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SERUM LYSYL OXIDASE, NITRIC OXIDE AND IL-8 AS POSSIBLE BIOLOGICAL MARKERS OF TRANSITIONAL CELL CARCINOMA OF THE BLADDER

[1]Shaimaa M. Mohammed, [1]Shatha H. Ali, [2]Mohammed S. Kamal
[1]Clinical Laboratory Science Department –College of Pharmacy – Baghdad University –Iraq.
[2]Immunohistochemistry Department - AL-Shaheed Gazi AL- Hariri Hospital- Medical City – Baghdad /Iraq

ABSTRACT
Cancers are complex clinical conditions that generally require invasive, laborious, expensive and time-consuming investigations for their diagnosis, treatment and follow-up. There is therefore an increasing need for exploring non-invasive markers for these conditions. Lysyl oxidase (LOX) an important modulator of extracellular matrix has an important role in the tumor development and progression, LOX has not previously been estimated in the serum of bladder cancer patients. We therefore sought to determine the clinical usefulness of serum LOX test, in addition to IL-8 and nitric oxide (NO), which are involved in carcinogenesis of transitional cell carcinoma of the bladder at different grades by comparing the results with that of controls. Forty six patients with bladder cancer (BC) and twenty apparently healthy individuals; (age, sex and ethnicity matched) were selected as controls to be enrolled in this study. Serum LOX, IL-8 and NO levels were determined. Serum LOX, IL-8 & NO were significantly elevated in BC over that of the control ($p < 0.05$). Data analysis according to the grade of the disease show a significant difference between high grade, low grade & control groups yet, there was no significant differences in LOX & IL-8 levels between high & low grade groups ($p= 0.846$ & 0.386 respectively) whereas, NO levels were significantly elevated by comparing high grade & control groups , also between high & low grade groups, but there was no significant difference between low grade & control group ($p= 0.203$) .Moreover, no significant correlation were observed among the studied parameters. Hence, LOX and IL-8 could be considered as promising markers for bladder cancer diagnosis, nevertheless, large scale studies are needed to substantiate the accuracy and outcome of their usefulness as biomarkers for transitional carcinoma of the bladder (TBC).While, NO seems to play an important role in BC, yet further studies will be necessary to elucidate how and to what extent NO and BC of different grades and stages are functionally interrelated.

KEY WORDS: lysyl oxidase, interleukin-8, nitric oxide, transitional cell carcinoma, bladder cancer.

INTRODUCTION

Transitional cell carcinoma of urinary bladder (TBC) is the most common genitourinary cancer [1]. Men are three times more likely than women to suffer from TBC; and the incidence rises with age, with peak levels between the age of 50 years and 70 years [2]. Bladder cancer is one of the top five cancers in the eastern Mediterranean region, in which it ranks the 3rd in the order of incidence in Iraq [3]. The most common risk factors for bladder cancer are the exposure to industrial carcinogens, cigarette smoking and possibly diet [4,5]. Another major etiological factor is the infection by the parasite *Schistosoma hematobium* [6]. In 70% of bladder cancer cases, gross hematuria is the main symptom that leads patients to seek for urologic consultation. Most TBCs are superficial at the time of diagnosis and can be treated effectively by a combination of transurethral resection and adjuvant chemo- or immune therapy[7].Diagnosis of TBC currently relies on cystoscopy and urine cytology, both examinations methods have certain limitations. Urethrocytoscopy is expensive, invasive and mostly associated with post cystopic pain and/or risk of urinary infection. Cystoscopy, also has a tendency to miss flat lesions such as carcinoma in situe, while urine cytology is prone to missing well-differentiated low grade lesions[7,8], both methods are dependent on observer expertise, and much effort has therefore been made to improve methods for diagnosis of TBC and for follow up testing. Thus, continuous efforts that are still seeking for convenient diagnostic markers for bladder cancer aiming in identifying new biological markers with good predictivity and sensitivity; to overcome the un necessity of cystoscopies and biopsies [9]. The majority of efforts in cancer research have focused on the functional consequences of oncogenesis and tumor suppressor gene mutations, however, cancer is a heterogeneous entity depends on the reciprocal interactions between cancer cells and their dynamic microenvironment provided by fibroblasts , endothelial cells, pericytes, inflammatory cells, and extracellular matrix (ECM)[10]. The microenvironment of solid tumors are now recognized as fundamentals in tumor pathogenesis and progression, related to from the role of ECM and matrix rigidity in determining polarity and growth potential of tissue, to the extracellular metabolism of growth factors and matrix molecules during cancer progression and metastasis [11,12]. Lysyl oxidase (LOX) one of the ECM components, a copper-dependent amine oxidase, that catalyzes the cross-linking of collagen and elastin in the ECM, thereby increasing insoluble matrix deposition and tensile strength [13]. Lysyl oxidase has been

