Association of Neo Angiogenesis by CD34 Expression and Clinicopathologic Features in Squamous Cell Carcinoma of Cervix

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Abstract- Tumor angiogenesis is one of the most important factors in tumor progression. In this study, the angiogenesis of cervical squamous cell carcinoma (SCC) and its association with prognostic factors was assessed by using CD34 immunostaining marker. The microvessel density in 40 patients with cervical SCC was studied in three areas of the tumor; stromal and peripheral tumor area (combined) central stromal tumor area and peripheral tumor area and the relationship of microvascular density and survival was also evaluated. The count of CD34 is correlated with younger age, the presence of perineural invasion and metastasis to lymph nodes. High peripheral tumor angiogenesis is also correlated with lower disease-free tumor survival. According to the findings of the present study, CD34 expression, especially in peripheral tumor areas, can be used as a prognostic marker in cervical SCC.

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Keywords: Cervical squamous cell carcinoma; Angiogenesis; CD34; Prognostic marker

Introduction

Cervical squamous cell carcinoma is the second leading cause of cancer related death among women worldwide (1). Despite complex therapeutic opportunities, relapse still occurs in about 40% of women with cervical cancer (2). Several factors have more important roles in tumor progression, such as unbridled proliferation accompanied by evasion of cell death's ability to invade, migrate and metastasis and tumor neoangiogenesis(3).

Angiogenesis is a well-known process during which new vessels developed from pre-existent capillaries. It may be physiologic as occurred in wound healing or pathologic as seen in diverse circumstances from diabetes to cancer. Angiogenesis has two major effects; first supplying nutrients and oxygen and removing waste products and the second one is producing growth factors that promote the growth of nearby tumor cells (1).

The angiogenic activity has been correlated with many tumors outcomes (4), and it is evident by evaluating the new microvessels formation in tumor tissue and can be quantitated by counting the expression of diverse molecules involved in angiogenesis such as VEGF, bFGF, VEA -1, CD31 and CD34 (3-6) .

The CD34 antigen is a member of sialomucin family and expressed on the human hematopoietic stem and progenitor cells and vascular endothelial cells especially on neoplastic ones with deeper shade. It has been widely used and studied in tumor angiogenesis (3).

As far as we know, in cervical cancer despite of well-known clinicopathologic factors (including age, histological type, FIGO stage, depth of stromal invasion, tumor size, parametrial involvement, positive resection margin and lymph node states) as well as modern prognostic factors (tumor and immune markers), very few studies evaluated the correlation of angiogenesis with recurrence and survival of patients (3).

In previous studies which worked on the possible prognostic value of tumor angiogenesis in cervical cancer, they have suggested rather conflicting data regarding the potential prognostic value of neoangiogenesis (1).

In the present study, we studied the angiogenesis (by evaluating the expression of CD34) and investigate the relationship between the prognostic factors and

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microvessel density in cervical cancer.

Materials and Methods

This is a cross-sectional study on 40 women with invasive cervical squamous cell carcinoma (SCC) who underwent radical hysterectomy and lymphadenectomy in the Women Hospital, affiliated with Tehran University of Medical Sciences, in Tehran, Iran, between 1999 to 2009.

Hematoxylin and eosin stained slides and subsequently selected paraffin embedded tissues were collected from the archive files, and histopathologic data including the histologic type of tumor, size, depth of stromal invasion and other relevant information's are investigated. The information regarding 3 to 5 years overall survival and disease-free survival was retrieved from the patients' oncology files and follow up notes.

Immunohistochemistry

Tissue sections were immunostained for CD34 biomarker (CD34 class II mouse monoclonal antibody, clone IR632/IS632, DAKO, Denmark) to identify neoangiogenesis. A vascular unit was identified based on criteria established by Weidner, who described it as a cell or group of endothelial cells of a brownish color which obviously separated from adjacent microvessel, tumor cells and other connective tissue (4).

Assessment of microvessel density was performed in three areas; stromal and peripheral tumor areas (combined), central stromal tumor area and peripheral tumor area, as described below:

In stromal and peripheral tumor areas calculation, hotspot areas of central stromal tumor and peripheral tumor areas (combined) are selected, and in each area, CD34 positivity was counted in five high power fields. Then counted average microvessel per examined field was classified in two groups (according to median): low microvessel density (MVD) (less than 22) and high MVD (more than 22) (Figure 1).

