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RESEARCH ARTICLE

Correlation between Serum Cytokines Level (IL-6 , IL-10 , TNF-α and MIF) of Urinary Bladder Carcinoma Patients

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Abstract

Bladder cancer is abnormal cells multiply without control in the bladder arising from the epithelial lining (the urothelium) of the urinary bladder has the highest recurrence rate of any malignancy. Rarely the bladder is involved by non-epithelial cancers, such as lymphoma or sarcoma. Bladder cancer generates the highest medical cost per patient and it is the fifth most expensive cancer in terms of total medical care expenditures. Cytokines represent a large family of proteins molecules that have a broad range of functions produced by more than one type of cell. cytokines signaling are thought to be contribute in bladder tumor environment via two mechanism: stimulation of cell growth and inhibition apoptosis of damaged cells. The aim of our study was to estimate serum level of (IL-6,IL-10,TNF-α and MIF)before TURBT surgery. Blood have been obtained from 135 subjects which divided into three groups (UBC, UBD an healthy control)then measured the serum level of four cytokines by Sandwich ELISA .Results showed that all cytokines expressed high serum level in UBC group than UBD and healthy group with significant difference . The highest level of cytokines strongly associated with advanced stage characterized by high grade.

INTRODUCTION

Cytokines are low molecular weight soluble proteins have a fundamental role in communication within the immune system and involved in a wide array of biological activities specially that regulate growth , differentiation and activation of immune cells (Dranoff, 2004 and Chokkalingam *et al*., 2013).Each cytokine binds to specific surface receptor followed by subsequent cascades of intracellular signaling that altered cell function and include the up regulation of several genes and their transcription factors result in production of other cytokines (Horacek *et al*.,2014). Disregulation of cytokine production is thought to play an important role in the development of diseases such as autoimmune disorder and cancer . Various cytokines are involved in interactions between malignant cells of tumors and immune cells , which may influence tumor progression directly by acting on tumors cells as growth promoting or inhibiting factors or indirectly by attracting inflammatory cell types and affecting angiogenesis(Brumatti *et al*., 2010)Gene expression of cytokines and cytokine receptors is tightly regulated and aberrant expression has been implicated in susceptibility to a range of infectious diseases and some cytokine Singl-Nucleotide polymorphisms(SNPs) have been demonstrated to be important in altering expression or function of the cytokine gene. Genetic alterations in cytokine genes may lead to high or low production of certain cytokines that

may influence native antitumor immune responses or tumor progression by acting on pathways of tumor angiogenesis (Cardillo and Ippolitl ,2006 ; Chen *et al*., 2013 ;Sjödahl *et al*., 2013 and Karra *et al.,* 2015).

MIF enhancement of macrophage transcription, activation and viability, coupled with its inhibitory effects on anti-tumor cell cytotoxic lymphocytes, suggests that MIF overexpression in developing malignancies may act in concert to facilitate increased tumor growth which present an important link between inflammation and cancer due to its pro-inflammatory role. Its molecular mechanisms involve, among others, the inhibition of p53 which promote tumor cell proliferation, cell survival and tumor-associated neoangiogenesis (Meyer *et al*., 2010 and Souza *et al.,*2014).

Interleukin-6 a glycoprotein of a molecular weight of 26 kDa, composed of 184 amino acids. The human gene for IL-6 is located on 7p15-p21 chromosome and has the structure similar to the gene for granulocyte colony-stimulating factor, which explains the functional similarity of both cytokines IL-6 produced by many different cell types Interleukin-6 plays a major role in pathogenesis and development of malignancies. It helps tumor to grow through inhibiting cancer cells apoptosis and the induction of tumor angiogenesis(Mastorakos and Ilias, 2007; Abdulmohymen and Ashoor, 2010 and Chen *et al*., 2013). . IL-6 may be involved in the regulation of solid tumor growth in paracrine and autocrine ways. Interleukin‑ 6 contributes to the proliferation of bladder cancer cells and other cancers, especially those at the advanced stage of development and It's concentrations depends on the tumor stage and histological grade (Tsui *et al*., 2013 and McBeth *et al*., 2015)

