RESEARCH ARTICLE

Modulatory Effects of Chemoradiation on Angiogenic Factors and Laminin in Cervical Cancer: Link with Treatment Response

Manoj Sharma[1], Rehan Khan[2], Mayank Aggarwal[1], Alpana Sharma[2]*

Abstract

Objective: Carcinoma of the uterine cervix is either the first or second most common malignancy in Indian women, depending on the registry. Tumor growth and metastasis primarily are determined by angiogenesis and parameters of the molecular environment including extracellular matrix elements, growth factors and cytokines. Effects of chemo-irradiation on biomarkers like vascular endothelial growth factor (VEGF), angiopoietin-2 (Ang-2) and laminin in patients with carcinoma cervix therefore need to be explored. **Methods:** Circulatory and mRNA levels of VEGF, Ang-2 and laminin in patients with stage III carcinoma cervix (n=40) were compared with those of normal healthy women (n=20). Measurement was prior to treatment, and after chemotherapy and teleradiation, using high sensitivity ELISA kits and Q-PCR. Clinical response was evaluated as per WHO criteria and was assessed for correlation with the biochemical markers. **Results:** Levels of all the studied molecules were significantly (p<0.001) higher in patients than in controls. After treatment significant decline (p<0.001) was noted. Out of 40 patients, 33 were complete responders and 7 were non-responders on clinical assessment. On comparison of before and after treatment levels of these molecules complete responders showed significant decline whereas non-responders showed non-significant decrease. Follow-up of the responders for 3 years, revealed 28 of 33 patients to still be disease free, the other 5 demonstrating recurrence. **Conclusions:** Higher levels of angiogenic factors along with laminin indicate roles played in disease progression aiding angiogenesis. These markers may serve as useful tools in post treatment disease mapping, for which available imaging methods may not provide a true picture.

Keywords: Cervical cancer − VEGF − Ang-2 − laminin − chemotherapy

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Introduction

Cervical cancer (CaCx) is the 4th most-common cause of cancer in women. About 80% of cervical cancers occur in developing countries (Globocan, 2012). Nearly 90% of the deaths from cervical cancer occurred in developing countries, suggesting that cancer diagnosis and treatment services are insufficient in regions with the highest prevalence of the disease (Globocan, 2012). It is ranked as the most frequent cancer in women in India. It occurs in a multistep process, a sequential transition from a cervix with a normal epithelium to cervical intraepithelial neoplasia (CIN) and invasive cervical cancer (WHO, 2008).

Angiogenesis is the physiological process that involves the growth of new blood vessels from pre-existing vessels (Vermeulen et al., 2010). The progression from in situ to invasive and metastatic solid tumors is accompanied and enhanced by the switch from avascular to vascular phase (Bryan and Kathy, 2005). Regulation of angiogenesis is signalled by surrounding environment, including growth factors/cytokines and extracellular matrix (ECM). In addition, excessive angiogenic stimulus and altered balance between stimulators and inhibitors, results in activation of an angiogenic switch that increases the formation of blood vessels in an unregulated manner (Bryan and Kathy, 2005). Clinical studies have shown a direct correlation between the density of tumor vessels and an adverse prognosis in patients with a variety of solid tumors (Bryan and Kathy, 2005). Tumor growth, which employs a number of regulators, requires the formation of new blood vessels. The most important regulators are VEGF and Ang-2. DNA sequence variations in VEGF and Ang-2 genes may lead to altered productions and/or activities of these genes(Konac et al., 2007).

VEGF is one of the most potent and well documented among all angiogenic factors. Endothelial cells express VEGF receptors, and upon stimulation, they are able to produce various haematopoietic growth factors. It plays a central role in endothelial cell survival, differentiation, vascular permeability and assembling of their progenitor cells (Hicklin and Ellis, 2005). Increased VEGF expression

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results inaugmented microvessel density, tumor expansion and has been associated with poor prognosis for various solid tumors, including cervical cancer (Guidi et al., 1995). Some studies have confirmed that the expression of VEGF increases in a stepwise manner in normal tissue and cervical cancer tissue, and shows a higher expression in the late stage of cervical cancer (Chen et al., 2007). Several studies have regarded the VEGF family and its corresponding receptors as anti-angiogenic target molecules (Qi et al., 2014).