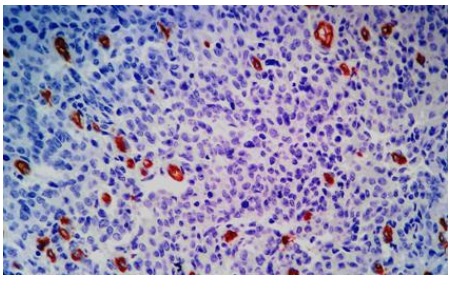

Figure 1. CD34 expression in cervical squamous cell carcinoma in central tumor area, IHC (ob.x10).

In the assessment of MVD in central stromal tumor area, five representative tumoral hotspot areas were chosen. Then, CD34 was counted, and MVD in tumoral tissue was classified as in three groups: low (less than 33), moderate (33-66), and high (more than 66) (Figure 2).

In the survey of MVD in peripheral tumor area, also five representative peripheral tumor hotspot foci were determined, and CD34 was counted and classified into three groups: low (less than 90), moderate (90-190) and high (more than 190).

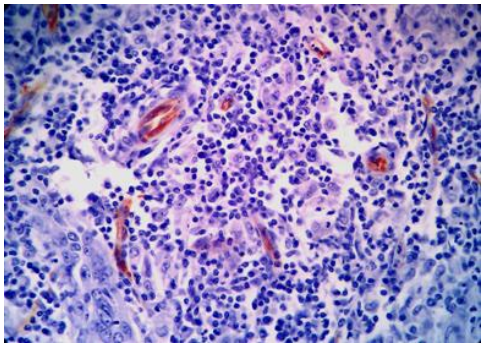

Figure 2. CD34 expression in cervical squamous cell carcinoma in peripheral and stromal tumor area, IHC.(ob.x10)

Statistical analysis

Descriptive statistics, Spearman rank correlation and Kaplan-Meier survival analysis were performed using SPSS-21 software and *P* less than 0.05 considered significant.

Results

A total of 40 patients with mean age of 46.8±10.0 (range: 26-74) years were studied. The topographic characteristics of the cases in this study are summarized in Table 1.

In stromal and peripheral tumor areas counting of CD34, 19 cases (47.5%) have the count of less than 22, labeled as low MVD, and 21 (52.5%) have the count more than 22, labeled as high MVD.

When CD34 was evaluated in central tumor areas, 14 cases (35%) have the count less than 33, labeled as low MVD, 10 (25%) with count between 33-66, labeled as moderate MVD, and 16 (40%) have the count more than 66, labeled as high MVD.

CD34 density in peripheral tumor areas shows 12 cases (30%) of low, 16 (40%) cases with moderate and 12 (30%) cases with high MVD.

Table 1. Topographic characteristics of patients and tumors

Cases (%)		Topographic characteristics
Age	<35	7(17.5)
	>35-54	25(62.5)
	>55	8(20)
Tumor type	Large cell keratinizing	25(62.5)
	Large cell non-keratinizing	15(37.5)
Depth of tumor (stromal invasion)	≤9 mm	10(25)
	>10 mm	30(75)
Tumor size	≤39 mm	22(55)
	>40mm	18(45)
Vascular invasion	Yes	31(77.5)
	No	9(22.5)
Perineural invasion	Yes	20(50)
	No	20(50)
Lymph node involvement	Yes	8(20)
	No	32(80)
Tumor stage	IB	28(70)
	IIA	6(15)
	IIB	4(10)
	IIIA	1(2.5)
	IIIB	1(2.5)

Association between CD34 expression in stromal and peripheral tumor areas and clinicopathological variables were classified as follow:

1) High microvessel count in three age groups was detected as 71.4% under 35-year-old, 56% between 35 to 55-year-old and 25% above the age of 55 years. No significant correlation between high stromal and peripheral tumor areas microvessel count and age of the cases (*P*=0.169) was identified.
2) High CD34 count in up to 52% of keratinized SCC versus in more than half of the non-keratinized SCC is seen. There was no statistically significant difference in CD34 count among two histologic types of SCC (*P*=0.935).
3) High angiogenesis in the majority (60%) of SCC with less than 9 mm stromal invasion and in 50% of SCC with more than 9 mm stromal invasion is seen ,which showed no statistically significant correlation (*P*=0.593).
4) No significant correlation was identified between tumor size and high count of CD34 in stromal and peripheral tumor areas. The high microvessel density was 63. 6% and 38.9% in tumors less than and more than 4 cm, respectively (*P*=0.119).
5) High microvessel density was detected in about 58.1% of tumors with lymph-vascular invasion. Whereas, 33.3% of cases without lymph-vascular invasion show high CD34 expression. However, no significant correlation was identified between high MVD and lymph-vascular invasion (*P*=0.191).
6) High CD34 stromal and peripheral tumor areas expression in up to 80% of cases with perineural invasion versus 25% of tumors without perineural invasion is seen which was statistically significant correlation (*P*=0.001).
7) High angiogenesis in 87.5% and 43.8% of

cases with and without lymph node metastasis, respectively, were detected which showed significant correlation (*P*=0.027).