IL-10 is also known as cytokine synthesis inhibitory factor (CSIF) that functions as a positive or negative mediator in innate and adaptive immunity under different circumstances. IL-10 is coded by a gene located on chromosome produced by numerous cell types including T cells (Th1, Th2 and Treg), B cells, monocytes/macrophages, keratinocytes and epithelial cells and binds to its receptor (IL-10R) expressed on the cell surface, which consists of R1 and R2 subunits**.** IL-10 has been shown to inhibit cellular immune responses via a number of mechanisms. IL-10 can block the accumulation of macrophages and DC at the tumor site, down-regulate the expression of MHC class II on these cells, thus suppressing the induction of specific immune responses (Saraiva and O-Garra, 2010; Luo *et al*., 2012 and Chan *et al*; 2013).

In contrast, TNF-*α* production levels can induce a tumor phenotype A TNF-*α* tumor promotion mechanism is based on reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation, which can induce DNA damage, hence facilitating tumorigenesis. TNF- *α*-mediated inflammation has been linked to cancer. TNF-α is one of the major mediators of inflammation and has been linked to all steps involved in tumorgenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis. Tumor cells secrete their own TNF-α in anticrime manner which further enhance the expression of other growth factors such as transforming growth factor-alpha (TGF-a) and epidermal growth factor receptor (EGFR), both of which mediate proliferation (Rama *et al*., 2012; Landskron *et al*., 2014 and Thompson *et al*., 2015).

Materials and Methods

Subjects

One hundred and thirty five subjects divided into three groups .seventy three patients of urinary bladder carcinoma (UBC), 60 male and 13 female with an average age 65.2 years and a range from (43 to 85) years, 32 patients hade urinary bladder disorder (UBD) and 30 healthy control .Subjects have been collected through the period from March 2014 to the November 2014. They attended to Urology Unit at Al-Yarmook hospital and Al-Jabchi privet hospital. The tumors were graded as low or high on the basis of WHO classification criteria.

Determination Serum Level of Cytokines

Serum level of(IL-6 , IL-10 , TNF-α and MIF) measured by using ELISA kit (R&D, USA), based upon coating wells of a high protein binding ELISA plate with monoclonal antibody specific for human cytokine . standard and sample were added to appropriate well followed by covering the plate with the adhesive strip and incubates for 2 hours at room temperature then washed four times with wash buffer . 200µl of cytokine conjugate was added to each well and covered with a new adhesive strip , incubated for 2 hours at room temperature on the shaker then washed four times . 200 µl of substrate solution was added to each well and incubated for 30 min at room temperature in dark. Finally,50 µl of Stop Solution was added to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing. The optical density of each well was measured within 30 minutes, using a microplate reader set to 450 nm.

Statistical analysis

The Statistical Analysis System-SAS(2012) program was used to effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage and least significant difference – LSD test was used to significant compare between means in the study.

Results and Discussion

The importance of secreted cytokines and growth factors in the development and promotion of malignancies is often underestimated. Many different soluble, extracellular gene products participate in processes that collectively contribute to the growth and survival of a developing neoplasm. These secreted molecules can, directly or indirectly, play a central role in uncontrolled tumor cell division, angiogenic stimulation or suppression of tumor cell immune surveillance (Walsh *et al.*,2012).

Results revealed that cytokines levels were higher in patients with UBC and UBD than in healthy controls with mean level (55.91, 90.14 , 35.84, and 36.74) pg/ml and (39.08,61.5,27.95 and 15.31) pg/ml in UBD than in healthy control(18.53,8.4,19.26 and 10.15)pg/ml with mean level respectively with highly significant differences as shown in table (1)

Table (1): Serum level of four cytokines in UBC,UBD and Healthy

Groups	Mean ± SE			
	MIF	IL-6	IL-10	TNF-α
UBC	55.91±2.97	90.14±7.29	35.84±1.83	36.74±2.25
UBD	39.08±2.37	61.5± 5.09	27.95±1.17	15.31±0.81
Healthy	18.53±2.26	8.4±0.45	19.26±0.87	10.15±0.55
LSD value	6.721	16.063	5.026	3.977
P-value	0.001	0.0001	0.00293	0.00041

The mean level of serum MIF in UBC patients was significantly higher than that observed in UBD patients and healthy control. Interestingly, MIF is found overexpressed in a large variety of human neoplasias Prostate, bladder, breast, colon, brain, skin and lung-derived tumors have all been shown to contain significantly higher levels of MIF protein than their non-cancerous cell counterparts(Takahashi *et al*., 2007 and Choudhary *et al*., 2013) .

