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CROSSTALK BETWEEN DECREASED ANTI-OXIDANT AND INCREASED SERUM ANGIOGENESIS IN RENAL CELL CARCINOMAS

Sana Javaid Awan*[1], Rubyia Yousaf[1], Maaida Ajmal[1], Ammad Tanvir[2], Kanza Tariq[1], Saba Boota[1], Fahim Hadi[1], Tahir Maqbool[1] and Ayesha Tariq[1]

[1]Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Pakistan.

[2]Department of Health and Medical Sciences, Khawarizmi International College. Abu Dhabi, UAE.

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***Corresponding Author**
Sana Javaid Awan
Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Pakistan.

ABSTRACT

Background: Renal cell carcinoma (RCC) is known to the hazardous of all urogenital manifestations. The growth, invasion, and metastasis of RCC is critically depends upon angiogenesis. Further, cells of RCC are known to be in extreme oxidative stress. Based upon these facts, this study was design to evaluate the potential of RCC patient derived serum for tumor angiogenesis via measuring its vascular endothelial growth factor (VEGF) levels and anti-oxidative enzyme index in order to comprehend the metastasis of RCC in Pakistani Population. **Methodology:** Serum samples of 100 patients of RCC and 100 normal person were taken from Agha Khan Laboratory of Lahore, Punjab after due consent of patients and lab authorities. Tissue samples were collected from 50 RCC patient biopsies. VEGF levels were measured by immunostaining and Sandwich ELISA method. Estimation of anti- oxidative enzymes in serum samples was done by estimating the levels of superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase. **Results:** Levels of VEGF were increased in RCC group than in normal ones representing augmented angiogenesis. Moreover, increased lactate dehydrogenase levels a valuable biomarker for monitoring the prognoses of RCC indicates lower overall survival of the patients and improper functionality of kidney cells. On the other hand tumor metastasis resulted in the decreased levels of anti-oxidative enzymes indicating increased oxidative stress in RCC patients. **Conclusion:** Our results show that the enhanced angiogenic property

of serum and a downfall in anti-oxidative enzymes status could play a crucial role in renal cell carcinoma progression and metastasis in Punjabi population.

KEYWORDS: Vascular endothelial growth factor, Sandwich ELISA, Lactate dehydrogenase and Glutathione reductase.

INTRODUCTION

Kidney is a vital organ of human body and its structure is bean shaped made up of two lobes.[1] Kidneys perform a large number of important functions such as controlling body fluids volume and composition, as well as removal of metabolic wastes from body.[2, 3] Renal cell carcinoma also called renal adenocarcinoma or kidney cancer, it is a disease in which malignant cells are found in the lining of tubules in the kidney. It is the eighth most common malignancy which affect adults, and accounts for 3% to 4% of new cancer cases arising in U.S and is the 7th most common cancer in men and 9th most common cancer in women. Renal cell carcinoma (RCC) accounts for over 80% of the kidney cancers.[4-7]

For treating the renal cell carcinoma patient immunotherapy is considered as the core therapeutic option. High dose produce 15-18% response, also 6-7% remission along with 4-5% of cure rate. Bevacizumab and interferon-α as a combined dose and Immunotherapy with heavy-dose of interleukin 2 [IL-2] are approved to be effective treatments.[8, 9]

Vascular endothelial growth factor (VEGF) is a principal controller of vascular development and lymphatic vessel function during health and disease in the adult. The growth of new vessel formation is known as angiogenesis. VEGF is the key player in tumor angiogenesis.[10] Inhibition of VEGF stimulates angiogenesis that may give rise to tumor growth and metastatic potential in RCC.[11, 12] For the treatment of RCC VEGF inhibitors have been established.[10, 13]

Oxidative stress initiates the instruction of DNA damage and insert its effects on intracellular signal transduction pathways because it plays a significant part in carcinogenesis.[14] There is an increase in Oxidative stress in renal cell carcinoma. Based upon these facts, this study was design to evaluate the potential of RCC patient derived serum for tumor angiogenesis via measuring its vascular endothelial growth factor (VEGF) levels and anti-oxidative enzyme levels in an attempt to understand the metastasis of RCC in Pakistani Population.

METHODOLOGY

Sample Collection

Serum samples from the patients diagnosed with RCC were randomly collected from Agha Khan Laboratory, Lahore, Pakistan. A total of 100 normal subject and 100 RCC patients' serum and biopsies of 50 patients were included in the study who belong to province Punjab.