8) No significant correlation was identified between CD34 expression in stromal and peripheral tumor areas and tumor stage (r=0.275 and *P*=0.886).

Association between CD34 expression in central tumor area and clinicopathological variables:

1) Significant correlation was identified between angiogenesis in central tumor area and age of the patients. High microvessel density was detected in 85.7% of cases under 35-year-old, 46% between 35-55-year-old and 0% above the age of 55 years of patients more than 55years (*P*=0.017).
2) When the correlation between high MVD and histologic type of tumor were evaluated, no significant association was detected. High microvessel density was 44% and 33.3% in keratinized and non-keratinized SCC, respectively (*P*=0.487).
3) No significant differences between high CD34 count and depth of stromal invasion were found. In tumors with less than 9 mm stromal invasion, high microvessel density was noted in 60% of cases versus in 40% of cervical carcinoma with more than 9 mm stromal invasion (*P*=0.443).
4) High CD34 expression was detected in 45.5% and 33.3% of tumors with size less and more than 4 cm, respectively. No statistically significant correlation was identified (*P*=0.739).
5) High angiogenesis was found in 45.2% of central tumor area with lymph-vascular invasion versus in 22.2% of cases without invasion. No differences between lymph-vascular invasion and high angiogenesis were detected (*P*=0.308).
6) High CD34 expression in central tumor area was seen in up to 60% of SCC with perineural invasion and 20% without perineural invasion. This showed statistically significant correlation (*P*=0.014).
7) High angiogenesis in central tumor area was noted in cases with (75%) and without (31.2%) lymph node metastasis which was statistically significant (*P*=0.038).
8) There was no statistically significant correlation between stage of the tumor and CD34 expression in central tumor area (*P*=0.125, r=0.247).

Association between CD34 expression in peripheral tumor area and clinicopathological variables were classified as follow:

1) Peripheral tumor area with high CD34 expression was noted in cases; 28.6% under 35-year-old, 40% between 35-55-year-old and 0% above the age of 55 years and no significant differences was detected (*P*=0.197).
2) No significant differences between keratinized and nonkeratinized SCC were found. High CD34 count in peripheral tumor area in 32% and 26.7% of two either histologic types (*P*=0.185) were seen, respectively.
3) High microvessel count in peripheral tumor area with less or more than 9 mm depth of stromal invasion were 50% and 23.3%, respectively. No difference was identified between high angiogenesis and depth of stromal invasion (*P*=0.279).
4) High CD34 expressions were detected in 40.9% and 16.7 % of tumors with the size of less and more than 4 cm, respectively. No statistically significant correlation was identified (*P*=0.237).
5) High angiogenesis was seen in 32.3% of peripheral tumor area with lymph-vascular invasion and in 22.2% of cases without invasion. No differences between lymph-vascular invasion and high angiogenesis was found (*P*=0.557).
6) High CD34 expression in peripheral tumor area was noted in up to 40% of SCC with perineural invasion and 20% without perineural invasion. This showed statistically significant correlation (*P*=0.044).
7) High angiogenesis in peripheral tumor area was seen in cases with (75%) and without (18.8%) lymph node metastasis, respectively, which was statistically significant (*P*=0.006).

No correlation was identified between high CD34 expression in peripheral tumor area and tumor stage (r=0.199, *P*=0.217). See also Table 2.

