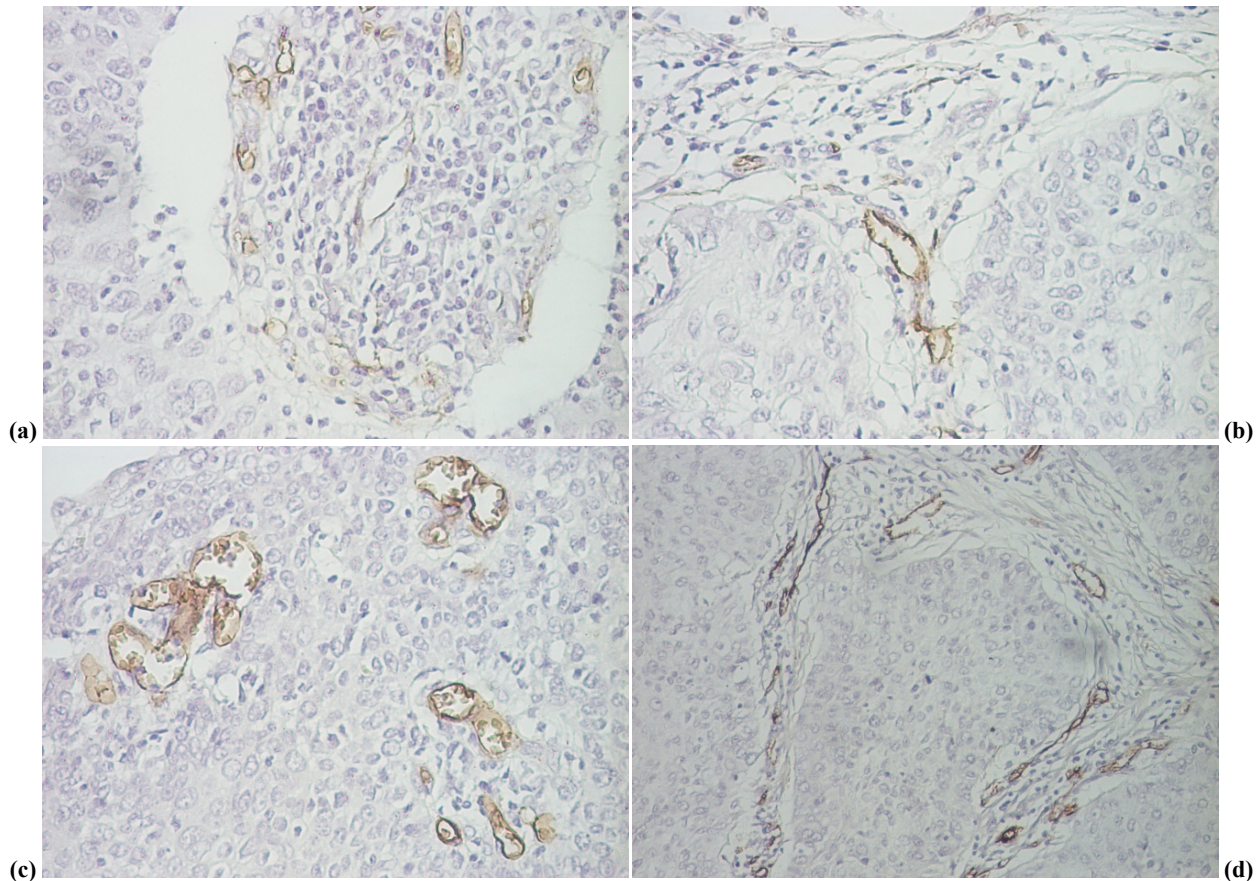


Figure 1 – CD34 expression in CC, IHC (DAKO monoclonal mouse antibody, brown staining, ob. 10×): (a) median tumor region micro-vessels (ob. 10×); (b) tumor stromal micro-vessels (ob. 10×); (c) tumor area micro-vessels (ob. 10×); (d) stromal and peritumoral micro-vessels (ob. 10×).

