

## ORIGINAL ARTICLE

# Vascular Endothelial Growth Factor Levels in Relation to Oxidative Damage and Antioxidant Status in Patients with Breast Cancer

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**Purpose:** Oxidative stress and angiogenesis are important elements in the pathogenesis of inflammatory diseases and cancer. Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic cytokines and is up-regulated by conditions associated with the generation of free radicals and reactive oxygen intermediates. In this study, we investigated the association between oxidative stress and serum VEGF status in patients with breast cancer. **Methods:** Forty patients with breast carcinoma, of which 21 were stage II and 19 were stage III, along with 40 age- and gender-matched healthy controls were enrolled. Oxidative stress, total antioxidant status, and VEGF levels in serum were evaluated by spectrophotometric procedures. Malondialdehyde (MDA) levels were measured and antioxidant status was assessed by measuring total antioxidant status (TAS) to assess oxidative

damage. **Results:** VEGF and MDA levels were significantly higher in patients with breast cancer than those of controls ( $p < 0.005$ ). Total antioxidant level decreased significantly in patients compared to that in controls. MDA, TAS, and VEGF levels were also analyzed based on menopausal status and different clinical disease stages. MDA and TAS level significantly different in the postmenopausal group than the premenopausal group, whereas VEGF level remained unchanged. **Conclusion:** Increased VEGF level and its positive correlation with oxidative stress level and decreased antioxidant status suggest a link between oxidative stress and malignant transformation.

**Key Words:** Antioxidants, Cytokines, Malondialdehyde, Oxidative stress, Vascular endothelial growth factors

## INTRODUCTION

Genetic instability, inactivation of tumor suppressor genes, activation of oncogenes, as well as expression of growth factors, cytokines, and angiogenic factors promote tumor progression and invasion. Many angiogenic factors, including fibroblast growth factor, vascular endothelial growth factor (VEGF), and platelet-derived endothelial cell growth factor have been identified [1]. One of the most potent and specific angiogenic cytokines is VEGF. It is a 23 to 45 kDa heparin-binding glycoprotein that exerts multiple effects on tumors, including stimulating the formation of new blood and lymphatics vessels and increasing vascular permeability [2]. Binding of VEGF to one of several tyrosine kinase receptors triggers their autophosphorylation resulting in the activation of mitogen-activated protein kinases. Breast cancer has overtaken cervical cancer to become the leading cause of cancer-related mortality in females world-

wide. The etiology of breast cancer is multifactorial. Hormonal, genetic, and environmental factors appear to interplay in the pathogenesis of breast cancer [3], and oxidative stress has been suggested to play a role in malignant transformation [4].

A number of studies have unraveled the role of estrogens as well as the imbalance in oncogenes and tumor suppressor genes in breast cancer, but only a few reports are available on the oxidant-antioxidant profile in patients with breast cancer. Although reactive oxygen species (ROS), normal byproducts of aerobic metabolism, are essential for various defense mechanisms in most cells, they can also cause oxidative damage to DNA, proteins, and lipids resulting in enhanced cancer risk [5].

Malondialdehyde (MDA), the end-product of lipid peroxidation (LPO) arising from the free radical degradation of polyunsaturated fatty acids, can cause cross-linking in lipids, proteins, and nucleic acids [6,7]. VEGF is up-regulated by conditions associated with the generation of free radicals and reactive oxygen intermediates [8]. However, the exact pathomechanism causing this malignant transformation is still unclear. The present study investigated the possible association between oxidative stress status and serum VEGF levels in patients with breast cancer.

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## METHODS

The study included 40 patients with clinically diagnosed breast carcinoma, who were transferred to the department of general surgery, Banaras Hindu University Hospital, for treatment. Patients who did not undergo chemotherapy or radiotherapy were included. Clinical breast cancer staging was performed according to the TNM-UICC classification [9]. The controls consisted of 40 age and gender-matched healthy volunteers with a socio-economic status similar to that of the patients. They had no acute or chronic diseases such as diabetes, parasites, or any immune dysfunction. Additionally, the controls had normal leukocyte levels and other blood cell counts and were not under any pharmacological therapy. Venous blood was collected from patients and healthy volunteers into sterile tubes and the serum was used for various biochemical and hematological analyses.

The Institutional Review Board of the Institute of Medical Sciences, Banaras Hindu University approved the study (no. Dean/2010-11/65). Informed consent was obtained from each patient and the healthy volunteers to collect blood samples.

### Reagents

All chemicals and reagents were of analytical grade from Sigma-Aldrich Co. (St. Louis, USA).

### Lipid oxidative damage assay

Oxidative damage in the serum of patients and healthy controls was assessed by measuring LPO products in serum using the thiobarbituric acid (TBA) method [10]. MDA, which is a stable end-product of fatty acid peroxidation, reacts with TBA under acidic conditions to form a complex that has a maximum absorbance at 532 nm.