Table 2. Association between high-density angiogenesis by CD34 expression in various areas of tumors and clinicopathologic features

Clinicopathologic characteristics		Stromal and peripheral	Central	Peripheral
Age	**<35** **>35-54** **>55**	NS*	*P* = 0.017	NS
Tumor type	**Large cell keratinizing** **Large cell non-keratinizing**	NS	NS	NS
Depth of tumor (stromal invasion)	**≤9 mm** **>10 mm**	NS	NS	NS
Tumor size	**≤39 mm** **>40 mm**	NS	NS	NS
Vascular invasion	**YES** **NO**	NS	NS	NS
Perineural invasion	**YES** **NO**	*P* = 0.001	*P* = 0.014	*P* = 0.044
Lymph node involvement	**YES** **NO**	*P* = 0.027	*P* = 0.036	*P* = 0.006
Tumor Stage	**IB** **IIA** **IIB** **IIIA** **IIIB**	NS	NS	NS

*NS= non-significant

CD34 expression in association with patients' survival:

The survival rate in stromal and peripheral tumor areas in low MVD was 46.47±19.87 months and in high MVD were 40.62±20.54 months. No significant correlation was detected (*P*=0.4) (Figure 3).

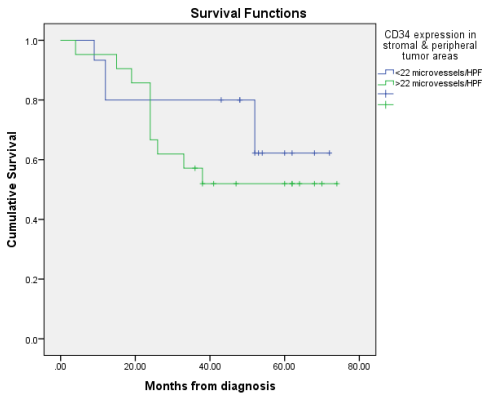

Figure 3. Kaplan-Meier survival analysis based on CD34 expression in stromal and peripheral tumor area (*P*=0.4)

The survival rate in central tumor area in low MVD was 51.73±21.43 months, moderate MVD: 37.78±22.08 months and high MVD: 40.06±17.5 months. No significant correlation was detected (*P*=0.2) (Figure 4).

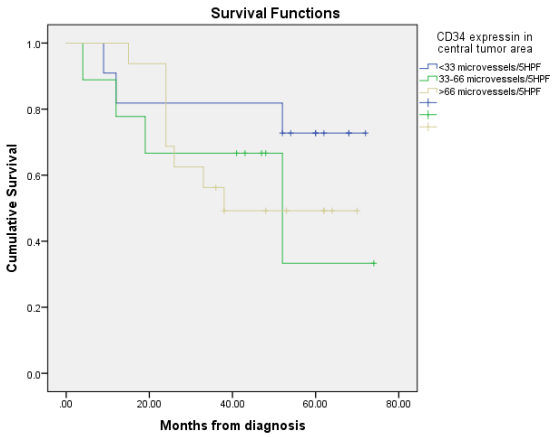

Figure 4. Kaplan-Meier survival analysis based on CD34 expression in central tumor area (*P*=0.2)

Significant differences were detected between low, moderate and high MVD in the peripheral tumor area. The survival rate in low MVD was 54.82±16.55 months,

moderate MVD: 42.50±22.51 months and high MVD: 32.00±14.54 months (*P*=0.025) (Figure 5).

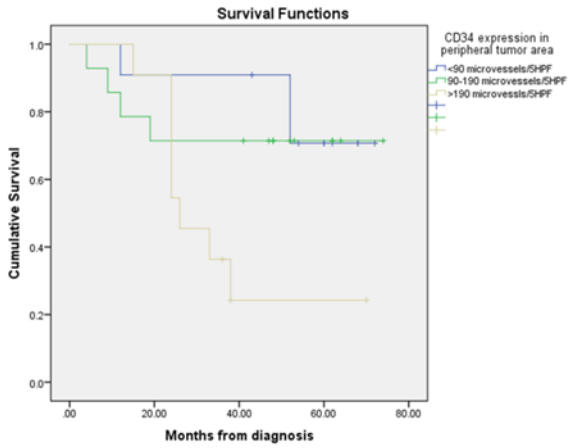

Figure 5. Kaplan-Meier survival analysis based on CD34 expression in peripheral tumor area (*P*=0.025)

Discussion

Angiogenesis is the process of new blood vessel formation from pre-existing ones, and it has been shown to be associated with the growth and progression of malignant tumors. Evidence showed that microvessel counts might be one of the most important clinical prognostic factors.

Although the importance of angiogenesis in tumor progression of many solid tumors has been well-recognized however there is a little information about the clinical significance of MVD in cervical SCC (5).

Previous studies in cervical SCC evaluating the prognostic significance of MVD as measured by IHC-detection of non-specific vascular markers (Factor-8, CD34, CD31) have reported conflicting results (3,1-2,6).