The angiopoietin (Ang) family of proteins play an important role in the regulation of blood vessel formation, maturation, repair, and permeability and is closely associated with cancer cell invasion and metastasis(Khan et al., 2009; Joshi et al., 2011). Recently, it has been revealed that VEGF and the angiopoietins display both distinct and overlapping expression patterns during angiogenesis (Thurston et al., 2000). Angiopoietin-2 (Ang-2) destabilizes vascular architecture and helps endothelial cells to gain access of VEGF (Holash et al., 1999). Afterward, VEGF elicits endothelial cell migration and proliferation leading to angiogenesis. Promotion of angiogenesis and vessel regression by Ang-2 in the presence or absence of VEGF, respectively, articulates how the cooperation between Ang and VEGF is crucial for the initiation of angiogenesis (Maisonpierre et al, 1997; Holash et al., 1999). Their co-expression promotes new vessel sprouting (Tait and Jones, 2004). There are studies which showed the higher levels of Ang-2 and showed its correlation with higher angiogenic activity(Tsutsui et al., 2006).

Interactions amid tumor cells and the extracellular matrix (ECM) play an important role in tumor invasion and metastasis. Many cellular processes such as adhesion, migration, differentiation, gene expression and apoptosis were controlled by basement membrane (Ashkenas et al., 1996). Major components of the basement membrane including laminins and nidogens, helps in cell adhesion and provides it the mechanical stability (Dziadek, 1995). ECM components and their proteolytic digestion products have been shown to stimulate in vitro migration of normal and malignant cells (Klomenek et al., 1993). Laminin-1 is involved in a number of biologically significant processes such as cell differentiation, migration, adhesion and signalling (Malinda and Kleinman, 1996). In a study, exposure of different neoplastic lymphocytes with laminin isoforms results in various cellular responses (Spessotto et al., 2001). Interaction of tumor cells with laminin via specific cell surface receptors helps them to invade sub-endothelial basement membrane (Ramos et al., 1991).

Studies have shown that VEGF, Ang-2 and Laminin in cervical cancer plays an important role in tumor progression and prognosis (Kohlberger et al., 2003; Andersson et al., 2005; Noel et al., 2005; Konac et al., 2007; Kopczyńska et al., 2009; Imura et al., 2012), but specific data to prove the diagnostic value of their serum level and its impact on therapeutic effect is lacking. This study examined the serum levels and mRNA expression of VEGF, Ang-2 and Laminin in cervical cancer patients before and after treatment. Further, we followed them up and combined with clinicopathological features, aiming to explore the prognostic potential and significance of serum level changes of these molecules in patients with advanced cervical cancer.

Materials and Methods

To obtain 80% study power and significance level (α) of 5%, sixty subjects (40 CaCx patients and 20 healthy controls) were recruited for this study. Inclusion criteria consisted of Patients with histopathologically proven squamous cell carcinoma of cervix, Federation of Gynecology and Obstetrics (FIGO) stage III, age 30 to 60 years, Karnofsky performance scale rating of > 50% and patient with normal hemogram, Kidney Function Test (KFT), Liver function test (LFT), Serum Electrolyte (SE), Random Blood Sugar (RBS) and cardiac function. Exclusion Criteria consisted of previously treated or recurrent case of carcinoma cervix, pregnant and lactating females, co-existing second malignancy, and co-morbid conditions uncontrolled diabetes mellitus, hypertension, tuberculosis etc. The study was carried out in the Department of Radiotherapy, Maulana Azad Medical College and associated Lok Nayak hospital, New Delhi and the Department of Biochemistry, All India Institute of Medical Sciences, New Delhi. Blood was withdrawn from 20 age matched, non-pregnant healthy females after taking their consent to be enrolled in this study. They have no history or laboratory evidence of malignancy or any other medical or surgical disease or gynaecological pelvic inflammatory disease as evidenced by haematological, colposcopic, ultrasonographic and mammographic evidence. Institute's Ethical Committee has approved this study (F.11/IEC/MAMC/10; Dated: 12/09/2010).