Assessment of Angiogenesis

Levels of VEGF (an angiogenesis marker) were evaluated via solid phase sandwich enzyme-linked immunosorbent assay (ELISA) and immune-histochemical analysis.

ELISA

ELISA was done as reported previously [15, 16]. Solid phase sandwich ELISA was done for anti-VEGF antibbody. Briefly, 96-well plate (Corning, USA) was coated with rabbit polyclonal anti-VEGF antibody (Santa Cruz biotechnology, USA) and incubated for 24 hours at 4 ºC. Washing with tris buffered saline containing tween 20 (TBST)] was done for three time and 200ul of blocking agent bovine serum albumin (BSA 1%) was added in the wells for 30 minutes. Again three time washing was given with TBST, 100 ul serum from both groups (Normal and RCC) was added and incubated for 24 hours. Wells were then washed again with TBST and incubated overnight with HRP conjugated donkey anti rabbit secondary antibody (Santa Cruz biotechnology, USA). After washing with TBST, 100ul of chromogenic solution 3,30,5,50-Tetramethylbenzidine (TMB) (Invitrogen Inc., USA) was added for 15 minutes and 0.18 M sulphuric acid (H_2SO_4) was added to stop the reaction after 15 minutes. Using a micro-titer plate reader, absorbance was taken at a wavelength of 450 nm along with 650 nm as a reference value.

Immunohistochemical Analysis

Tissues slides were taken from paraffin embedded biopsy samples. Immunohistochemical staining protocol was followed as reported.[17, 18] Briefly, three sections per biopsy were deparafinized and fixed in 4% paraformaldehyde (PFA). To block nonspecific binding, tissue sections were treated with 5% BSA and cells were stained with primary rabbit polyclonal VEGF antibody (Santa Cruz biotechnology, USA). After incubation, cells were washed and incubated with fluorescein isothiocyanate (FITC) conjugated goat anti rabbit secondary antibody (Santa Cruz biotechnology, USA). Fluorescent images were taken by microscope with DP-70 camera (Meiji, Japan).

Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase (LDH) activity was calculated in the serum of both groups. The assessment of LDH release was done according to the manufacturer's instructions (AMP Diagnostics, GmbH). The assay was performed by incubating the mixing 5ul serum from each group and 95ul of working reagent for 5 min. The absorbance was taken at 340 nm.

Assessment of Anti-Oxidative enzymes

Evaluation of anti-oxidative enzymes in both groups was done via measuring the levels Superoxide dismutase (SOD), Ascorbate peroxidase (APOX), Glutathione peroxidase (GSH) and Catalase (CAT) in serum.

Superoxide dismutase (SOD) Assay

SOD assay was performed with slight modifications in the reported protocol.[19] For preparation of reaction, the following components were added, 0.1 mM EDTA, 100 mM KH2PO4 buffer (pH 7.8), 60 mM riboflavin, 13 mM methionine, 2.25 mM NBT (Nitro blue tetrazolium) and remaining was serum. After mixing all the components, the reaction plate was exposed to light for 10 minutes. Optical density was taken at 560nm.

Ascorbate peroxidase (APOX) Assay

Apox assay was performed with slight modifications in the reported protocol.[19] For preparation of reaction the added components are, 0.5 mM ascorbate, 0.3 mM H_2O_2, 100 mM KH_2PO_4 buffer (pH 7.0) and remaining was serum. Optical density at 290 nm was taken after 3 minutes.

Glutathione (GSH) Assay

GSH assay was performed as reported previously[19] with slight modifications. Reaction was prepared by adding 1 mM EDTA, 0.5 mM oxidized glutathione, 100 mM KH_2PO_4 buffer (pH 7.5) and remaining serum were added. For starting the reaction 0.2 mM NADPH was added in the end. The reaction was permitted to proceed for 3 min. Absorbance of reaction was taken at 340 nm.

Catalase (CAT) Assay

CAT assay was performed with slight modifications in the reported protocol.[19] Buffer used for reaction contain 12.5 mM H_2O_2 in 50 mM KH_2PO_4 (pH 7.0). 100ul serum was added in it. In this assay plates were placed in dark and absorbance was taken at 240nm after 45 and 60seconds. CAT activity was estimated by using the difference between 45 and 60seconds.