MIF is the initial inflammatory mediator to stimulate the expression of other cytokines such as TNF-α and IL-1 via suppression of the anti-inflammatory actions of glucocorticoids . MIF has the potential to inhibit action of the tumor suppressor gene p_{53} and suppress transcriptional activity of p_{21}. Macrophages lacking MIF are sensitized to p53-dependent activation-induced apoptosis while cells containing MIF are significantly more resistant. In the tumor microenvironment, bypass of p53 by high concentrations of MIF expressed intrinsically by transformed cells or provided by surrounding inflammatory cells would enhance cell proliferation, extend lifespan, create a deficient response to genotoxic damage and allow for the accumulation of oncogenic mutations(Nishihira *et al*., 2003; Siegler *et al*.,2007 and Bach et al., 2009).

Another study reported IL-6 is a pleiotropic cytokine with varied systemic functions , secreted by a number of different cell types and has been implicated in various disease processes, including bladder cancer which implicated in proliferation pathways, because it acts with other factors and recorded elevated mean serum level in sera of bladder cancer patients when compared to serum IL-6 levels of non-cancerous patients thus suggested that plays a significant role in bladder carcinoma (Abdulmohymen and Ashoor,2010)

IL-10 suggested may play an important role in the progression of tumor. This high IL-10 level was suggested to contribute to a relative state of immunosuppression in patients with urinary bladder carcinoma with reduced host immunity thus high circulating and local levels of IL-10 may be produced by tumor cells or other cells, and may contribute to the development of an environment favorable to neoplastic cells and by enhancing the metastatic potential of neoplasticcells.

Also, these results are in harmony with Metwally *et al*.,(2011) who found a significant increase in TNF-a level (37.7 pg/ml) in sera of bladder cancer patients versus normal controls (8.7 pg/ml).Also with Fan *et al*.,(2012) They recorded Serum IL-6 and TNF-α concentrations in UBC patients were significantly higher than those in the control group which increased according to the severity of the disease, and differed greatly among different types of the disease , Significant positive relation of TNF -α and IL-6 was found.

The relationship between serum mean level of MIF and tumor stages of UBC patients showed that the highest level was observed in sera of UBC patients had T3(91.48 pg/ml),then T4(86.62 pg/ml), T2(64.21 pg/ml) and T1(43.06 pg/ml) while Ta (37.63 pg/ml) MIF play a central role in uncontrolled tumor cell division, angiogenic stimulation or suppression of tumor cell immune surveillance

Table (2): Association between MIF, IL-6 IL-10and TNF-alpha serum level and tumor stage.

Stage	Mean ± SE			
	MIF	IL-6	IL-10	TNF-alpha
Ta	37.63 ± 5.06	45.25 ± 5.06	29.97 ± 1.81	17.72 ± 1.09
T1	43.95 ± 3.78	70.48 ± 7.04	31.44 ± 1.35	30.44 ± 3.18
T2	64.21 ± 3.66	91.99 ± 9.59	37.19 ± 3.29	46.68 ± 3.78
T3	91.48 ± 5.19	155.71 ± 29.29	40.57 ± 7.82	54.65 ± 3.79
T4	86.62 ± 6.84	237.47 ± 26.31	68.61 ± 15.2	51.92 ± 2.73
LSD value	14.84 *	25.04 *	9.71 *	13.65 *
P-value	0.0014	0.0001	0.0152	0.0027

The MIF and the MIF receptor (CD74) when they bound ,initiate survival pathways and cell proliferation thus were highly expressed in invasive stages than non invasive tumors . Overexpressed in most tumor types has been shown to promote malignant cell transformation, inhibit tumor cell-specific immune cytolytic responses and strongly enhance neovascularization (Bai *et al.*,2012 and Morris *et al.*, 2014).