Patients diagnosed with carcinoma cervix who presented to Radiotherapy OPD, Lok Nayak Hospital, New Delhi after satisfying the aforementioned inclusion criteria were evaluated and enrolled after taking an informed consent. Pre-treatment staging and evaluation included history, physical and complete gynecological examination, routine laboratory investigations, chest roentgenogram, electrocardiogram, and computed tomography of the abdomen and pelvis. Other investigations such as cystoscopy, proctoscopy and Positron emission tomography (PET) Scan were also done, when indicated and feasible. Patients were staged as per International Federation of Gynecology and Obstetrics (FIGO) classification. Patients with stage III carcinoma of uterine cervix were given Neoadjuvant Chemotherapy i.e Inj. Cisplatin 20mg/m2 i.v. (Day-1 to Day-4), Inj. 5-FU 350mg/m2i.v. (Day-1 to Day-5) and Inj. Bleomycin 10 IU/m^2i.v.(Day-1 and Day-5). After completion of 2 cycles of induction chemotherapy with an interval of 21 days in-between, the patients were taken up for radiotherapy. Three weeks after completion of two cycles of chemotherapy, radical pelvic irradiation was given by means of Tele-cobalt machine (Theratron 780E, AECL Ltd) upto a dose of 5,000 cGy using conventional fractionation. Patients were then reviewed on 7th day for the suitability for Intracavitary Radiation Therapy (ICRT). Patients were evaluated clinically by thorough general and gynecological examination to assess response to chemo-radiation,

response evaluation was done through contrast-enhanced computed tomography (CECT; Somatom Definition FLASH, Siemens Healthcare, Forchheim, Germany) abdomen and pelvis after two cycles of chemotherapy and at the end of tele-radiation therapy while comparing it with pretreatment CECT abdomen and pelvis images. Similar evaluation was done as per WHO guidelines at 4 and 8 weeks of completion of treatment. CECT images were processed for tumor volume evaluation using syngo. via software (Siemens Healthcare, Forchheim, Germany). Our Treatment policy is evidence based improvement on existing traditions of "Standard of Care".

Ten milliliters of blood were withdrawn from patients before initiation of chemotherapy and 2 weeks after completion of 2 cycles of chemotherapy and after radiotherapy. Plain sterile tubes free of endotoxins were used to withdraw blood for isolating serum (4 mL), whereas EDTA vials were used to collect blood for peripheral blood mononuclear cells (PBMC) isolation (6 mL). For serum isolation, blood was kept at room temperature for 10 min, and then, centrifugation was done for 10 min at 3,000 rpm. Isolated serum was stored at -80 °C for further use, while the Ficoll gradient was used for PBMC isolation.

Angiogenic Factors and Laminin Enzyme Linked Immunosorbent Assay

High-sensitivity enzyme-linked immunosorbent assay (ELISA) kits were used for determining the circulatory levels of VEGF, Ang-2 and Laminin. Each sample was analyzed in triplicates to enhance the precision of the test. Absorbance was read on BioTek ELx800 absorbance reader and washing steps were performed on BioTek ELx405 Select Deep Well Washer. Gen5 data analysis software from BioTek was used to calculate biomarker concentration.VEGF ELISA kit was supplied by RayBiotech (Catalog #: ELH-VEGF; Standard Concentration Range- 8.23 - 6000 pg/mL; Intra-Assay CV%: <10%, Inter-Assay CV%: <12%) and serum samples were diluted 5 times before assay. The minimum detectable dose of Human VEGF-A was determined to be 10 pg/ml. Minimum detectable dose is defined as the analyte concentration resulting in an absorbance that is 2 standard deviations higher than that of the blank (diluents buffer). Ang-2 ELISA kit was procured from R and D, Minneapolis, USA (Catalog Number DANG20; Standard Concentration Range- 46.9 - 3000 pg/mL) and serum samples were diluted 5 times before assay. Thirty-eight assays were evaluated and the minimum detectable dose (MDD) of human Ang-2 ranged from 1.20-21.3 pg/mL. The mean MDD was 8.29 pg/mL. The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration. Laminin ELISA kit was supplied by USCN Life Science Inc., USA (Cat. No.: E0082Hu; Standard Concentration Range- 6.25 - 400 ng/mL). The minimum detectable dose of human Laminin is typically less than 2.2 ng/mL. The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined from the mean O.D. value of 20 replicates of the zero standard added by their three standard deviations.

Quantitative mRNA expression by real-time reverse transcription PCR

The mRNA levels of VEGF, Ang-2 and Laminin [laminin alpha 1 (Lα1), laminin beta 1 (Lβ1), laminin gamma 1 (Lγ1)] were analyzed through relative quantitation using ABI 7500 real-time PCR (Applied Biosystems Inc.). Total RNA was isolated by ethanol–chloroform precipitation from PBMCs isolated from blood using TRIZOL reagent. One microgram of the total RNA was used to prepare cDNA using random hexamers (Thermo Scientific) that were used as template in real-time PCR. Twenty microliters of reaction mixture included the Maxima SYBR Green master mix (Thermo Scientific), cDNA, and the nuclease free water. The conditions for PCR were initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. β-Actin was used as the endogenous control for quantitation.