### Total antioxidant status (TAS) assay

Serum TAS was determined using an assay kit (Randox Laboratories Ltd., Crumlin, UK) [11]. The assay was based on the principle that ABTS (2, 2'-azino-di-[3-ethylbenzthiazoline-6-sulfonate]) produces the radical cation ABTS<sup>+</sup> upon incubation with a peroxidase and H<sub>2</sub>O<sub>2</sub>. This reaction produces a relatively stable blue green color that is measured at 600 nm. Antioxidants in the serum suppress color production proportional to their concentration.

### VEGF levels

VEGF levels were measured with a commercially available enzyme-linked immunosorbent assay kit (Thermo Scientific, Rockford, USA), according to the manufacturers protocol. The microplate used for the assay was coated with anti-human VEGF

antibody, which captures VEGF in the standard and samples added to the plate. After removing of unbound proteins, a biotinylated detecting antibody is added and allowed to bind to the VEGF. Excess antibody is removed and streptavidin-horse-radish peroxidase is added, which reacts with 3,3',5,5'-tetramethylbenzidine (TMB) to produce a colorimetric signal. The lower limit of detection for VEGF was < 8.0 pg/mL.

### Statistical analysis

The statistical analyses were conducted with the commercial SPSS version 16.0 package for Windows (SPSS Inc., Chicago, USA). All data are expressed as the mean ± standard error of the mean (SEM). Data were analyzed with the independent Student's t-test. All statistical analyses were two-tailed and a *p*-value < 0.05 was considered statistically significant.

Correlation between oxidative stress levels and VEGF was evaluated with Pearson's correlation coefficient. A *p*-value < 0.05 was considered as statistically significant.

## RESULTS

MDA, TAS, and VEGF were estimated among the patients with breast cancer in relation to their clinical stages and menopausal status, respectively. The patients were clinically classified as stage II (21 patients) and III B (19 patients), according to the UICC TNM system (Table 1). Of the 40 patients with breast cancer, 23 were premenopausal and 17 were postmenopausal. Table 2 shows the changes in VEGF levels as a result of oxidative damage and associated total antioxidant levels in patients with breast cancer with respect to their corresponding controls. The results revealed that the VEGF and MDA levels were significantly higher in patients with breast cancer than those in the controls (*p* < 0.05). The total antioxidant level decreased significantly in patients compared to that in controls. Table 3 shows the correlations between levels of oxidative stress, TAS and VEGF in patients with breast cancer. The VEGF levels were significantly and positively correlated with oxidative stress, whereas total antioxidant level was significantly and negatively correlated with

**Table 1.** Patients' characteristics (n=40)

Variable	No. of patients (%)
Age (mean ± SD, yr)	46.5 ± 11.23
Menopausal status	
Premenopausal	23 (57.5)
Postmenopausal	17 (42.5)
Stage	
II A	7 (17.5)
II B	14 (35)
III A	11 (27.5)
III B	8 (20)

VEGF. At the same time, MDA and TAS were significantly and negatively correlated. MDA, TAS, and VEGF levels measured in patients with breast cancer in relation to their menopausal status are depicted in Table 4. The levels of VEGF and TAS were not altered significantly in relation to menopausal status but there were significant changes in the MDA levels in the patients. MDA, TAS, and VEGF levels were studied in relation to their clinical stage (stages II and III) (Table 5). VEGF and MDA levels tended to increase at higher clinical stages of cancer, whereas the total antioxidant levels decreased with increasing stage.

### DISCUSSION

At the onset, the process of angiogenesis requires a change in the local equilibrium between proangiogenic and antiangiogenic factors [12]. Oxidative stress, after causing nonlethal injury to cells, may initiate a signal transduction cascade leading to tissue repair and angiogenesis [13]. Endothelial and inflammatory cells release high concentrations of angiogenesis activators, such as VEGF, angiopoietin-1, fibroblast growth factor, and transforming growth factor. Excess generation of oxygen derived radicals can cause oxidative damage [14]. Data also indicate that ROS are involved in initiating and promoting cancer. Our results showed a correlation between serum levels

of oxidative stress and VEGF in patients with breast cancer prior to treatment initiation. MDA levels were significantly higher in the blood of patients with breast cancer compared to those in the controls. Simultaneously, TAS concentrations were reduced significantly in patients with breast cancer compared to those in the controls. Our findings agree with most previous studies suggesting that ROS may accumulate due to decreased antioxidant levels in the blood, which may cause significantly higher LPO at the cellular and molecular levels.

MDA increased significantly in patients with breast cancer,

**Table 3.** Correlation between VEGF, MDA, and TAS in breast carcinoma patients

	VEGF (n=40)	MDA (n=40)	TAS (n=40)
Pearson correlation			
VEGF	-	0.376*	0.534†
MDA	0.376*	-	0.312*
TAS	0.534†	0.312*	-
Significance (two tailed)			
VEGF	-	0.017	<0.001
MDA	0.017	-	0.044
TAS	<0.001	0.044	-

VEGF=vascular endothelial growth factor; MDA=malondialdehyde; TAS=total antioxidant status.