shown to enhance tumor cell proliferation and invasion [14,15], particularly by increasing ECM rigidity and stiffness [16]. On the other hand the microenvironment of solid tumor, is exposed to low oxygen form (hypoxia) as a result of in adequate and chatotic blood supply, a key regulator of the cellular response to oxygen deprivation is the hypoxia inducible transcription factor-1α (HIF-1α), which activate the transcription of target genes involved in angiogenesis, energy, metabolism, adaptive survival and apoptosis [17,18]. Meanwhile, LOX is one of the hypoxia gene signatures[19]. Among the anti-inflammatory chemokines that contribute to human cancer progression is interleukin-8 (IL-8) in autocrine and paracrine manner through multiple mechanisms that are involved in its action, including direct effect on angiogenesis, tumor cell growth and migration[20]. Other important signaling molecule is nitric oxide (NO); which is a potent biological molecule that could participate in the pathogenesis of cancer by induction of apoptosis & promotion of angiogenesis; also it could play an important role in TBC [21]. The aim of this study is to investigate the significance of assessment of LOX, IL-8 & NO serum levels in patients with different grades of bladder cancer.

SUBJECT & METHODS

Forty-six patients (12 females and 34 males) with age range of 31- 85 years (mean±SE was 65.826±1.686), with histologically confirmed transitional bladder carcinoma (TBC) after transurethral resection, who were attending Ghazi AL-HaririHospital for Specialized Surgery/Baghdad Medical City (during the period of January-July / 2013) were enrolled in the study . None of the patients had any other malignant disease and all were newly diagnosed with no prior chemotherapeutic or radiation therapy. From each patient, a full medical history for diseases and previous laboratory finding was obtained, besides a cystoscopic examination by which transurethral resection (TUR) biopsies were taken from the apparent lesion, processed by standard oncological procedures, studied and graded by a specialist pathologist using the WHO/IUPS grading system [22] into:

A) High grade patient study group (designated as High grade TBC).
B) Low grade patient study group (designated as Low grade TBC).

In addition to twenty apparently healthy individuals whom age, sex and ethnicity matching that of the selected patients, were included as a control group. Table -1 illustrate the demographic data for the subjects included in the study. The study was approved by the Local Research Ethics Committee & all the subjects were given a written informed consent to participate in this study.

Venous blood specimen were obtained from each subject included in the study, immediate assessment of NO was done using the method of Miranda et al (2001)[23]. LOX & IL-8 serum levels were estimated by quantitative sandwich enzyme immunoassay technique (ELISA kit- CusaBio; China)[24,25]. Analysis of data was carried out using the available statistical package of SPSS-20, all the results were expressed as mean ± SE, The significance of difference of different percentages (qualitative data) were tested using chi-square test (χ^2-test) , whereas parametric variables were compared using student t-test between 2 groups & ANOVA among more than two.