Mean Ct values were calculated for each molecule using $2^{-\delta Ct}$ method, where Ct values of the molecules were normalized to that of β-actin and compared with Ct values of normal healthy controls to observe fold change in expression.

Statistical Analysis

Stata 11.2 was used for statistical assessment. Data were presented as mean ± SD. Continuous data was compared by independent t-test (following normal distribution) and Wilcoxon rank sum (skewed data) in two groups. Comparison between the groups according to treatment was done by repeated major ANOVA followed by multiple comparisons by Bonferroni test. Karl Pearson correlation coefficient was calculated to find the correlation of different molecules with each other. A p value of <0.05 was considered statistically significant.

Results

Significant ($p<0.001$) increase was observed in levels of all the studied molecules as compared to healthy controls. Their levels were significantly ($p<0.001$) declined after treatment (Table 1). On clinical assessment, 33 patients were complete responders and 7 patients were non-responders out of total 40 patients. Complete responders showed significant decline on comparison of before and after treatment levels whereas non responders showed insignificant decline. Upon follow up of the responders for 3 years, 28 patients were found to be disease free while 5 patients showed recurrence (9-26 months) out of total 33 patients (Sharma et al., 2015). Interestingly, we have observed that the relapse patients have significantly lower difference between pretreatment and post treatment levels as compared to patients with disease free survival (Table 2).

Circulatory levels of VEGF, Ang-2 and Laminin

Mean values of VEGF, Ang-2 and Laminin studied in cervical cancer patients (Pre Treatment, Post Chemotherapy

Table 1. Comparison between Patients' Pre-Treatment, Patients' Post-Chemotherapy and Post radiotherapy and Healthy Control's Values of Serum VEGF, Ang-2 and Laminin levels

		Parameters			
		VEGF (pg/mL)	Ang-2 (pg/mL)	Laminin (pg/mL)	Radiological Tumor Volume (cm^3)
Pre Treatment (S1; n=40)		2097.5 ± 522.8	1945.6 ± 501.9	1447.7 ± 348.8	144.3 ± 124.9
Post Chemotherapy (S2; n=40)		1645.6 ± 397.1	1443.4 ± 562.4	979.8 ± 385.8	40.6 ± 81.5
Post Radiotherapy (S3; n=40)		1159.7 ± 465.7	1025.2 ± 677	510.3 ± 379.3	10.5 ± 20.5
Controls (C; n=20)		441 ± 61.5	304.5 ± 89.8	311 ± 65.2	----------
P value	S1 vs S2	0.001	0.001	0.001	0.001
	S1 vs S3	0.001	0.001	0.0001	0.001
	S1 vs C	0.0001	0.0001	0.0001	----------
	S2 vs S3	0.001	0.001	0.0001	0.002
	S2 vs C	0.0001	0.0001	0.0001	----------
	S3 vs C	0.0001	0.0001	0.0027	----------

S1, Pre-Treatment levels; S2, Post-Chemotherapy levels; S3, Post-Radiotherapy; C, Healthy Controls; VEGF, Vascular Endothelial Growth Factor; Ang-2: Angiopoietin-2

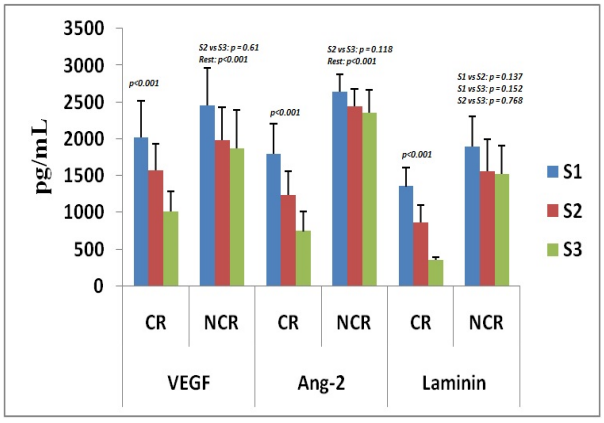

Figure 1. Comparison between Patient's Pre-Treatment, Patients's Post-Chemotherapy and Post Radiotherapy Values of Serum VEGF, Ang-2 and Laminin Levels in Complete Responders or Non-Responders. S1, Pre-Treatment levels; S2, Post-Chemotherapy levels; S3, Post-Radiotherapy; CR, Complete Responders; NCR, Non- Complete Responders; VEGF, Vascular Endothelial Growth Factor; Ang-2, Angiopoietin-2

