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Research Article

Role of PD-1 and TIM-3 expression in urinary bladder cancer

Hiba Qassem Ali[1], Nibras S. Al-Ammar[1], Murtadha Almusafer[2]

[1]*Microbiology Department, College of Medicine, University of Basrah*
[2]*Urology Consultant, FICMS, FRCS (Glasgow), FACS, Iraq*

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Abstract

Bladder cancers are a tumor cluster which considered to be an immunologically conserved. Programmed death 1 (PD-1), and T cell immunoglobulin mucin-3 (TIM-3) has been shown to be expressed by T lymphocytes and tumor cells in the tumor microenvironment in several malignancies. Many studies observed an important role for both PD-1 and TIM-3 expression in patients with cancer but still, the clinicopathological influence of these immunological markers has not yet been confirmed. In the present study, gene expression assessment by real-time polymerase chain reaction (qRT-PCR) was performed using paired normal and cancerous bladder cancer tissue to investigate gene expression.

Keywords Bladder cancers, Immunological markers**,** Microenvironment, Malignancies

Introduction

Bladder cancer is the fourth most common cancer in Iraq and the most common cancer in adult males (Abood et al.,2020) Immune checkpoint blocking mechanism by using monoclonal antibodies has developed to be a hopeful immune therapy for advanced urothelial cancer. Bladder cancer has been found to have a relatively conserved immune response. PD-1, a cell surface protein their major ligands (PD-1and PD-2), expressed by activated T cells, and significantly highly expressed by tumor-infiltrating T lymphocytes (Pardoll, 2012), also expressed by activated B cells, monocytes, and natural killer cells, it has been found to modulate immunity in a T cell-independent pattern (Hellmann et al., 2018). The monoclonal antibodies targeting the Anti-(PD-1) have exhibited a strong anti-tumor effect and a controllable outline of safety in many advanced cancers, like urothelial cancer. The activity of the (PD-1) binding to PD-L1 play a vital function in tumor immunological responses in both mouse and human. (Addeo and Banna, 2018). The ligation activity of PD-1 to the PD-L1 mediates the regulation of in situ T cell-mediated immune responses and tolerance. This pathway was found to suppress T cell activation (Hamid et al., 2019).

TCR stimulation induces (PD-1) expression in naive T-cells. This transitory expression would be declined if the TCR signaling was reduced while it would be sustained when chronically activated with a persistent target epitope as in malignancy. When PD-1-(PD-L1 and PD-L2) ligation occurs, this will result in impaired TCR signaling and CD28 co-stimulation. The tumor-specific T cell's constitutive expression of PD-1 at first has been defined to be related to the expression of other inhibitory immune checkpoints like LAG-3, TIGIT, or Tim-3, as it binds to its ligand (PD-L1) this will inhibit T-cell activities and tumor immune evasion as well, the PD-L1 expressed within tumors microenvironment by immune infiltrating cells and tumor cells as well (Ahmadzadeh et al.,2008). The Constitutive expression of PD1 gives a type of immunological tolerance to persistent activation, resulting in physiological restraint of immune responses and a reduction in autoimmune symptoms. The tumor has therefore taken over this system. This mechanism is therefore captured by the cells of the tumor to promote peripheral tolerance (Gros et al., 2014). Recently, many therapeutic studies targeting PD-1 with anti-PD-1 antibodies found to be valuable for patients with urothelial carcinoma. tumor immune escape, on the other hand, is an energetic mechanism including the creation of an immunosuppressed tumor microenvironment, in which the (PD-1/PD-L1)

signaling pathway has a complex mechanism. The monoclonal antibodies targeting PD-1 have changed patient care with a rising number of solid tumors, resulting in exceptional treatment responses in a wide range of advanced-stage tumors. It has been demonstrated that PD-1 level of expression only can't distinguish between fatigued and active T lymphocytes, which are the product of unique genetic programming governed by TCR signaling intensity and the microenvironment (Wang et al., 2015; Singer et al., 2016). Tim-3 receptors one of the immune checkpoints acts as an inhibitor for type 1 immunity Both immune and tumor cells express TIM-3 in the tumor microenvironment. The tumor infiltrated lymphocytes CD8+ T cells, T-helper1 cells, regulatory T cells, Th17, and innate immune cells all express TIM-3 (Sakuishi et al., 2010). Galectin-9 is the ligand of TIM-3 when bound together, triggering apoptosis and weakening the cellular immune responses in TIM3-expressing T cells. TIM-3 can induce immune tolerance when it binds to its ligands, and also it inhibits NK cell-mediated cytotoxicity and Th1 and Th17 responses, resulting in immune tolerance (Saleh R et al., 2020; Ndhlovu et al., 2012).

