Lysyl Oxidase, Hypoxia Inducible Factor –1 alpha and Inducible Nitric Oxide Synthase as Possible Biological Markers in Bladder Cancer

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Introduction

Bladder Cancer (BC) is the commonest malignancy of the urinary tract ; it is a heterogeneous disease, with 70% of patients presenting with superficial tumors, that tend to recur and are generally not life threatening, About 30% of BC cases presenting as muscle-invasive disease associated with a high risk of death from distant metastasis. Several molecules and pathways have been identified to be linked to the pathogenesis of bladder cancer; while major efforts was directed to characterize molecular alterations to improve disease prognostication, only a few biomarkers of potential clinical relevance have been identified.

The hallmark of cancer comprise of six biological capabilities acquired during the multistep development of human tumors, forming an organizing principle for rationalizing the complexities and diversity of neoplastic disease. They include sustaining proliferative signaling, evading growth suppressor, enabling replicative immortality and resisting cell death. In the past decades, majority efforts of cancer research have focused on the functional consequences of oncogene and tumor suppressor gene mutations. However tumors are complex tissues composed of multiple distinct cell types and the extracellular matrix (ECM), these participate in heterotypic interactions with one another in the genesis of tumors by influenceing the tumor microenvironment . Features of the tumor microenvironment that are significantly different from normal tissue are the reduction in oxygen pressure (PO_2) which is indicated by hypoxia and pH reduction (acidosis) . Two important means by which cancer cells adapt to their microenvironment, by reprogramming cellular glucose / energy metabolism to use pathways that generate ATP in the absence of oxygen, and by stimulating angiogenesis to increase oxygen delivery .

The microenvironment of solid tumors, is exposed to low oxygen tension (hypoxia), a key regulator of the cellular oxygen - signaling pathway is the Hypoxia Inducible Factor-1α (HIF-1α) , a transcription factor that facilitate adaptation to oxygen deprivation by regulating the expression of genes that control cell metabolism, angiogenesis, cell proliferation and apoptosis. On the other hand, lysyl oxidase enzyme (LOX), an important modulator of extracellular matrix, is one critical HIF-1α targets having an important role in the tumor development and progression. Furthermore, hypoxia associated with the maintained inflammatory state, resulted in activation of inducible nitric oxide synthase (iNOS), resulting in nitric oxide (NO) release, a further interesting affecter in the tumor microenvironment. Hypoxia-inducible factors (HIFs) are important angiogenic molecules, as they control a cell's response to a hypoxic stress. Over expression of hypoxia inducible factor -1 alpha (HIF-1α) has been demonstrated in many types of cancer with poor prognosis; its prognostic value is even more significant when combined with p53 .

Aim of the study

The present study is aimed to estimate the possible contribution of LOX (Lysyl Oxidase), HIF-1α (Hypoxia Inducible Factor -1 alpha) , and iNOS (Inducible Nitric Oxide Synthase) isoform , to transitional bladder carcinoma (TBC) , by studying both tissue expression bladder cancer patients with different grades and comparing results to that of controls. In addition to evaluating the correlation between studied markers.

Methodology

This clinical study was conducted at AL-Shaheed Gazi AL-Hariri Hospital for Specialist Surgery / Baghdad Medical City.The study was performed on 2 study groups :

1- Disease Study Group: consist of 46 patients with histologically confirmed TBC , this further subdivided into:

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

2- Control Study Group: This group is also divided into two subgroups :

- **A) Disease control study group (designated as tissue control group): who had no documented malignancy.**
- **B) Control Group (designated as healthy control group)**

Table I : Demonstrates the demographic description among different studied groups :

Study group	Age (years) Mean ± SE	Sex
High grade TBC n=26	67.73±1.949	69.2 % ♂ 30.8% ♀
Low grade TBC n=20	63.35±2.903	80.0 % ♂ 20.0 % ♀
Tissue Controls n=20	64.10±2.034	75.0 % ♂ 25.0 % ♀
Healthy controls n=20	63.10±2.118	75.0 % ♂ 25.0 % ♀