RESULTS

Assessment of Angiogenic Potential

The potential of angiogenesis in RCC was determined by measuring the VEGF levels in the serum obtained from diseased (RCC) and normal groups. Immunostaining of RCC section was done via VEGF antibody in order to determine cellular levels of angiogenesis while ELISA methods was used to investigate the levels of VEGF in serum of RCC patients as well as normal subjects. Staining of tissue section of RCC revealed high level of angiogenesis which is a prerequisite for tumor progression and growth (Fig 1). Moreover, VEGF levels were increased significantly in diseased group (0.735±0.023) as compared to normal group (0.387±0.015) as evidenced from the graph (Fig 1B).

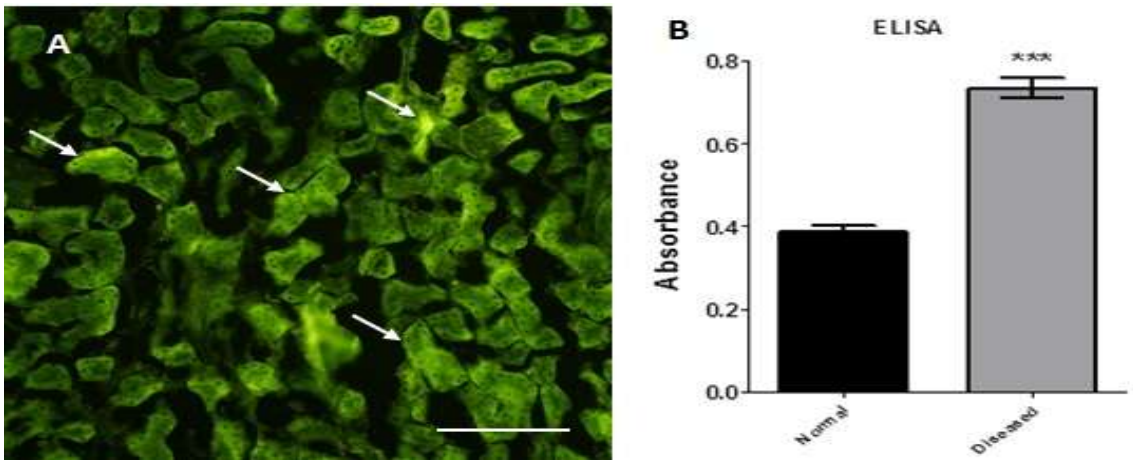

Fig 1: Estimation of VEGF levels in normal and diseased groups. A) Tissue section stained with anti-VEGF antibody. Staining show high number of VEGF positive cells as shown by white arrows. Green color of cells is due to FITC conjugated antibody. B) Bar graph shows high level of VEGF in diseased groups as compared to normal group. Values were expressed as mean ± SEM (normal vs. diseased * *p< 0.05)*.

LDH Release Assay

Lactate dehydrogenase (LDH) is common injury marker and disease, it is released as a response to tissue damage. The levels of LDH were measured in the serum obtained from normal and diseased groups. It is evidenced form the graph that levels of LDH were significantly higher in diseased group (12.9±0.3) as compared to normal group (8.25±0.1) (Fig 2).

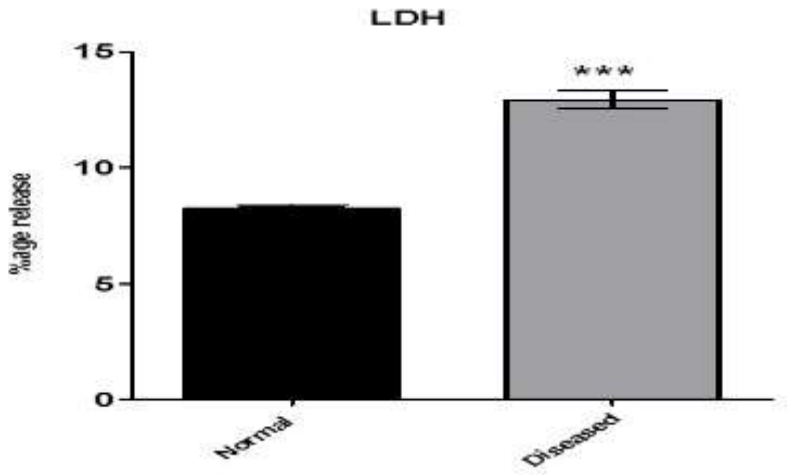

Figure 2: Percentage analysis of LDH release by normal and diseased groups. LDH release was significantly increased in diseased group as compared to normal group. Values were expressed as mean ± SEM (normal vs. diseased * *p< 0.05)*.