\*Correlation is significant at the 0.05 level (2-tailed); †Correlation is significant at the 0.01 level (2-tailed).

**Table 2.** Levels of VEGF, MDA, and TAS in breast carcinoma patients and control cases

	Control (n=40)	Cases (n=40)	p-value	T-value
VEGF (pg/mL)	464.223±31.352	622.3119±22.211	0.0001*	4.114
MDA (mM/L)	3.0258±0.189	3.94467±0.267	0.006*	2.802
TAS (mM/L)	1.815±0.0692	1.419±0.1039	0.002*	3.176

Values are presented as mean ± SEM.

VEGF=vascular endothelial growth factor; MDA= malondialdehyde; TAS=total antioxidant status.

\*Statistical analysis was done by independent Student's t-test. All statistical analyses were two-tailed and a value of p<0.05 was considered statistically significant.

**Table 4.** Levels of VEGF, MDA, and TAS in breast carcinoma patients in relation to their menopausal status

	Premenopausal (n=22)	Postmenopausal (n=18)	p-value	T-value
VEGF (pg/mL)	600.1214±30.274063	649.43351±32.4456	0.275	-1.108
MDA (mM/L)	3.4146±0.3040367	4.5925±0.4246482	0.026*	-2.309
TAS (mM/L)	1.58376±0.1697	1.21798±0.085366	0.080	1.800

Values are presented as mean ± SEM.

VEGF=vascular endothelial growth factor; MDA=malondialdehyde; TAS=total antioxidant status.

\*Statistical analysis was done by independent Student's t-test. All statistical analyses were two-tailed and a value of p<0.05 was considered statistically significant.

**Table 5.** Levels of VEGF, MDA, and VEGF in breast carcinoma patients in relation to the clinical stage

	II A (n=7)	II B (n=14)	III A (n=11)	III B (n=8)
VEGF (pg/mL)	511.021±37.676	550.532±31.266	703.547±35.604	733.605±33.309
MDA (mM/L)	2.800±0.466	3.307±0.536	4.576±0.261	5.192±0.439
TAS (mM/L)	1.828±0.201	1.633±0.227	1.186±0.093	1.005±0.131

Values are presented as mean ± SEM.

VEGF=vascular endothelial growth factor; MDA=malondialdehyde; TAS=total antioxidant status.

which represents the oxidation of polyunsaturated fatty acids in membranes induced by free radicals. Lipid peroxides are well-characterized mutagens and are cytotoxic to the endothelial cells.

Therefore, an increase in MDA, a LPO product, could indicate involvement of endothelial cell dysfunction in tumor pathophysiology. Accompanying the accumulation of oxidative damage products, a significant reduction in total antioxidant capacity measured in the sera of patients with breast cancer was observed. Changes in TAS levels also reflected disease progression [15]. VEGF is widely recognized as a significant stimulator of tumor angiogenesis [16], and its cellular expression is regulated by a plethora of external factors. Cytokines, growth factors, and gonadotropins, which do not stimulate angiogenesis directly, can modulate angiogenesis by modulating VEGF expression in specific cell types and, thus, exert an indirect angiogenic or anti-angiogenic effect. The exact pathomechanism of this transformation is unknown.

Previous studies have shown that oxidative stress stimulates angiogenesis in cultured endothelial cells [17], which may activate angiogenesis for cancer. In this study, VEGF and MDA levels increased simultaneously and were positively correlated, which might indicate a cause or effect of the disease. The angiogenic response in vascular tissue is triggered by reactive oxygen species signaling. In cancer cells, a chronic imbalance in redox status, results in excessive ROS production, including  $H_2O_2$ . Additionally, low concentrations of  $H_2O_2$ , play various physiological roles, such as regulating gene expression and endothelial cell proliferation. Furthermore,  $H_2O_2$  up regulates nuclear factor- $\kappa$ B, which, in turn, regulates VEGF mRNA in breast cancer cells, supporting its role in angiogenesis and breast cancer metastasis [18,19]. Therefore, release of  $H_2O_2$  might be triggers for the angiogenic process in cancer cells. Potent suppressors of ROS formation in humans may be a target for new cancer therapy.

A significant increase in MDA level was found in the postmenopausal group associated with decreases in TAS, whereas VEGF levels were not altered significantly. The decreased TAS levels in postmenopausal women agree with a previous study [20].

Taken together, our results suggest a functional interplay among oxidative stress, antioxidants, and VEGF in patients with breast cancer. High levels of VEGF in patients with breast cancer were paralleled by elevated oxidative damage and decreased antioxidant levels.

## CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

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