Original Article

Correlation of serum vascular endothelial growth factor with clinicopathological parameters in cervical cancer

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Summary **Angiogenesis plays an important role in cervical cancer progression. Currently among several factors known to promote angiogenesis, vascular endothelial growth factor (VEGF) is most important. To evaluate the effect of treatment on VEGF levels and their correlation with other predictive factors, pre-and post treatment levels of VEGF were estimated in cervical cancer patients. 110 cases of frank cancer and 50 controls were enrolled for the present study: 18 in Stage I, 32 in Stage II, 48 in Stage III, and 12 in Stage IV. Serum VEGF levels were estimated by ELISA in patients on the day of recruitment and post treatment follow-up at a fixed time interval of 6-8 weeks. VEGF levels were highly significant among patients as compared to controls ($p = 0.001$). The pre-treatment VEGF levels among different stages of the disease were marginally insignificant ($p = 0.07$). However, they were significantly different for (i) various grades ($p < 0.001$), (ii) tumor size ($p = 0.026$), and (iii) smoking habits ($p = 0.018$). Post treatment levels were highly significant, as compared to pre-treatment values ($p = 0.001$). The pre-treatment and post-treatment VEGF levels were associated with (i) disease stage ($p = 0.002$), (ii) grade ($p = 0.001$), and (iii) tumor size ($p = 0.001$). In conclusion, VEGF is a potent angiogenic factor and can be considered as an effective prognostic marker in cervical cancer.**

Keywords: VEGF, cervical cancer stages, prognostic markers

1. Introduction

Cervical cancer is the most common cancer affecting women in India (*1*). Human papilloma virus infection has been determined as the main risk factor for cervical cancer (*1*). Pap smear screening is still the most reliable means of diagnosing cytopathological changes leading to cancer development in the developing world (*2*,*3*). Pap smear screening, though simple is not very common among the Indian population (*2*,*3*). Therefore, detection is late and thus the patients reaching hospitals are in a higher stage of disease and in a wide age group, with the median age of the patients being 50 years. Primary surgery on the one hand and chemo radiation on the other are the most effective means of treatment depending on the stage of cancer (*4*). Surgery also determines the number of positive lymph nodes which in turn indicates the prognosis of the tumor.

Angiogenesis, the formation of new blood vessels from pre-existing capillaries, is essential for both tumor growth and spread (*4*,*5*). The existence of angiogenic factors was initially postulated on the basis of the strong neovascular response induced by transplanted tumors (*6*). Tumor growth beyond 1-2 mm is strictly dependent on angiogenesis (*5*). Tumor tissues secrete angiogenic factors that activate neovascularization in and around tumors (*7*). Tumor angiogenesis in cervical cancer is a complex process controlled by numerous cytokines which relate to prognosis (*8-10*). These factors include

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vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), basic fibroblast growth factor (b FGF), and IL-8.

Vascular endothelial growth factor, also called vascular permeability factor (VPF) is an endothelial cell mitogen with angiogenic activity (*11*). VEGF currently includes five members, in addition to the prototype VEGF, which mediates angiogenic signals *via* high-affinity receptor tyrosine kinases. Three of the receptors are known as VEGFR-1, VEGFR-2, and VEGFR-3. VEGFR-1 with VEGFR-2 is expressed in vascular endothelium, whereas VEGFR-3 is expressed in lymphatic endothelium (*12*).

Vascular endothelial growth factor (VEGF-A, also called as VPF) has emerged as the single most important regulator of blood vessel formation in health and disease. It is important for embryogenic vasculogenesis and angiogenesis as a key mediator of neovascularization (*13*). Inhibition of VEGF results in increased endothelial cell killing by radiotherapy and produces a supra-additive anti-tumor effect in a murine mouse model (*14*). VEGF is expressed in precursor lesions of the cervix and invasive cervical cancer (*15*). VEGF expression has recently been determined also by semi-quantitative immuno-histochemistry (*16*). Loncaster *et al.* (*16*) has reported a positive and inverse correlation between increased MVD or VEGF and disease free survival, while some workers have found no such correlation (*17*).