Assessment of anti-oxidative enzymes

Super Oxide Dismutase (SOD)

Percentage of Super Oxide Dismutase (SOD) was measured in blood serums that were obtained from the normal and disease groups. SOD percentage was compared in both groups. SOD percentage was lower in disease group (57.4±1.07) and higher in normal group (91.0±0.41) shown in (Fig 3A).

Glutathione (GSH)

Percentages of Glutathione were estimated in blood serums that were obtained from the normal and disease groups. Percentages of GSH were compared in both groups. GSH percentage was lower in disease group (7.53±0.25) and higher in normal group (28.5±1.07) (Figure 3B).

Catalase (CAT)

For the estimation of percentages of Catalase obtained from the blood serums of normal and RCC subjects. Catalase percentage was lower in disease group (5.89±0.49) and higher in normal group (18.0±0.07) (Figure 3C).

Ascorbate peroxidase (APOX)

APOX assay was done by measuring the levels of APOX from the blood serums of diseased group and normal one. APOX percentage was decreased in disease group (11.4±0.4) and increased in normal group (17.8±0.9) (Fig 3D).

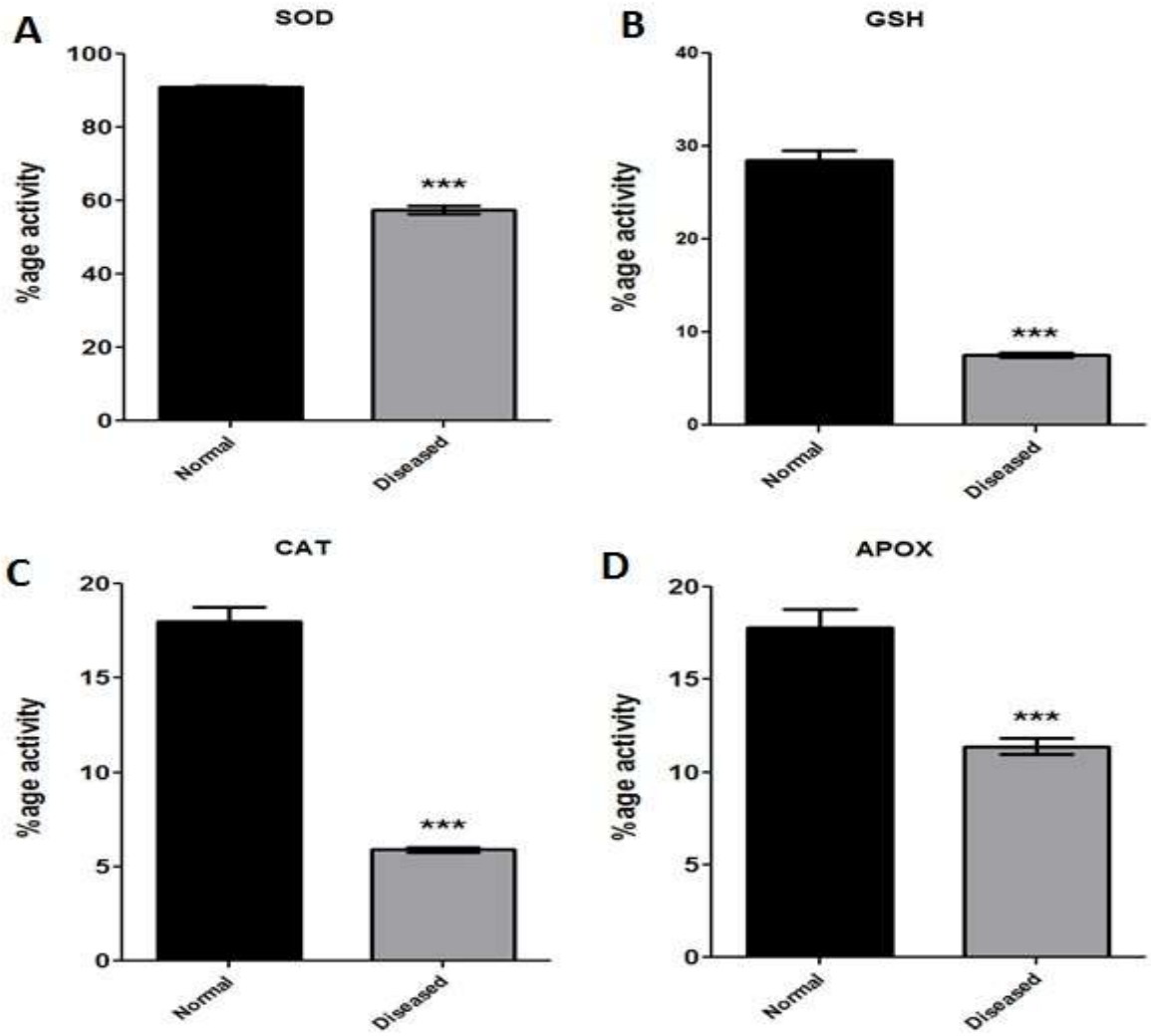

Figure 3: Assessment of anti-oxidative enzymes. A) Representation of the percentage activity of Super Oxide Dismutase in normal and diseased group. B) Percentage activity of GSH in normal and diseased groups. C) Percentage activity of catalase in normal and diseased groups. D) Bar graph shows the percentage activity of APOX in normal and diseased groups. Values were expressed as mean ± SEM (normal vs. diseased * *p< 0.05)*.

DISCUSSION

Renal cell carcinoma (RCC or hypernephroma) is one of the most common type of kidney cancer that initiates in the parenchyma of very small tubules in the kidney and accounts for most of the renal malignancies.[11, 20-22] Angiogenesis and oxidative stress play an significant role in the pathogenesis of RCC, therefore, this study investigates the anti-oxidative status and angiogenic potential of the RCC patients derived serum was determined via determining their vascular endothelial growth factor and anti-oxidative enzyme levels. Blood samples were collected from 100 renal cell carcinoma (RCC) patients and 100 normal subjects and serum was isolated for anti-oxidative enzyme assays and ELISA.