TIM-3 binding to galectin-9 causes the death of Th1 cells and subsequently loss of interferon-Ɣ produced by these cells. The CD8+ and CD4+ T-cells which are found to be highly expressing TIM-3 become less reactive to antigen stimulation. During chronic inflammation, TIM-3 receptors are found to have a vital effect on the exhaustion of the T cells as in the case of cancer (Sakuishi et al., 2010). Tim-3^{+} Foxp3^{+} Treg cells were found to be regulated by TIM-3 receptors (Zhong et al., 2021) as in the case of ovarian cancer, colorectal cancer, and cervical cancer the amount of T-cells expressing TIM-3 Foxp3^{+} CD4^{+} found to be high (Olguin et al., 2018, Guo et al., 2013). In prostatic cancer, Tim-3 expression was found to be greater in the tumor microenvironment than their expression in neighboring non-cancerous tissues (Piao et al., 2013).

The TIM-3 expression in tumors, specifically those expressed on immune cells can act as a biological marker for cancer state prediction. The CD8+ and CD4+ T cell TIM-3 expression was shown to be elevated in individuals with hepatocellular carcinoma due to the hepatitis B virus. Tim-3+ T cells were replicative senescent, expressing senescence markers on the surface and in the genome (Hakemi et al., 2020). The tumor immunopathogenesis might be affected by TIM-3 expression level (Cong et al., 2020). The failure of PD-1 blockade therapy was found to be associated with TIM-3 expression therapy and subsequent disease control in preclinical models (Barrueto et al., 2020).

There is a high expression of TIM-3 receptors on cytotoxic CD8+ T lymphocytes in the tumor microenvironment (Fourcade *et al.*,2010). In colonic cancer cytotoxic CD8+ T cells which express Tim-3 and PD-1 together make up the largest percentage of T- cells, while those who express PD-1 receptors only or those express none of Tim-3 and PD-1 make up a lesser percentage (Sakuishi et al., 2010). The Tim-3+ PD-1+ T-cell in the tumor microenvironment was found to be the weakest and malfunctioned, which was illustrated by diminished multiplication and cytokine generation (Sakuishi et al., 2013). Bladder cancer microenvironment was found to have a high expression of TIM-3 and a significant association was observed between the expression level and advanced pathological grades and tumor stage (Yang et al., 2015).

Methods

Patients and Samples

Autologous tissue (normal and tumor) samples were taken from patients 35 undergoing tumor resection surgery for primary non-recurrent urinary bladder cancer at the Basra Teaching Hospital after written informed consent. Follow up period for assessment of disease recurrence was 1-2 years. Excluding patients with recurrent tumors or those who are on immune-modulating therapy. Written consent was obtained from all patients. Tissue samples were transferred immediately to the molecular laboratory at Al Bayan group for the advanced lab. Diagnostic, the expression of PD-1 and TIM-3 was assessed by RT-qPCR.

Real time PCR for RNA expression assessment of both PD-1 and TIM-3 immune checkpoints

RNA extraction

Tissue homogenization

1 ml of Wizol™ Reagent from (Wizbiosolution, Republic of Korea) has been added to 50 -100mg of tissue (tumor and normal).

Homogenization of both tumor and normal tissue samples by a homogenizer.

Total RNA extracted as described in the manufactured kit.

cDNA synthesis

The kit used for cDNA synthesis from WizScript™ RT FDmix (Hexamer) from (Wizbiosolution, Republic of Korea). RT FDmix (Hexamer) tube placed on PCR tube rack then the reaction component added to the RT FDmix (Hexamer) tube which prepared by adding < 5 µg of Template RNA added, then complete the volume by adding RNase free water to 20 µl.

The RNA template was amplified by a thermal cycler and programmed as the following

(25°C / 10 min.),
(42°C / 30 min.),
(85°C / 5 min.),
(4°C / Hold).

Synthesized cDNA is immediately used as a template for PCR or stored at-20°C.