Serum VEGF has become an efficient means to determine VEGF levels as a surrogate marker of tumor angiogenesis (*18*); particularly in females, in breast, vulvar and ovarian cancer, VEGF has been found to be associated with tumor progression (*19-21*).

The relationship between tumor and serum VEGF has been a focus of a number of studies and the results vary greatly. Tissue expression of VEGF has been found high in squamous cell carcinoma as compared to CIN (*22*). Cheng *et al.* (*8*) showed that VEGF levels in tumors were high and correlated well with tumor progression; where as Lee *et al.* (*23*) and Tjalma *et al.* (*17*) found VEGF levels of no prognostic value. Studies have shown that VEGF levels correlate to successful treatment (*24,25*). Serum VEGF offers the possibility of an early available biomarker with prognostic potential (*4*).

The purpose of the present study was to determine serum VEGF concentration in pre- and post-treatment cases of cervical cancer and correlate it with established clinicopathological characteristics. Serum VEGF levels relate with progression of stage of cancer. The levels of VEGF rise with the higher stage of the disease. How serum VEGF levels vary in patients who have received conventional treatment; that is, whether the regular and routine treatment itself affects or modifies the level of VEGF in cervical cancer patients is still far from established.

A positive answer will act as a response predictor in cases who have received treatment, thus signifying the measurement of post-treatment levels of VEGF. This will be specifically true for patients who have received only regular treatment and not added anti-angiogenic therapy.

2. Materials and Methods

2.1. *Subjects*

A total of 110 histologically confirmed cervical cancer cases and 50 controls were enrolled for the study. All the controls enrolled for the study were healthy subjects free from any cervical pathology and who after physical examination showed no symptoms of any debilitating disease. The median age of the patients was 50 years (range, 26-80 yrs) and the median age of the controls was 40 years (range, 24-70 yrs). The patients were classified using FIGO staging according to which there were 18 patients in stage I, 32 in stage II, 48 in stage III, and 12 in stage IV. Out of 110 cases enrolled in the study, 8 patients underwent primary surgery, 10 underwent surgery and radiotherapy, 80 had chemo radiation and 12 had surgery and chemo radiation. Blood samples from patients and controls were collected only from those who consented to be a part of the study. The protocol of informed consent had already been approved by the Ethical Committee of Chattrapati Shahuji Maharaj Medical University.

2.2. *Treatment*

All the patients under study received the following radiation treatment. Patients were given radiotherapy by external beam radiotherapy EBRT followed by brachytherapy. EBRT was delivered by telecobalt therapy machine (Theratron 780 E, AECL, Ottawa, Canada). A total dose of 50 Gy in 5 weeks at 5Fc per week was delivered to the whole pelvis. This was followed by high dose rate (HDR) brachytherapy after a gap of 2 weeks of completion of EBRT. Patients were also given chemotherapy in the form of injection of cisplatin 30 mg/m^2 *i.v.* weekly throughout the course of EBRT with *i.v.* hydration and antimetic prophylaxis.

2.3. *Serum assay for VEGF estimation*

Blood samples of all cases were obtained by peripheral venous puncture both on the day of recruitment of the patient and after 6-8 weeks of chemo-radiation. The samples were centrifuged at 3,000 rpm for 10 min. The samples were aliquoted and immediately stored at –80°C. VEGF-A levels were determined in serum samples using a quantitative human VEGF immunoassay kit (Bender Med Systems, Vienna, Austria) using the manufacturers protocol. In brief, the samples were diluted using sample

diluent. Biotin conjugate was then added to the wells and the plate was incubated at room temperature for 3 h. The plate was then thoroughly washed with the wash buffer provided in the kit. The plate was then coated with streptavidin HRP. After incubation for 15 min the reaction was stopped with wash solution and the absorbance was read at 450 nm.