RESULTS

There were no significant statistical difference between BC patients and control group in respect to age and sex (table -1). Significant variations were detected concerning smoking among different studied groups ($p < 0.0001$) suggesting smoking as a risk factor for BC. Serum LOX, IL-8 & NO were significantly higher in BC than control ($p < 0.05$) as demonstrated in table- 2. Data analysis according to the grade of the disease, show significant differences between high grade, low grade & control groups (table-3). Yet, LOX & IL-8 levels were not significantly different between high & low grade groups (p= 0.846 & 0.386, respectively), while their concentrations were significantly higher verses that of control group. However, NO levels were significantly higher between high grade & control groups , also between high & low grade groups , yet , there was no significant difference concerning its concentration between low grade & control group (p= 0.203) .

TABLE 1: Demographic description among different studied groups

Parameter	BC Total N=46		High grade TBC N=26		Low grade TBC N=20		Control N=20		P value
	N	%	N	%	N	%	N	%	
Age (years) <60	9	19.6	3	11.5	6	30.0	5	25.0	0.421
60--69	15	32.6	9	34.6	6	30.0	10	50.0	
≥70years	22	47.8	14	53.8	8	40.0	5	25.0	
Gender Male	34	73.9	18	69.2	16	80.0	15	75.0	0.365
Female	12	26.1	8	30.8	4	20.0	5	25.0	
Smoking	33	71.7	21	80.8	12	60.0	5	25.0	0.0005*
Smoker	13	28.3	5	19.2	8	40.0	15	75.0	
Not									

* Significant using Pearson Chi-square test ($p<0.05$)
TBC: Transitional Bladder Carcinoma, BC: Bladder Cancer, N: Number.

TABLE 2: Serum Levels of LOX, IL-8 and NO in Bladder Cancer Patients and Controls

parameter	BC N= 46	Control N=20	P value
LOX (ng/ml)	2.048±0.184	0.717±0.122	0.0001*
IL-8 (pg/ ml)	69.190±5.332	39.125±1.620	0.0001*
NO (µmol/L)	42.666±2.830	27.96±3.394	0.003*

Values are mean ± SE

*Significant using student t- test at $p < 0.05$ level

BC: Bladder Cancer, LOX: Lysyl Oxidase, IL-8: Interleukin-8, NO: Nitric Oxide.

TABLE 3: Serum Levels of LOX, IL-8 and NO in Different Grades of TBC Patients compared to Controls

Parameter	High grade TBC N=26	Low grade TBC N=20	Control N=20	P value ANOVA
LOX (ng/ml)	2.016 ± 0.241 [a]	2.089 ± 0.291 [a]	0.717 ± 0.122	0.0001*
IL-8 (pg/ ml)	73.297 ± 8.093 [a]	63.851 ± 6.337 [a]	39.125 ± 1.620	0.0011*
NO (µmol/L)	48.889 ± 3.663 [ab]	34.575 ± 3.820	27.963 ± 3.394	0.0001*

Values are mean ± SE

* Significant difference among different groups (ANOVA test $P< 0.05$)

[a] Significant difference as compared to control group (Student t- test $P< 0.05$)

[b] Significant difference between high & low grade groups (Student t- test $P< 0.05$)

TBC: Transitional Bladder Carcinoma, LOX: Lysyl Oxidase, IL-8: Interleukin-8, NO: Nitric Oxide.