Assessment of PD-1 and TIM-3 expression qRT-PCR

SYBR Green Master mix from Promega USA was added as the manufactured kit.

mRNA	Primer sequence
PD1	Forward-(5′-CCAGGATGGTTCTTAGACTCCC-3′), reverse-(5′-TTTAGCACGAAGCTCTCCGAT-3′)
TIM-3	Forward-(5′-GCTACTACTTACAAGGTCCTCAG-3′) reverse-(5′-ATTCACATCCCTTTCATCAGTC-3′)
GAPDH	Forward-(5′-GTGGACATCCGCAAAGAC-3′) reverse-(5′-AAAGGGTGTAACGCAACTA-3′)

Statistical analysis

PSS software (2020) was used for statistical analysis. Wilcoxon signed rank; Kruskal Wallis Test**s** used for assessment of results**:** We present the value as the mean ± standard deviation. The statistically significant difference was considered at P<0.05. Gene expression of PD-1 and TIM-3 was assessed as $2^{-\Delta\Delta Ct}$.

Results

The major characteristics of the patients

The general characteristics of the patients included in this study are shown in Table 1. Their age range was (17-78) years with a mean age of about, (56%) of them between (60-70) years old. Males represent about 85% of the cases in this study while females were about (14%), and about (48.6%) of them were smokers.

Table 1. Patients character

No. of Patients		35
Age (yr)		35-82
Gender	Male	30(85.7%)
	Female	5(14.3%)
Smocking	Yes	17(48.6%)
	no	18(51.4%)
Pathological Grade	High	8(23%)
	Low	27(77%)
Tumor size	T1	13(37%)
	>T1	22(62%)
Pathological Stage	I	14(42%)
	II	13(31%)
	III	8(22%)

PD-1 expression in the tumor was higher (1.82±2.4) than their expression by the autologous normal tissue (1.22±1.68) but this difference is statistically not significant P value (0.342) Table 2. At the same time, there is a significantly higher expression of PD-1 in high-grade (4.31±2.78) tumors than in low-grade tumors (0.62±1.26) P value (0.027) Table 3.

Table 2. Comparison of PD-1 and TIM-3 expression between tumor and autologous normal tissue

PD-1	Tumor	1.82±2.4	0.062
	Normal	1.22±1.68	
TIM-3	Tumor	1.327±1.65	0.044*
	Normal	0.53±0.67	

- ***Related sample Wilcoxon signed rank test,***
- ****Significant difference at 0.05 level***

TIM-3 expression is significantly higher in bladder cancer higher stages (stage I (0.35± 0.62), stage II (0.64± 0.57), stage III (2.7± 1.79)), P value (0.009). according to the disease grade, the TIM-3 expression was also significantly higher in the high grade (3.96 ± 2.9) than in the low-grade tumor (0.62± 1.1) P value (0.002) Table 3.

Table 3. PD-1 and TIM-3 tumor tissue expression correlation with disease stage and grade

PD-1	Stage	I	1.191±2.161	0.342
		II	2.51±2.321	
		III	3.54 ± 4.29	
	Grade	Low	0.62±1.26	0.027*
		High	4.31±2.78	
TIM-3	Stage	I	0.35± 0.62	0.009*
		II	0.64± 0.57	
		III	2.7± 1.79	
	Grade	Low	0.62± 1.1	0.002*
		High	3.96 ± 2.9	

- **Kruskal Wallis Test *level of significance ≤ 0.05.**

According to disease recurrence both PD-1 and TIM-3 expression, it is significantly higher in urinary bladder cancer patients who develop disease recurrence during the first year after the diagnosis than in patients who did not (PD-1(1.021±1.78) P value 0.04, TIM-3 (0.98±1.62) P value 0.03) Table 4.