The process of new blood vessel formation is called angiogenesis which is necessary for embryologic development, tissue repair and normal growth.[23] Moreover, it is a prerequisite for tumor growth, progression, invasion, metastasis and prognosis.[24-26] Therefore, targeting the angiogenesis of a tumor may result in inhibition of tumor growth and progression.[25, 27] In this study increased angiogenesis has accounted as a marker for tumor progression, therefore, RCC patients have more levels of VEGF in their serum compared to normal ones (Fig: 1). Anti-angiogenesis is one of the most popular clinical interventions for cancer chemotherapy.[23-26] The key factor for angiogenesis is vascular endothelial growth factor (VEGF), therefore VEGF has been believed to be a central target for anti-angiogenic therapies.[28-30]

Most cancer cells including RCC use aerobic glycolysis to fuel their growth and targeting LDH is promising for tumor inhibition.[31] Increased LDH is an undesirable risk factor for survival of renal cell carcinoma.[32, 33] Therefore, serum sample taken from RCC patients show elevated serum level of LDH (Figure: 2).

Oxidative stress-mediated DNA damage has been reported to inhibit RCC and play a pivotal role in RCC pathogenesis.[33-35] It has been reported that oxidative stress play a significant positive effects on the growth and progression of RCC.[36-39] Strong anti-oxidants are reported to have strong anticancer abilities.[40] Moreover, agents that causes reduction in anti-oxidative enzymes are reported to have significant effects on tumor progression markers.[41] Previously reported data suggest a significant decrease in the levels of anti-oxidative enzymes in serum of RCC patient.[36, 42] Decreased levels of anti-oxidative enzymes (SOD, CAT, GSH and APOX) in the present finding can be a cause of progression of RCC in patients (Figure 3).
Thus, this study will helps in understanding the angiogenesis and anti-oxidative status of RCC patients as evidenced from their VEGF and anti-oxidative enzyme level profiling. Further, this information will aid in investigating the mechanism of progression of RCC. Our results show that the enhanced angiogenic property of serum and a downfall in anti-oxidative enzymes status could play a crucial role in renal cell carcinoma progression and metastasis in Punjabi population.

Competing interests

The authors have no financial conflicts of interest.

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