For each subject of the disease study group and tissue control group, a transurethral resection biopsy were taken from the apparent lesions, from which, paraffin-embedded tissue blocks then prepared , stored at room temperature until time of immunohistochemical analysis, these included the following:

1. **Investigating the expression of HIF-1α in each tissue embedded block.**
2. **Investigating the expression of LOX in each paraffin embedded block.**
3. **Investigating the expression of iNOS in each paraffin embedded block.**

Analysis of data was carried out using the available statistical package of SPSS-20. Data were presented in simple measures of frequency, percentage, mean, standard error, and range . Student t-test was used for testing the significant difference between two groups, and ANOVA among three. Person's correlation was calculated for the correlation between two quantitative variables with its t-test for testing the significance of correlation. The correlation coefficient value (r) either positive (direct correlation) or negative (inverse correlation). Statistical significance was considered whenever the P value was equal or less than 0.05.

Results and Discussion

1- Case Control Difference in the Studied Serum Parameters Stratified by Different Grades of TBC Patients

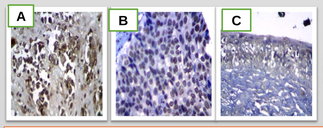
Parameter	High grade TBC (n=26)	Low grade TBC (n=20)	Healthy Controls (n=20	P value ANOVA
LOX (ng/ml)	2.016±0.241a	2.089±0.291a	0.717±0.122	0.0001
TGF-β(ng/ml)	33.342±1.494 a	30.243±2.136 a	22.110±2.236	0.0001
TNF-α (pg/ml)	17.857±1.392a,b	11.173±0.866 a	6.383±0.556	0.0001
IL-8 (pg/ ml)	73.297±8.093a	63.851±6.337 a	39.125±1.620	0.0011
NO (µmol/L)	48.889±3.663ab	34.575±3.820	27.963±3.394	0.0001

a Significant difference as compared to control group using Student t- test (P< 0.05), b Significant difference between high & low grade groups using Student t- test (P< 0.05) , Significant differences among different groups are considered using ANOVA test whenever (P< 0.05).

2- The Description of HIF-1α Immunoreactivity Scoring Components among the Different Studied Groups

HIF-1αIRS Components		TBC (Total)		High grade TBC		Low grade TBC		Tissue Controls	
		n	%	n	%	n	%	n	%
Staining Intensity	0	11	23.9	-	-	11	55.0	20	100.0
	Low	8	17.4	4	15.4	4	20.0	-	-
	Moderate	12	26.1	7	26.9	5	25.0	-	-
	High	15	32.6	15	57.7	-	-	-	-
Percent of positive cells (Nuclear Expression)		38.348±5.482		64.615±5.505		4.200±1.815		0	
Percent of positive cells (Cytoplasmic Expression)		18.891±3.531		30.385±5.098		3.950±1.636		0	
IRS/ N		5.630±0.847		9.423±0.969		0.700±0.206		0	
Expression Type	0	12	26.1	-	-	12	60.0	20	100.0
	Low	15	32.6	7	26.9	8	40.0	-	-
	High	19	41.3	19	73.1	-	-	-	-

3- Immunohistochemical Analysis of HIF-1α Nuclear Expression in TBC & control tissues :

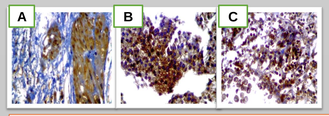


A : High grade TBC
B : Low Grade TBC
C: tissue control

4- The Description of LOX Immunoreactivity Scoring Components in the Different Studied Groups :