According to results as shown as in table (2) of this study showed the highest mean levels of IL-6 were observed in sera of UBC patients had T4 (237.47pg/ml) when compared with patients had T3 stage with mean level (155.70 pg/ml) then T2(91.99 pg/ml), T1(70.48 pg/ml) and Ta(45.24) with significant difference. Kirti *et al.*, (2015) documented that the function of IL-6 in the pathogenesis and development of showed an association between serum IL‑6 concentrations and the bladder cancer stage. IL-6 expressions level in patients with bladder cancers and controls examined by ELISA analysis showed higher level in UBC subjects than other had no bladder tumors. Also, in sera of patients with more advanced invasive stage (T2-T4) expressed high level than patients had early stage tumors (Ta-T1) thus reflected significantly correlated with clinical stage (Chen *et al.*,2013). A significant higher level of IL-10 was recorded in stage T4 (68.6)pg/ml followed by (40.57 , 37.19, 31.43 and 29.97)pg/ml in T3, T2, T1 and Ta respectively. As shown as that serum TNF alpha levels correlated with the clinical staging of urinary bladder carcinoma with higher levels in T3(54.65 pg/ml) ,T4 advanced-stage patients (51.92 pg/ml) while low levels in T1(29.01 pg/ml) and T2 early-stage patients. (46.68 pg/ml) Serum TNF alpha levels might be one of the factors contributing to the progression of disease. Szaflarska *et al*., (2009) showed that proinflammatory cytokine levels increased in in comparison to healthy volunteers, but no clear association with the stage of disease was observed. TNF was higher in stages T1-T2 (80.9 pg/ml) and T3-T4 (52.2 pg/ml) of the disease. TNF-α is a very well-known cytokine frequently seen in several cancers and several studies reported that the cancer stage and grade were significantly associated with the GA genotype in the TNF-α promoter region. Moreover, the serum concentration of TNF-α was significantly higher in bladder cancer patients had advanced invasive stage and has been implicated in tumor invasion and metastasis (Kakehi *et al*.,2010 and Chen *et al*., 2013). Mean serum level of MIF is significantly elevated with higher grade of advanced stage (69.26 pg/ml) than low grade of primary stage (38.98 pg/ml). Above result opposed with Ys *et al*., (2011)who reported that the expression of MIF protein was found predominantly in tumor cell and inversely correlated with tumor stage and grade . The expression of MIF in non muscle invasive bladder cancer was more frequently than in the muscle invasive disease.

The relationship between mean level of IL-6 in sera of patients and grade of urinary bladder tumors reflected positive correlation because the highest mean IL-6 levels were observed in sera of bladder cancer patients with high grade (118.16 pg/ml) and in sera of patients had low grade (59.98pg/ml) with significant difference as shown in table (3). Yeh *et al*., (2015) compared IL-6 expression in muscle-invasive and non-muscle invasive bladder cancer samples and their data revealed that the expression level of IL-6 was significantly correlated with higher clinical grade , higher recurrence rate after treatment, and reduced survival rate. Actually, IL-6 plays dual roles in bladder cancer progression.

Results in table (3) showed mean serum level of IL-10 is significantly elevated with higher grade of advanced stage (40.98 pg/ml) than low grade of primary stage (29.61 pg/ml). Since IL-10 plays an important regulatory role in bladder cancer immunosurveillance and BCG immunotherapy, blocking IL-10 activity could enhance BCG induction of Th1 immunity and therapeutic control of bladder cancer . IL-6 and IL-10 expressed elevated levels d in high-grade and in more advanced stage tumors (Luo *et al.*, 2012 and Redelman *et al*., 2014).

Table (3) Association between MIF, IL-6, IL-10 & TNF- alpha serum level and tumor grade.