and Post Radiotherapy) and the control subjects are shown in Table 1. We have found significantly higher (p<0.0001) mean pretreatment levels (S1) of VEGF, Ang-2 and Laminin in patients (2097.5, 1945.6 and 1447.7 pg/mL, respectively) as compared to healthy controls (441, 304.5 and 311 pg/mL, respectively). Post chemotherapy levels (S2), when compared with healthy controls, also showed significantly higher (p<0.0001) levels of VEGF, Ang-2 and laminin, but when compared with S1 levels, their levels were significantly lower (p<0.001). Post radiotherapy levels (S3), when compared with healthy controls, showed significantly higher (p<0.0001, <0.0001 and <0.0027, respectively) levels of VEGF, Ang-2 and Laminin, but when compared with S2 levels, their levels were significantly lower (p<0.001, <0.001 and <0.0001, respectively). Radiological Tumor volume also showed significant decline after chemotherapy (p<0.001) as well as after radiotherapy (p<0.001). When Tumor volume after chemotherapy and radiotherapy were compared, it showed significant (p<0.002) decline. Figure 1 shows

Table 2. Comparison between the Difference in Pre Treatment and Post Treatment Levels of Serum VEGF, Ang-2 and Laminin in Relapsed and Disease Free Patients Out of Total Responders

	Responders (n=33)						
	Relapse Cases (n=5) Mean ± SD (Range)			Disease Free (n=28) Mean ± SD (Range)			p value
	PT	POT	Difference	PT	POT	Difference	
VEGF (pg/mL)	1391.8 ± 114.1 (1282.4-1571.9)	610.2 ± 124.7 (467.4-797.0)	781.7 ± 73.4 (657-833.9)	2133.2 ± 456.4 (1296.0-4123.8)	1081.2 ± 237.1 (410.6-1463.4)	1052 ± 339.3 (883-2660.4)	0.0008
Ang-2 (pg/mL)	1479.1 ± 135.9 (1334.7-1644.4)	621.9 ± 93.2 (538.0-768.0)	857.3 ± 58.3 (796.7-932.9)	1854.5 ± 416.2 (1370.5-3009.6)	765.7 ± 288.5 (379.0-1515.0)	1088.8 ± 206.2 (937-1961.6)	0.0001
Laminin (pg/mL)	921.8 ± 74.5 (862.2-1030.9)	319.3 ± 14.7 (300.5-336.0)	602.5 ± 72.2 (546.2-694.9)	1429.5 ± 183.1 (1210.2-2291.1)	359.8 ± 36.6 (260.8-430.0)	1069.7 ± 179.1 (892.1-1907.1)	0.0001

VEGF, Vascular Endothelial Growth Factor; Ang-2, Angiopoietin-2

Table 3. Comparison between Patients's Pre-Treatment, Patient's Post-Treatment and Healthy Control's Values of VEGF, Ang-2 and Laminin mRNA Levels

		Parameters (Relative mRNA Fold Expression)				
		VEGF	Ang-2	Lα1	Lβ1	Lγ1
Pre Treatment (PT; n=40)		0.591 ± 0.263	0.076 ± 0.027	0.350 ± 0.108	0.056 ± 0.007	0.004 ± 0.001
Post Treatment (POT; n=40)		0.151 ± 0.109	0.017 ± 0.022	0.046 ± 0.061	0.017 ± 0.008	0.001 ± 0.002
Controls (C; n=20)		0.0021 ± 0.001	0.0008 ± 0.0003	0.0002 ± 0.0001	0.0007 ± 0.0003	0.0004 ± 0.0001
	PT vs POT	0.0001	0.0001	0.0001	0.0001	0.0001
P value	PT vs C	0.0001	0.0001	0.0001	0.0001	0.0001
	POT vs C	0.0001	0.0001	0.0001	0.0001	0.0255

PT, Pre Treatment; POT, Post-Chemotherapy + Post-Radiotherapy; C, Healthy Controls; VEGF, Vascular Endothelial Growth Factor; Ang-2, Angiopoietin-2; Lα1, Laminin Alpha 1; Lβ1, Laminin Beta 1; Lγ1, Laminin Gamma 1

Table 4. Correlation between Serum Levels of Angiogenic Factors, Laminin and Radiological Tumor Volume in Cervical Cancer Patients