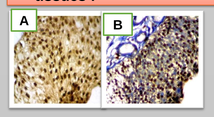
LOX IRS Components		TBC (Total)		High grade TBC		Low grade TBC		Tissue Controls	
		n	%	n	%	n	%	n	%
Staining Intensity	0	-	-	-	-	-	-	-	-
	Low	5	10.9	-	-	5	25.0	19	95.0
	Moderate	16	34.8	6	23.1	10	50.0	1	5.0
	High	25	54.3	20	76.9	5	25.0	-	-
Percent of positive cells (Nuclear Expression)		55.000±3.554		63.077±4.633		44.500±4.672		0	
Percent of positive cells Cytoplasmic Expression		65.217±2.793		73.462±2.414		54.500±4.672		22.500±1.230	
IRS/N		7.804±0.618		9.885±0.751		5.100±0.661		0	
IRS/C		8.652±0.578		10.808±0.474		5.850±0.841		1.300±0.193	
Expression Type/N	0	-	-	-	-	-	-	20	100.0
	Low	21	45.7	5	19.2	16	80.0	-	-
	High	25	54.3	21	80.8	4	20.0	-	-
Expression Type/C	0	-	-	-	-	-	-	1	5.0
	Low	13	28.3	-	-	13	65.0	19	95.0
	High	33	71.7	26	100.0	7	35.0	-	-

5- Immunohistochemical Analysis of LOX Cytoplasmic Expression in TBC & control tissues :



A : High grade TBC
B : Low Grade TBC
C: tissue control

6- Immunohistochemical Analysis of LOX Nuclear Expression in TBC tissues :

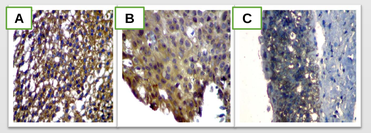


A : High grade TBC
B : Low Grade TBC

7- The Description of iNOS Immunoreactivity Scoring Components in the Different Studied Groups :

iNOS IRS Components		TBC (Total)		High grade TBC		Low grade TBC		Tissue Controls	
		n	%	n	%	n	%	n	%
Staining Intensity	0	-	-	0	0.0	0	0.0	16	80.0
	Low	15	32.6	5	19.2	10	50.0	4	20.0
	Moderate	21	45.7	13	50.0	8	40.0	-	-
	High	10	21.7	8	30.8	2	10.0	-	-
Percent of positive cells(Nuclear Expression)		0		0		0		0	
Percent of positive cells(Cytoplasmic Expression)		78.478±2.014		76.154±2.991		81.500±2.43		2.000±0.918	
IRS/C		8.109±0.507		8.961±0.711		7.000±0.649		0.200±0.092	
Expression Type	0	-	-	-	-	-	-	16	80.0
	Low	17	37.0	6	23.1	11	55.0	4	20.0
	High	29	63.0	20	76.9	9	45.0	-	-

8- Immunohistochemical Analysis of iNOS Cytoplasmic Expression in TBC & control tissues :



A : High grade TBC
B : Low Grade TBC
C: tissue control

9- Tissue Immunoreactivity Scoring of Studied Parameters in Different Grades of Transitional Bladder Carcinoma (TBC) Patients compared to Controls :

Parameter	High grade TBC (n=26)	Low grade TBC (n=20)	Tissue Controls (n=20)	P value ANOVA
HIF-1α /N	9.423±0.969[b]	0.700±0.206	0	0.0001
LOX /C	10.808±0.474[a,b]	5.850±0.841[a]	1.300±0.193	0.0001
LOX /N	9.885±0.751[b]	5.100±0.661	0	0.0001
iNOS /C	8.961±0.711[a]	7.000±0.649[a]	0.200±0.092	0.0001

Conclusion

1. **Lysyl oxidase, HIF-1α, and iNOS could be considered as promising biomarkers in TBC.**
2. **Tissue expression of LOX , show an interesting possible rols in TBC, it can discriminate between different grades , correlates with hypoxia state and inflammation, thus giving the possibility of using it as prognostic or follow up marker.**
3. **Hypoxia have a positive link to TBC, by its HIF-1α tissue expression. Interesting link is observed between HIF-1α and both LOX and iNOS, suggesting important roles in regulating angiogenesis and cancer metastasis**

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