2.4. *Statistical analysis*

Normal distribution of serum VEGF levels was measured using the Shapiro-Wilk test. Due to the skewed distribution of VEGF levels and the small number of observations in certain groups non parametric tests were used and for description of baseline characteristics median, range and interquartile range were used. Comparisons between two independent groups were made using the Mann Whitney U test. Comparisons between multiple groups were made using one-way ANOVA on ranks with Dunn's test as a multiple comparison procedure. For comparing two groups a one-tailed test was used and a p value of < 0.05 was considered to be significant. The statistical analysis was performed using SPSS version 16.0.

3. Results

A total of 110 cases and 50 controls were enrolled in the present study. Table 1 summarizes the clinical baseline characteristics of patients. The median age of the patients was 50 years (range, 26-80 yrs) and the median age of the controls was 40 years (range, 24-70 yrs). The patients were classified using FIGO staging according to which there were 18 (16.4%) patients in stage I, 32 (29.1%) in stage II, 48 (43.6%) in stage III, and 12 (10.9%) in stage IV. The lymph nodes of 29 (26.4%) patients were positive and those of 81 (73.6%) were negative. Out of all the 110 cases enrolled for the study 27 (24.5%) were smokers and 83 (75.5%) were non smokers. The tumor size was (i) < 2 cm in 10 (9.1%) cases, (ii) in the range of 2-4 cm in 19 (17.3%) cases, and (iii) > 4 cm in 81 (73.6%) cases. Out of 110 cases enrolled in the study, 8 patients underwent primary surgery, 10 underwent surgery and radiotherapy, 80 had chemo radiation and 12 had surgery and chemo radiation.

Table 2 gives the results of (i) comparison of Serum VEGF levels between controls and cancer patients, and (ii) comparison of VEGF levels between different clinicopathological categories of patients. It is seen that statistically VEGF levels are neither associated with different status of lymph nodes in a significant manner ($p = 0.23$) nor with different stages of cervical cancer ($p = 0.07$). The VEGF levels were significantly different between controls and patients ($p < 0.001$). The VEGF levels were significantly different (i) among various grades ($p < 0.001$) and (ii) among various tumor size ($p = 0.026$), and (iii) between smokers and non smokers ($p = 0.018$).

Table 1. Clinicopathological characteristics of cervical cancer cases (n = 110)

Characteristic	Number (%)
Age (years)	50.0 (26-80)[a]
Stage	
I	18 (16.4%)
II	32 (29.1%)
III	48 (43.6%)
IV	12 (10.9%)
Grade[b]	
WD	92 (83.6%)
MD	5 (4.5%)
PD	13 (11.8%)
Lymph node	
Positive	29 (26.4%)
Negative	81 (73.6%)
Smoking status	
Smoker	27 (24.5%)
Non smoker	83 (75.5%)
Tumor size	
< 2 cm	10 (9.1%)
2 - 4 cm	19 (17.3%)
> 4 cm	81 (73.6%)
Treatment	
Surgery	8 (7.3%)
Surgery + Radiotherapy	10 (9.1%)
Chemo radiation	80 (72.7%)
Surgery + Chemo radiation	12 (10.9%)

[a] The values are median (range); [b] WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

The post-treatment level of serum VEGF concentrations were taken into consideration at a period of 6-8 weeks after treatment. The VEGF level decreased uniformly among post-treatment patients.

Table 3 summarizes (i) the correlation between pre- and post-treatment serum VEGF levels in squamous cell carcinoma and (ii) association of pre- and post-treatment VEGF levels with different clinicopathological categories of patients. The pre-treatment and post-treatment VEGF levels of patients were significantly different ($p < 0.001$) and both levels were significantly associated (i) with various stages of cervical cancer ($p = 0.002$), (ii) with various grades ($p < 0.001$), and (iii) with various tumor sizes ($p < 0.001$). However, the VEGF levels were neither associated (i) with the patients having a history of smoking ($p = 0.07$) nor (ii) with the lymph node status ($p = 0.44$).