Parameters		Pearson Correlation	Significance (p)
VEGF vs. Ang-2	S1	0.541	0.0003
	S2	0.5311	0.0004
	S3	0.5904	0.0001
VEGF vs. Laminin	S1	0.6406	0.0001
	S2	0.6508	0.0001
	S3	0.421	0.0068
VEGF vs. RTV	S1	0.2125	0.1881
	S2	0.3258	0.0402
	S3	0.5595	0.0002
Ang-2 vs. Laminin	S1	0.7798	0.0001
	S2	0.7072	0.0001
	S3	0.638	0.0001
Ang-2 vs. RTV	S1	0.3216	0.043
	S2	0.5359	0.0004
	S3	0.3151	0.0476
Laminin vs. RTV	S1	0.4307	0.0055
	S2	0.5726	0.0001
	S3	0.2297	0.154

S1, Pre-Treatment levels; S2, Post-Chemotherapy levels; S3, Post-Radiotherapy; RTV, Radiological Tumor Volume; VEGF, Vascular Endothelial Growth Factor; Ang-2, Angiopoietin-2; Lα1, Laminin Alpha 1; Lβ1, Laminin Beta 1; Lγ1, Laminin Gamma 1

the comparison of VEGF, Ang-2 and Laminin between responders and non-responders after treatment.

mRNA expression levels of VEGF, Ang-2 and Laminin using quantitative PCR

The mean fold expression values for each molecule showed an increase in mRNA expression by real-time RT-PCR (Table 3). Post treatment levels showed significant (p<0.0001) decrease when compared to pretreatment levels but the levels remain significantly higher when compared to healthy control levels. When the pre and post treatment levels were compared in responders, each molecule showed significant (p<0.0001) decline. But in non-responders, only Lα1 and Lβ1 showed significant decrease when pre and post levels compared. VEGF, Ang-2 and Lγ1 levels decrease insignificantly (p=0.49, =0.058 and =0.733 respectively) after treatment in non-responders (Figure 2). Pretreatment levels when compared between responders and non responders, only VEGF showed significant (p<0.01) difference whereas Ang-2, Lα1, Lβ1 and Lγ1 shows insignificant difference. When the post treatment levels of responders and non-responders were compared, Ang-2, Lα1 and Lγ1 showed significant (p<0.0001) difference whereas VEGF and Lβ1 showed insignificant (p=0.066 and =0.364) difference (Figure 2).

Correlation between VEGF, Ang-2, Laminin and Radiological Tumor volume

On Pearson correlation analysis of ELISA results (Table 4), VEGF shows a significant positive correlation with Laminin (Pretreatment and post chemotherapy) with Pearson correlation coefficient greater than 0.5. Ang-2 also shows significant positive correlation with laminin. On Pearson correlation analysis of post treatment levels of quantitative PCR (Q-PCR) results (Table 5), VEGF showed a significant positive correlation with Ang-2, Lα1 and Lγ1. Ang-2 also showed significant positive correlation with Lα1, Lγ1 and tumor volume. Lα1 shows significant positive correlation with Lγ1 and tumor volume. When post treatment levels of Lγ1 were correlated it shows significant positive correlation with post treatment tumor volume.

Discussion

Although great progress has been achieved in chemotherapy and radiotherapy for the treatment of cervical cancer, the 5-year survival rate of these patients is still not satisfactory. Clinical observations show that radio-sensitivities of different cervical cancer patients differ significantly; therefore, an effective molecular marker is desperately needed to follow the treatment response and help select treatment regimen and judge prognosis so as to modify or amend treatment timely. The invasion of malignancies is closely related to angiogenesis and ECM

Table 5. Correlation between mRNA Levels of Angiogenic Factors, Laminin and Radiological Tumor Volume in Cervical Cancer patients