In the largest of these studies, Obermair *et al*., reported the worse outcome in women with high tumor MVD. In this study of stage IB patients, the 5 years survival rate for women with high MVD (>20 vessels/hpf) was 63% VS 90% for women with low MVD (7).

Additional studies have supported these findings (8-11), however high MVD has also been shown to be associated with improved survival (12-13) or not correlate with the outcome at all (14-15). While other studies bring into attention several biomarkers for cervical SCC including CD31, anti-VEGF,anti-BNH9 and factor VIII, we have applied only CD34 endothelial cell marker for the quantification of neoangiogenesis. Commonly, the information acquired admits the data from articles with certain differences.

Anti CD34 is a very sensitive antibody for endothelial differentiation, staining neoplastic endothelium more strongly than normal endothelium (15) and has been consumed and evaluated lonely or in association with other vascular biomarkers for evaluation of tumor angiogenesis.

Although our study was performed on a limited number of patients (40 cases) with cervical SCC and designated to evaluate angiogenesis in these cancers and to find out any possible relation between angiogenesis and clinical outcome, we have pointed out on several particular aspects of angiogenesis in cervical SCC.

We studied differences in CD34 distribution in three areas of neoangiogenesis; stromal and peripheral tumor areas (combined) central stromal tumor area and peripheral tumor area, according to age, histological type, depth of tumoral invasion, tumor size, lymphovascular and perineural invasion, lymph node metastasis and tumor stage.

Our study demonstrated a distinctive pattern of CD34 expression among three regions of interest , as follows: low mean of CD34 count in stromal and peripheral tumor areas (23.42 in 5/hpf), moderate angiogenic activity in central tumor area (65.28 in 5/hpf) , and highest neoangiogenesis in peripheral tumor area (138.68 in 5/hpf),while in study reported by Ancuta *et al*., they have demonstrated :low CD34 level in central tumor region, while high microvessel formation was reported in stromal and peritumoral areas (3).

We have identified several statistically significant correlations between in three areas of neoangiogenesis and perineural invasion as well as lymph node metastasis (*P*<0.05).

Ancuta *et al.*, have found the presence of a high angiogenesis in median tumor tissue associated with lymph node metastasis that dramatically affects free-disease survival (3).

Some studies demonstrated correlation between CD34 level and lymphatic invasion (3) however in the present study, no statistically significant differences between lymphatic invasion and three areas of neoangiogenesis was identified. Since according to the results attained in this study, patients with lymph node metastasis present with greater angiogenesis activity in all three areas of interest, we have noted that in the evaluation of H and E slides about the lymphatic invasion, strict criteria were used.

We have found high microvascular density assigned to peripheral tumor areas was associated with low five-year overall survival rate in cervical SCC as suggested by Kaplan-Meier survival analysis. In our study high

CD34 expression in the peripheral tumor, area is associated with decreased free survival; significant differences were detected between low, moderate and high MVD in peripheral tumor area. The survival rate in low MVD was 54.82±16.55 months, moderate MVD: 42.50±22.51 months and high MVD: 32.00±14.54 months (P=0.025), and also we have previously mentioned that in our study highest neoangiogenesis was demonstrated in peripheral tumor area (138.68 in 5/hpf).

In other word with increased count of CD34 in peripheral tumor area, the survival rate of patients declined, while in study by Ancuta *et al*., low microvascular density in central tumor areas is associated with high five-year overall survival and higher CD34 expression in stromal and peripheral tumor tissues advanced with decreased five-years overall survival (3).

Data from other literature also support the idea that the five-year overall survival rate for patient with high microvessel density was significantly worse than for those with low angiogenesis (3,16).

At the same time, Kaplan-Meier survival analysis for invasive SCC displayed a comparable pattern of CD34 expression in peripheral tumor areas, supporting the concept that high angiogenesis promotes cancer invasion and death.

In the present study, we noted significant correlation was identified between angiogenesis in central tumor area and age of the patient (P=0.017). High microvessel density was detected in 85.7% of cases under 35-year-old, 46% between 35 to 55-year-old and 0% of patients more than 55 years. Also Ancuta *et al*., have found that patients more than 55-year-old presented with low microvessel count in central tumor area (3).

Neoangiogenesis, which is defined by CD34 expression in cervical SCC suggests additional factors that promote tumor aggressiveness, and it can be helpful for evolution and prognosis of cervical SCC.

Consequently, it could be proposed that CD34 reactivity especially in peripheral tumor area as a prognostic marker. However larger cohort studies are required for confirming the results.

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