4. Discussion

Prognosis and progression are the key words for cancer management in this new era. Since the prognosis of cancer cannot be judged by any clear means, factors which indicate progression become important. Angiogenesis or neovascularization is important for

tumor growth and development. Several factors have come to light which promote tissue angiogenesis. These include basic fibroblast growth factor (b FGF), Interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α). Among the various angiogenic factors vascular endothelial growth factor has a pivotal role in tumor angiogenesis and promotes differentiation of endothelial cells thus increasing the permeability of capillaries (*6*,*26*).

Fukumura *et al*. (*27*) has shown that tumor associated stroma also produces VEGF. VEGF thus came into focus and has been extensively studied since then. Salven *et al.* (*28*) found that VEGF has a prognostic impact in non-Hodgkins lymphoma. Similarly, VEGF was associated with disease progression of carcinoma in ovary, esophagus, colon,

Table 2. Comparison of serum VEGF levels in controls and different clinicopathological categories of cancer patients

Characteristics	*n*	VEGF levels (pg/mL)		*p*
		Median	IQR	
Controls	50	225.0	252.75	< 0.001
Patients	110	786.8	506.9	
Stage				
I	18	684.4	591.9	0.07
II	32	665.4	577.6	
III	48	818.3	376.4	
IV	12	918.6	472.5	
Grade[a]				
WD	92	16.8	481.1	< 0.001
MD	5	975.6	266.2	
PD	13	1,200.7	943.4	
Lymph node				
Positive	29	900.8	724.6	0.23
Negative	81	775.0	485.1	
Tumor size				
< 2	10	497.0	616.6	0.026
2 - 4	19	561.9	594.5	
> 4	81	799.4	407.8	
Smoking				
Smoker	27	900.8	638.8	0.018
Non smoker	83	770.6	522.2	

[a] WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

Table 3. Correlation between pre- and post-treatment serum VEGF (pg/mL) levels in squamous cell carcinoma

	n	Pre-treatment median (IQR)	Post-treatment median (IQR)	*p*
Patients	110	786.8 (506.9)	510.5 (245.1)	< 0.001
Stage				
I	18	684 (591.9)	480.7 (452.4)	0.002
II	32	665.4 (577.6)	528.7 (300.4)	
III	48	818.3 (376.4)	510.5 (201.3)	
IV	12	918.6 (472.5)	485.2 (347.5)	
Grade[a]				
WD	92	16.8 (481.1)	502.2 (248.6)	< 0.001
MD	5	975.6 (266.2)	541.3 (156.8)	
PD	13	1,200.7 (943.4)	617.4 (360.5)	
Lymph node				
Positive	29	900.8 (724.6)	502.0 (320.4)	0.44
Negative	81	775.0 (485.1)	512.0 (234.9)	
Tumor size				
< 2	10	497.0 (616.6)	372.8 (466.5)	< 0.001
2 - 4	19	561.9 (594.5)	541.3 (360.4)	
> 4	81	799.4 (407.8)	511.0 (225.8)	
Smoking				
Smoker	27	900.8 (638.8)	515.8 (251.0)	0.07
Non smoker	83	770.6 (522.2)	502.0 (256.1)	

[a] WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

breast, head and neck, and lung cancers (*29-34*). VEGF has also been shown to be over expressed in cervical cancer (*12,19,35,36*).

In an original study by Kodama *et al.* (*37*) the highest levels of VEGF mRNA expression were observed in early invasive cervical cancer. Except for stage IVb the stage of the disease inversely correlated with the level of VEGF mRNA. There was no significant difference in the level of VEGF mRNA with respect to histological subtypes, tumor size, depth of stromal invasion, parametrial involvement and lymph node metastasis.

Kodama *et al.* (*37*) also found that VEGF mRNA closely correlated with tumor vascularity. This finding was in agreement with that of Tokumo *et al.* (*36*) in which the micro vessel density correlated significantly with VEGF expression in stage Ib-IIb immunohistochemical methods.