Parameters		Pearson Correlation	Significance (p)
VEGF vs. Ang-2	Pre	0.2066	0.2009
	Post	0.7797	0.0001
VEGF vs. Lα1	Pre	0.0393	0.8095
	Post	0.6021	0.0001
VEGF vs. Lβ1	Pre	0.1786	0.2703
	Post	0.0025	0.9878
VEGF vs. Lγ1	Pre	-0.2718	0.0897
	Post	0.6742	0.0001
VEGF vs. RTV	Pre	-0.0257	0.8749
	Post	0.3178	0.0456
Ang-2 vs. Lα1	Pre	0.1226	0.451
	Post	0.8847	0.0001
Ang-2 vs. Lβ1	Pre	0.0496	0.7611
	Post	0.0335	0.8373
Ang-2 vs. Lγ1	Pre	-0.3924	0.0123
	Post	0.8903	0.0001
Ang-2 vs. RTV	Pre	-0.1024	0.5294
	Post	0.7459	0.0001
Lα1 vs. Lβ1	Pre	-0.3322	0.0362
	Post	0.3966	0.0113
Lα1 vs. Lγ1	Pre	-0.2566	0.11
	Post	0.9646	0.0001
Lα1 vs. RTV	Pre	0.3903	0.0128
	Post	0.7128	0.0001
Lβ1 vs. Lγ1	Pre	0.4198	0.007
	Post	0.4025	0.01
Lβ1 vs. RTV	Pre	-0.0366	0.8228
	Post	-0.0097	0.9525
Lγ1 vs. RTV	Pre	-0.1389	0.3925
	Post	0.6324	0.0001

Pre, Pre-Treatment; Post, Post-Treatment; RTV, Radiological Tumor Volume; VEGF, Vascular Endothelial Growth Factor; Ang-2, Angiopoietin-2; Lα1, Laminin Alpha 1; Lβ1, Laminin Beta 1; Lγ1, Laminin Gamma 1

proteins. Currently, anti-angiogenic therapy is a major focus for the treatment of cancers.

To the best of our knowledge, no studies have been carried out to investigate the circulatory level as well as cellular expression of angiogenic factors and laminin and the interrelationship between these molecules in cervical cancer. Circulatory levels of angiogenic factors (Ang-2 and VEGF) and laminin, along with their mRNA levels, were estimated in a single study before and after chemo-radiotherapy in advanced cervical cancer patients and the patients have followed up for 3 years. The data generated in this study showed significantly higher circulatory levels of Ang-2 and VEGF, and elevated laminin levels was also observed. This data may suggest their role in disease progression as they may be involved in the process of angiogenesis and metastasis in cervical cancer.

VEGF plays an important role in cervical cancer angiogenesis and its inhibition results in reduced tumor angiogenesis, retarded growth and induction of apoptosis in tumor cells. Higher incidence of deep stromal andparametrial invasion along with lymph node metastasis was reported in tumors with overexpressed VEGF (Cheng et al., 1999). So far, there are many studies dedicated to the relationship between serum VEGF levels and tumor behaviors of cervical cancer patients, but the results are different. VEGF has been investigated in cervical carcinoma at different levels including serum level (Zusterzeel et al., 2009), mRNA level (Guidi et al., 1995; Nagy et al., 2011) and protein at tissue level (Loncaster et al., 2000). Interestingly, serum VEGF levels have been shown to be a prognostic marker for disease free survival in cervical cancer patients, whereby high pre-treatment VEGF levels were associated with worse survival (Loncaster et al., 2000; Zusterzeel et al., 2009).

Few studies observed correlation between serum VEGF levels and clinico-pathological features (Zusterzeel et al., 2009). On the contrary, there are studies which could not establish correlations between VEGF expression and disease stage (Loncaster et al., 2000) as well as its prognostic value in cervical carcinoma (Nagy et al., 2011). There is a study which utilized alterations in the serum concentration of VEGF to measure treatment response in

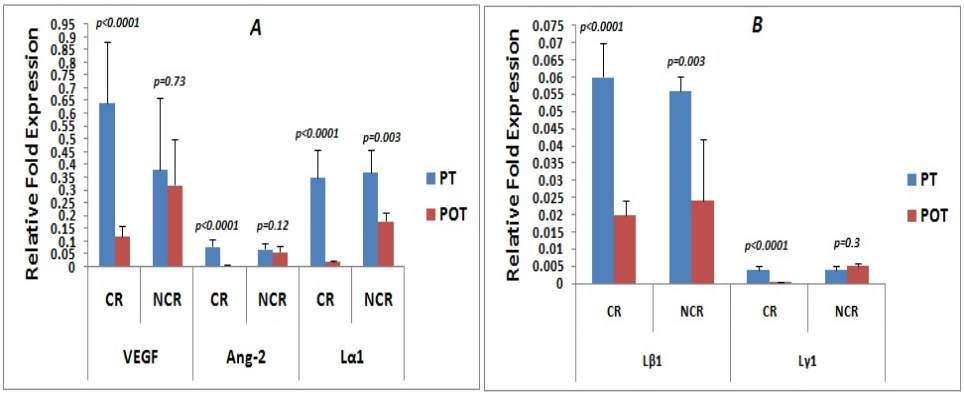

Figure 2. Comparison between Patient's Pre-Treatment and Patient's Post-Treatment mRNA Levels of (A) VEGF, Ang-2 and Lα1; (B) Lβ1 and Lγ1 in complete responders or non-responders. PT, Pre Treatment; POT, Post-Chemotherapy + Post-Radiotherapy; CR, Complete Responders; NCR, Non- Complete Responders; VEGF, Vascular Endothelial Growth Factor; Ang-2, Angiopoietin-2; Lα1, Laminin Alpha 1; Lβ1, Laminin Beta 1; Lγ1, Laminin Gamma 1