Grade	Mean ± SE			
	MIF	IL-6	IL-10	TNF-alpha
Low	39.74 ± 3.48	60.28 ± 5.89	29.61 ± 1.02	23.87 ± 2.66
High	69.26 ± 3.17	114.77 ± 11.01	40.98 ± 3.02	47.35 ± 2.33
LSD value	8.024 **	16.731 **	6.894 *	9.416 **
P-value	0.0048	0.0001	0.0372	0.0074

Since IL-10 plays an important regulatory role in bladder cancer immunosurveillance and BCG immunotherapy, blocking IL-10 activity could enhance BCG induction of Th1 immunity and therapeutic control of bladder cancer . IL-6 and IL-10 expressed elevated levels d in high-grade and in more advanced stage tumors (Luo *et al.,* 2012 and Redelman *et al*., 2014). The relationship between sera mean level of TNF-α and tumor grade of UBC patients showed a high significant increased mean level in UBC (47.35pg/ml) with high grade as compared with low grade(23.87)pg/ml. These results compatible with Zhu *et al*., (2012) who found a critical mediator of inflammation, tumor necrosis factor (TNF-α) represents one of the potential molecular links between chronic inflammation and cancer and expressed increased serum level for high grade tumors when compared with low grade tumors and normal urothelium..

According to correlation coefficient between some parameters test, there were several correlations between those biomarkers involved in bladder cancer as shown in table (3-18).Expression of MIF had a significant strong positive correlation with expression of other investigated cytokines IL-6, IL-10 and TNF-α (r=0.52 , 0.69 and 0.67) respectively . Among other cytokines , IL-10 expression was also found to be positively correlated with IL-6 and TNF-α (r= 0.68 and 0.48)respectively .Also positive association was observed between IL-6 and TNF-α expression (r=0.59)($p \leq 0.001$).

Table (4): Correlation between (MIF, IL-10, IL-6 & TNF-alpha) level in UBC.

Cytokine parameters	Correlation coefficient (r)	P-valuea
MIF & IL-10	0.52 **	0.0001
MIF & IL-6	0.69 **	0.0001
MIF & TNF-alpha	0.67 **	0.0001
IL-10 & IL-6	0.68 **	0.0001
IL-10 & TNF-alpha	0.48 **	0.0001
IL-6 & TNF-alpha	0.59 **	0.0001

** ($P<0.01$).

Above result agreed with results reported by Conroy *et al.,(* 2010) who found that MIF was the original cytokine, described almost 50 years ago and has since been revealed to be an important player in pro-inflammatory diseases and has specific biological activities related directly to cancer growth or contributing towards a microenvironment favoring cancer progression such as induce production of other inflammatory cytokine like IL-6,IL-8 and TNF-α.

Cytokines modulate the functional activities of individual cells and tissues both under normal and pathogenic condition. Cytokines affect nearly every biological process. Cytokines such IL-6 and IL-8 are now being regarded as main culprit molecules in the pathway of progression of chronic inflammatory process to carcinogenesis .Therefore evaluated expression of different cytokines (TNF-α, IL -6, IL-8 and VEGF) in bladder cancer. TNF-α is released in response to infection and inflammation produced by activated macrophages and lymphocytes which found overexpression of TNF-α with the advance stage and lymph nodal metastasis of the cancer. TNF-α showed a positive linear correlation with IL-6 with invasion of the cancer and expressed a higher serum level as compared to healthy control . Also, serum levels of IL-6 were found to be highly elevated and positively correlated to tumor load which indicates that it has significant role in carcinogenesis of BC and altered gene expression of IL-6 enhances tumor growth (Kerschbaumer *et al.,* 2012 kang *et al*., 2013 and Chaturmohta *et al.,* 2015) .

cytokine production capacity varies among individuals and depends on cytokine gene polymorphisms which associated with altered protein levels and/or transcription rates might influence cancer susceptibility by

altered inflammatory responses. IL-10 was elevated mostly in advanced disease. The increased levels of IL-10 were associated with significantly poorer survival of patients(Seifarta *et al.*, 2005; Landskron *et al.*, 2014) .

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