DISCUSSION

Nearly all types of cancer present both diagnostic and prognostic challenges; such difficulty can delay treatment resulting in excess mortality and high light the need for better biomarkers. Although both cystoscopy and urine cytology remain the golden standards for diagnosis and follow up of bladder malignancy, the search for more convenient sensitive reliable marker is going on [26] the ECM play a critical role in the development and invasion of primary tumors, however the function of specific ECM components and the nature of signaling between ECM components and tumor cells is not fully understood , LOX is an amine oxidase primarily studied for its involvement in the formation of the ECM. Following secretion by fibrinogenic cells, it oxidize lysine residue in collagen and elastin resulting in the covalent cross linking and stabilization of theses structural members of the ECM [13]. The role of LOX in cancer emerges from the up regulation of LOX expression in many types of tumor e.g. breast, prostate, head and neck, sequamous cell carcinoma [27,28,29]. In addition LOX is now more widely accepted as poor prognostic factor , especially in promoting cancer metastasis in breast [14,30,31], head and neck sequamous cells [14,28], lung[32] and bronchogenic carcinoma[33]. However, serum levels of LOX has not been measured in the serum of bladder cancer patients ,except for one study in colorectal cancer showed an increased serum LOX level but, it failed to reach the statistical level of significance [34]. This study shows significant increase in LOX level in TBC patients as compared with control group ($p= 0.0001$), also serum levels were significantly higher in high grade TBC and low grade TBC groups as compared with control group ($p = 0.0001$) yet, there was no significant difference in LOX level between the high and low grade groups, such results give an indication on the possibility of using this marker as a diagnostic but not a prognostic test. The importance of LOX in cancer came from many reasons; at first, its ability to modulated tumor behavior in part through ECM remodeling by producing a stiff micro environment. In solid tumors , there is evident pervasive growth of dense fibrous tissue, featured with accumulation of fibroblasts and excess and/or disordered ECM deposition a phenomena named desmoplasia which in clinical practice attracted much attention [35]. On the other hand, solid tumors are characterized by hypoxic condition within the tumor leading to activation of signaling pathways which initiate tumor cell invasion, migration, adhesion and subsequent angiogenesis which is a critical process in the development of metastatic tumor phenotype [36], of interest is the recent discovery which identifies LOX as an important regulator of hypoxia induced metastasis [36]. IL-8 which is an inflammatory chemokine originally discovered as chemo tactic factor for leukocytes, recently, it has been shown to play a critical role in cancer invasion, angiogenesis and metastasis[37] in that, it contribute to human cancer in an autocrine and paracrine manner, multiple mechanisms are involved in IL-8 action , including direct effect or angiogenesis , tumor cell growth and migration and indirect effects vice attracting host infiltration cells [38]. IL-8 have been evaluated as a pro-oncogenic effecter in various types of human cancers, including leukemia, astrocytoma, melanoma, breast cancer, ovarian cancer, lung cancer, prostate cancer, colon cancer, urinary system cancer, gastric & pancreatic cancer [39]. The results of the present study revealed a significantly higher IL-8 among bladder cancer patients as compared with controls, also significantly higher level observed as comparing high grade and low grade patients with that of controls. Yet , there was no statistically difference between high and low grade IL-8 serum levels ($p=0.386$), these results came in accordance with previous study done by Mahdi N.K. *et al*., 2013, which demonstrated a higher IL-8 levels in patients with Bladder cancer as compared with control [40]. Nitric oxide a short lived pleotropic molecule with multiple biologic function, since its discovery in the last 1980s, NO has been thought to play a role as a signaling molecule in many parts of the organism, in immunological and defense mechanism [41], and in carcinogenesis its role is complex and has both facilitatory and inhibitory effect on the tumor

growth [42,43], it has been reported that continuous NO production may be involved in the inflammatory processes associated with appearance of many human malignancies including bladder cancer [44] and its level is significantly increased in BC patients [43] , other study done by Eijan *et al*., 2002 showed elevated nitric oxide level in the urine of patients with BC [45]. Other studies didn't observe any significant differences in serum NO levels as compared with controls [46].

In the present study , a significant difference in serum NO level of patients with BC was observed as compared with that of controls in addition to that, high grade TBC patients show significant increase in NO level as compared with low grade TBC patients and control groups (p value of 0.011 and 0.0001, respectively), meanwhile, there was no significant difference between low grade level and control groups such results came in accordance with a previous studies by kilic *et al*., 2006 and Gecit *et al*., 2012 which demonstrated sign of higher NO level in BC compared with control and could suggest a conductive role of NO in tumor progression and metastasis [43,47]. In conclusion, LOX and IL-8 could be considered as promising marker for bladder cancer at least for diagnosis, nevertheless, large scale studies are needed to substantiate the accuracy and outcome of their usefulness and effectiveness as biomarkers for TBC. On the other hand , NO seems to play an important role in BC yet further studies using a variety of tumor markers including molecular genetics techniques will be necessary to elucidate how and to what extent NO and BC of different grades and stages are functionally interrelated .

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