cervical cancer patients (Dirix et al., 1997). Some studies also observed association between VEGF expression and microvessel density (MVD) (Barbu et al., 2013), while others did not find any association (Zijlmans et al., 2009). Our results are in concordance with the previous findings. We have observed statistically significant elevated pre-treatment levels of VEGF which goes down after chemo-radiotherapy. We have also observed elevated expression of VEGF at mRNA level which shows correlation with the treatment response. Post-treatment levels of VEGF also showed significant correlation with radiological tumor volume.

Ang proteins are a major focus for the treatment of cancer because of their involvement in angiogenesis during malignancy affecting cancer growth, metastasis and invasion (Liao and Johnson, 2007). With the presence of VEGF in cancer tissues, Ang-2 seems to be the strengthening factor for the initiation of angiogenesis and for vascular sprouting (Maisonpierre et al., 1997). In a study by Kopczyńska et al., (2009), plasma concentration of Ang-2 was significantly higher in cervical cancer patients than in controls. Other studies on prostate cancer, liver cancer, gastric cancer, and oral squamous cell carcinoma revealed upregulation of the protein and mRNA expression of Ang-2 in these tumors (Khan et al., (2009; Joshi et al., 2011). On the contrary, Konac et al., (2007) observed lack of association between VEGF and Ang-2 polymorphisms and cervical cancer. In this study, serum levels, as well as mRNA levels of Ang-2, were significantly elevated. After chemo-radiotherapy its serum as well as mRNA level declined significantly. Ang-2 post treatment levels showed significant correlation with tumor size. The strong correlation found between Ang-2 and VEGF in this study might add some light on the induction of neoangiogenesis by Ang-2 and VEGF synergistically. The findings in this study indicate that Ang-2 could be used as a factor to predict the prognosis and that it also may serve as a potential target to inhibit angiogenesis.

Laminins are a family of basement membrane proteins, which associate with cell differentiation, adhesion and migration proteins, as well as being structural components themselves (Gasparoni et al., 2007). Imura et al., (2012) reported that Laminin-5 can be used as a useful biomarker in the evaluation of invasiveness in cervical adenocarcinoma. There are studies which reported the involvement of laminin-5 γ2 chain in the process of neoplastic changes of uterine cervical squamous epithelium (Andersson et al, 2005; Noel et al., 2005). Maity et al., (2011) demonstrated that Laminin induces matrix metalloproteinase-9 expression and activation in human cervical cancer cell line. Kohlberger et al., (2003) observed significant correlation between the grade of cervical intraepithelial neoplasia and laminin-5 immuno-reactivity. In our study, we have observed the higher circulatory levels of laminin in pretreatment which got significantly decreased after chemo-radiotherapy. We have also observed elevated expression of laminin (Lα1, Lβ1 and Lγ1) at mRNA level which shows correlation with the treatment response. Pretreatment and post chemotherapy levels showed significant correlation with tumor size while post radiotherapy levels showed no significant correlation.

The responders were followed for 3 years, out of 33 responders 28 were disease free upto 3 years while 5 showed relapse (9-26 months). Interestingly, we have found that the relapsed patients have lower difference between pretreatment and post treatment levels of the molecules studied as compared to disease free patients. This study observed the serum VEGF, Ang-2 and Laminin levels of cervical cancer patients before and after treatment and found that their serum levels were declined after treatment and were closely related to tumor size. Thus, the biomarkers VEGF, Ang-2 and Laminin could be reliable markers for early diagnosis and monitoring of efficacy of therapy in cervical cancer. In conclusion, higher levels of angiogenic factors might suggest their role in disease progression through the induction of angiogenesis. These markers may serve as useful tools in post treatment disease mapping and response evaluation. Further studies in larger patient cohort and long-term follow up are needed to validate the data to implement in clinical settings.

Conflict of Interest

The authors declare that there is no conflict of interest.

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