CD34 expression in stromal and peripheral tumor tissue – association between immunostaining and clinico-pathological variables

CD34 expression in stromal and peripheral tumor tissue – association between immunostaining and clinico-pathological variables: (i) no difference in CD34 expression under 35-year-old, but highly vascularized CC after 35-year-old (57% between 35–55-year-old, 67% after 55-year-old); (ii) high micro-vessel density in about 40% of MICC and 60% of SICC; (iii) high CD34 expression in both G1 (47%) and G2 (53%) tumors; (iv) highly vascularized CC in the majority (72%) of tumors with pelvic lymph node invasion; (v) increased angiogenesis in relapse (68%), while (vi) abundant angiogenesis in CC with decreased survival (69%) have been reported in our research.

We have noted a distinctive pattern of CD34 distribution in selected areas resulting in intra-tumoral heterogeneity, as follows: low vascular density in central zone, moderate angiogenic activity in median areas, while highest neo-angiogenesis was demonstrated in stromal and peripheral tumor sites ($\chi^2=16.262$, $p<0.001$; $\chi^2=16.754$; $p<0.001$).

Correlations

Several statistically significant correlations, mainly related to micro-vessel count in stromal and peripheral tumor tissues and certain cancer variables have been established:

- indirect moderate correlation with E-cadherin expression (Spearman $r=-0.321$, $p<0.05$): higher is angiogenesis, higher is CC aggressiveness as defined by loss of E-cadherin expression; the same, but stronger tendency was reported in particular cases of types 16 and/or 18 HPV-positive cervical cancer (Spearman $r=-0.626$, $p<0.01$);
- direct mild correlation with lymph node metastases (Spearman $r=0.455$, $p=0.001$): high micro-vessel counts in stromal and peripheral tumor area are commonly associated with pelvic lymph node invasion;
- indirect moderate correlation type 16 and/or 18 HPV-infection (Spearman $r=-0.401$, $p=0.001$); positive HPV-infection is associated with higher vascular density in peripheral sites;
- direct moderate correlation with cancer type (Spearman $r=0.306$; $p=0.016$); the most important vascularization have been in endo-cervical and mixed CC;
- direct moderate correlation between CD34 expression in both central and median tumor areas and median and peritumoral tissues (Spearman $r_1=0.496$, $p<0.01$; $r_2=0.352$, $p<0.01$);

No statistically significant correlation have been demonstrated between CD34 expression and other classical prognostic factors, relapse or survival.

Furthermore, multiple regression analysis has revealed two additional factors for relapse in cervical cancer: E-cadherin and CD34 expression in median

tumoral area: $R^2=0.368$; ANOVA: $F=4.085$, $p=0.001$; t -Student (E-cadherin): $t=-2.227$, $p=0.030$; t -Student (CD34 median area): $t=2.233$, $p=0.030$; loss of E-cadherin expression combined with high micro-vessel count in median tumor compartment account for 36.8% of CC relapse.

Kaplan–Meier survival analysis

Low micro-vascular density assigned to central tumor sites is associated with high five-years overall survival rate in cervical cancer as suggested by Kaplan–Meier survival analysis (Figure 2a). Moreover, lower CD34 expression in median tumor area, higher

disease free survival; as we have shown predominantly moderate CD34 expression in this particular area, we have reported an increased number of deaths during the monitoring interval. Survival rate was settled at 40% in both moderate and high angiogenesis CC and increases at 60% for those with low vascularized CC (Figure 2b).

High CD34 expression in stromal and peripheral area is associated with decreased free survival; about 30% of CC with high tumor angiogenesis in the above mentioned area and up to 90% of low vascularized CC are still alive five years after diagnosis (Figure 2c).