In contrast to some studies, Jacobson *et al.* (*38*) showed that VEGF is not correlated with survival. Lebrecht *et al.* (*39*) also found no correlation of VEGF with patient's prognosis regarding disease free and over-all survival. In the study by Lebrecht *et al.* (*39*), VEGF correlated significantly with tumor stage whereas no significant conclusion could be drawn from lymphatic spread and tumor grade.

Loncaster *et al.* (*16*) evaluated immunohistochemically the low VEGF levels in cervical cancer and found that it was significantly associated with metastasis free survival. Cheng *et al.* (*8*) showed that intratumoral cytosol VEGF concentration in cervical cancer was an independent prognostic factor.

Serum VEGF levels were significantly higher in both patients of CIN and cervical cancer compared to healthy controls, as shown in the studies of Yang *et al.* (*25*) Mitsuhashi *et al.* (*40*), and Lebrecht *et al*. (*39*).

The present study was undertaken to estimate serum VEGF levels in healthy controls and various stages of cervical cancer in pre- and post-treatment states. This was further correlated with different stages of patients including: tumor size, smoking status, tumor grade, and lymph node status in descriptive analysis.

There was a monotonous increasing trend between VEGF level and the stages of the disease. The association between VEGF level and the various stages of cancer was marginally insignificant ($p = 0.07$). This might be due to the small number of observations in different categories of the disease (*e.g.* $n = 12$ for stage IV patients) and because of the use of non-parametric tests which are less powerful than the corresponding parametric tests. Clinically, the differences in median VEGF level among various stages seem to be important. In this case, statistical significance or insignificance has to be carefully interpreted (*41,42*).

The pre-treatment VEGF values of patients were correlated with their lymph node status, where the levels obtained were insignificant. However, the tumor size and histological grade along with smoking status of patients had a significant correlation.

A fixed time interval of 6-8 weeks was considered for the evaluation of post-treatment levels as shown in Table 3. The post treatment levels of VEGF decreased significantly after treatment.

Tumor grade and tumor size also showed a significant correlation in pre- and post-treatment levels of VEGF showing a statistically significant consistent and linear increase. However, neither smoking nor lymph node status was statistically different, though pre-treatment levels of VEGF correlated well with the smoking status of patients in other studies. VEGF data compared to smoking habits of patients is not available to the best of our knowledge in an indexed literature and it needs more in depth study.

The results of the present study compare well with the results of Mitsuhashi *et al.* (*40*), who demonstrated a significant difference in pre- and post-treatment VEGF levels in cervical cancer cases. Lebrecht *et al.* (*39*) also reported that serum VEGF was markedly elevated in patients with squamous cell carcinoma of uterine cervix when compared to healthy women. Here too, VEGF level correlated well with tumor stage, but not with lymph node status.

Thus the present study of pre- and post-treatment levels of VEGF in patients with cervical cancer showed an increased and positive correlation with tumor size, the stage of the disease and grade. The over-all difference in VEGF levels in cervical cancer patients of all stages at the time of diagnosis was statistically higher than that in healthy controls; indicating increased angiogenesis. The histological studies in the above cervical cancer cases showed a correlation with increased angiogenesis.

Zusterzeel *et al.* (*4*) in one of the largest studies correlated serum VEGF levels to establish prognostic factors in cervical cancer. They concluded that serum VEGF was highest in advanced tumor stage, large tumor size (> 2 cm) and was associated with overall disease free survival, thus speculating that it could act as a useful prognostic factor in patients with cervical cancer. The study was done in a western population and the present study has been done in Asian-Indian population where risk factors differ slightly. The findings of the present study appear similar to the Zusterzeel *et al.* (*4*) study despite the fact that the data are from two different ethnic populations.

The present study compares a series of patients and controls, correlating VEGF with other established risk factors associated with prognosis. However, further long term studies are required to correlate prognostic outcomes to further validate VEGF levels in cervical cancer patients and thus establish VEGF as an independent prognostic marker.

Moreover, whatever causes VEGF to stimulate endothelial cells to proliferate, needs more signal transduction studies.

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