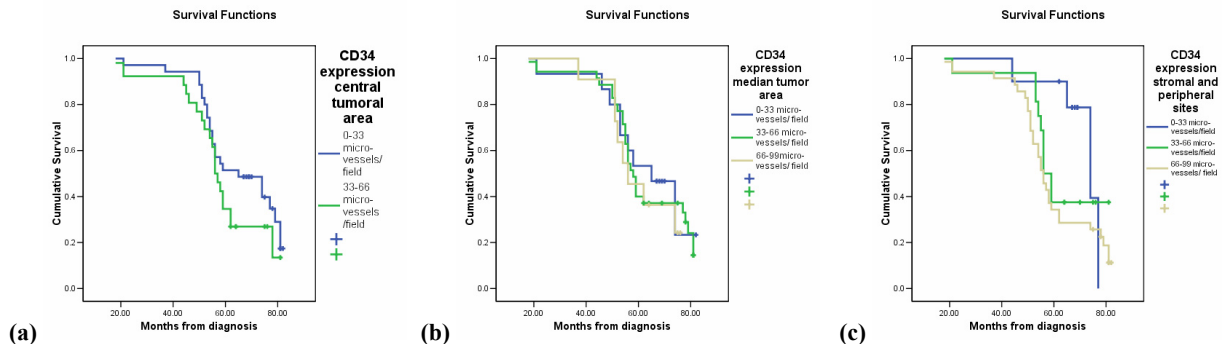


Figure 2 – Kaplan–Meier survival analysis based on tumor CD34 expression in three interest compartments: central tumoral area (a), median tumoral area (b), stromal and peripheral sites (c).

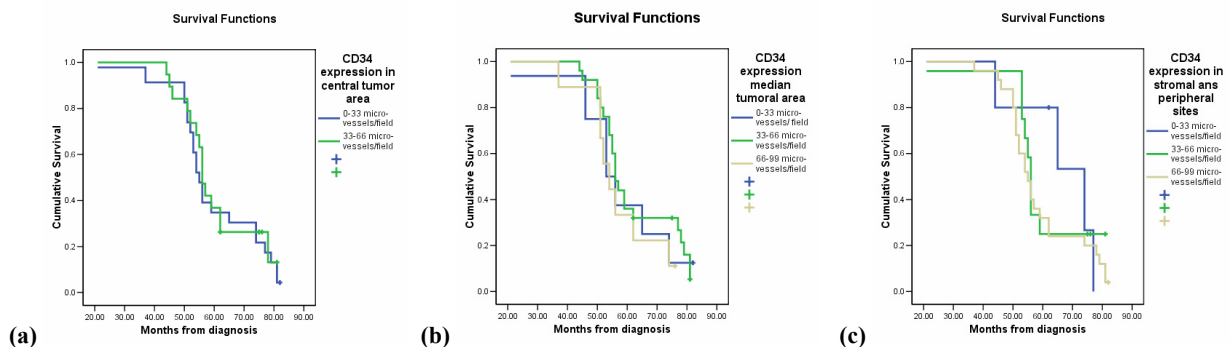


Figure 3 – Kaplan–Meier survival analysis based on tumor CD34 expression in squamous invasive cervical cancer: central tumor area (a), median tumoral area (b), stromal and peripheral sites (c).

In the particular case of squamous invasive CC, Kaplan–Meier survival analysis based on micro-vessel density in central tumor area has suggested a dramatic course of the disease. Five-years global survival rate achieve only 25% for moderate neo-angiogenesis tumors, while low-vascular density up to 30%, suggesting that certain specific factors stimulate such an aggressive pattern (Figure 3a). Also, highly vascularized median tumor region denoted significant decrease in survival rate (about 20%), while mild micro-vessel density account for 30% survival rate; CC with low angiogenesis featured the best survival rate (40%) (Figure 3b).

According to CD34 expression in the last region of interest (stromal and peripheral area), low vascularized SICC displayed a good survival rate (up to 80%), while both moderate and high micro-vessel density resulted in decreased survival (mean value of 20%) (Figure 3c).

Discussion

Although performed on a limited number of patients (61 cases) and designed to evaluate vascular density in cervical cancer and the possible relation to clinical outcome, this study has pointed out on several particular aspects of angiogenesis in cervical cancer.

While other studies bring into attention several biomarkers for cervical neoplasia including anti-BN9, anti-VEGF, anti-CD31 [3, 12, 13], we have applied only CD34 endothelial cell marker for the quantification of neo-angiogenesis. Commonly, the information acquired admit the data from literature with certain differences.

We have demonstrated a distinctive pattern of CD34 expression among three region of interest (low CD34 level in central tumor region, moderate angiogenesis in median area, while high micro-vessel formation was reported in stromal and peri-tumoral tissues) which is statistically significant (χ^2 , $p=0.001$). Micro-vessels

density significantly increases from central to peripheral area. In addition we have studied differences in CD34 distribution according to age, tumor grading, histological type, HPV-infection, pelvic lymph node metastases, relapse and 5-years overall survival rate.

Other studies have also reported major differences of CD34 expression among the above mentioned intra-tumoral regions [11]; moreover, intra-tumoral variation of the micro-vascular density has been demonstrated especially in poor differentiated carcinoma where the highest angiogenesis is reported in central and extra-tumoral area [11]. The same authors have also suggested significant discrepancy in CD34 expression based on tumor grading. Besides, the spatial variation of tumor angiogenesis is considered of predictive value for relapse and survival in women diagnosed with CC [11].

We have identified several relevant correlations between CD34 expression, mainly CD34 in stroma and peri-tumor tissues, and other factors of negative prognosis, such as *E-cadherin expression* (high peri-tumor neo-angiogenesis is associated with loss of E-cadherin expression) and *lymphatic invasion* (high neo-angiogenesis in peri-tumor tissues illustrated the direct spread of CC to lymph node). The same correlation between CD34 level and lymphatic metastases was suggested by Francu DL *et al.*; higher angiogenesis, higher potential of metastasis and poor prognosis [11].

As demonstrated by Vieira SC *et al.*, higher micro-vessel density is frequently reported in squamous invasive CC type and undifferentiated carcinoma [12, 13].

Furthermore, research studies in literature reported statistically significant correlation between the intensity of angiogenesis and the presence of lymphatic invasion [12–14] as well as vascular involvement [14]. Also, anti-CD34 antibody reactivity is associated with patho-anatomical features indicative of poorer prognosis in cervical carcinoma [12, 13], particularly in SICC [14].

As reflected by Kaplan–Meier analysis, five-year overall median survival in our study is clearly dependent on micro-vessel density in selected tumor sites: lower CD34 expression among central and median tumor areas is associated with increased survival, while higher CD34 in stromal and peripheral tumor tissues advanced with decreased five-years overall survival. Moreover, the presence of a high angiogenesis in median tumor tissue associated with lymph node metastasis dramatically affects free survival; the same tendency was reported by Francu DL *et al.* [11].

Data from literature also support the idea that the five-year overall survival rate for patients with high micro-vessel density was significantly worse than for those with low angiogenesis [11, 14].

At the same time, Kaplan–Meier survival analysis for squamous invasive CC displayed a comparable pattern for CD34 expression in stroma and peri-tumor tissues, supporting the concept that high angiogenesis promote cancer invasion and death; the same is valuable for micro-vessel density in median tumor, while central tumor vascularization lead to a different design; specific squamous invasive CC factors result in aggressive disease pattern.

Conclusions

(Neo)angiogenesis (assessed by CD34 expression) and tumor invasiveness (defined by E-cadherin) represent additional factors that promote tumor aggressiveness. Moreover, angiogenesis is essential for guiding cervical cancer evolution and prognosis, particularly in squamous invasive types. However, larger cohort studies are necessary for the validation of CD34 as prognostic biomarker in cervical cancer.

Acknowledgments

The authors would like to acknowledge the collaboration of C. Berteau-Hanganu, S.C. Profit, Medical Research & Human Development (biostatistics).

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Received: September 16th, 2009

Accepted: April 15th, 2010