Table 4. Expression of PD-1 and TIM-3 in correlation to disease recurrence

Molecular Marker	PD-1	TIM-3
No recurrence	1.021±1.78	0.98±1.62
Recurrence	3.69±2.79	2.36±1.38
P value	0.041	0.033

- **Kruskal Wallis Test *level of significance ≤ 0.05**

Discussion

Immune checkpoints monoclonal antibody targeting PD-1 or TIM-3 has made a revolution in the treatment of cancer by

enhancing anticancer immune responses (Korman et al., 2006; Pardoll et al., 2012). The expression of PD-1 along with its ligand (PD-L1) by various tumors acts as a mechanism for achieving immune evasion and making their environment immune tolerant (Blank et al.,2004; Keir et al.,2008); therefore PD-1/PD-L1 binding inhibition found to be an important strategy to retain and enhancing cancer immune surveillance by activating adaptive immune cell antitumor responses (Flemming, 2012). Yang et al., 2015 found a significantly higher expression of PD-1 in tumors than in normal urinary bladder tissue, at the same time he suggested PD-1 expression as an independent disease-free survival predictor (Yang et al., 2015). While this study shows no significant difference in the expression of PD-1 between the tumor and normal tissue or the pathological stage of urinary bladder cancer patients, this becomes consistent with the observations of Kawahara et al., 2018. At the same time. Kawahara et al found the expression of PD-1 in the tumor microenvironment with high grades was significantly higher than in low-grade tumors (Kawahara et al., 2018), so this study agreed with this. Since the PD-L1 is found to be expressed within the tumors microenvironment by immune infiltrating cells and tumor cells as well (Ahmadzadeh et al.,2009; Fourcade et al., 2009; Fourcade et al., 2010), in presence of higher PD-1 expression this will result in impaired TCR signaling and CD28 co-stimulation, thus microenvironments of urinary bladder tumors with higher grades became highly immune suppressed than low-grade tumors so this makes us suggest selecting high-risk patients by coupling PD-1 expression with high-grade tumors. But as PD-1 expression is not affected by tumor stage or tumor recurrence, therefore the finding of this study confirms Kawahara, 2015 opinion about the insufficient use of PD-1 expression as an independent molecular biomarker for predicting disease progression or recurrence (Kawahara et al., 2018). In view of the fact that the PD-L1 was found to be expressed on the cell surface by many tumors (Pardoll et al., 2012; Zou & Chen et al., 2008; Dong et al., 2002) and its expression was evaluated as a mediator of disease stage progression, so the assessment of PD-1 role in tumor growth and progression without integrating their ligands may result in a shortage in the real facts of the PD-1 role without the PD-L1.

TIM-3 receptors induce T cell exhaustion and immune tolerance (Saleh et al., 2020; Ndhlovu et al., 2012), they also have been found not only to be expressed by T cells microenvironment of the tumor but also expressed by a cell of the tumor as well (Kikushige et al., 2015). In prostatic cancer Tim-3 expression was found to be greater in the tumor microenvironment than their expression in neighboring non-cancerous tissues (Wu et al., 2017), this agrees with this study as we found TIM-3 expression by tumor tissue was significantly higher than their expression by normal tissue of the urinary bladder cancer, also there was a significant difference in TIM-3 expression between high grade and low-grade tumors, and its expression positively associated with tumor stage, these finding consistent with Yang et al., 2015 who have found that bladder cancer microenvironment highly expressing TIM-3, and its expression was positively associated advanced pathological grades and tumor stage (Yang et al., 2015) . These findings support Yang et al. opinion about the role of the TIM-3 assessment as an independent predictor for disease progression in urinary bladder cancer patients.

On the other hand, it has been found the (PD-1+ TIM-3+) CD8+ TIL represents the most abundant population and also implies the most exhausted and dysfunctional proportion of TILs in multiple solid tumors (Sakuishi et al., 2010). In the same context, a study of lung cancer-bearing mouse models treated with an anti-PD-1 monoclonal antibody revealed other immune checkpoints have been upregulated particularly Tim-3 on therapeutic antibody-bound TILs (Koyama et al., 2016). In this study, we have identified that the PD-1/TIM-3 co-expression was positively associated with urinary bladder cancer recurrence. The preceding findings reflect the importance of assessing PD-1 and TIM-3 co-expression as a predictor for urinary bladder cancer recurrence.

In the same context in presence of resistance to the anti-PD-1 monoclonal antibody as therapy, it would be useful to choose the target who are at great risk of recurrence and may not make benefit from treatment other than combining both anti-PD-1 and anti-TIM-3. Since both PD-1 and TIM-3 have a positive association with high-grade tumors, therefore they have an important role in tumor immunopathogenesis, and as they are both associated with T-cell exhaustion in the tumor microenvironment, particularly CD8+ this makes the synergetic assessment of both Tim-3 and PD-1 as a predictor for tumor growth disease progression and treatment response.

Conclusion

Immune checkpoints expression reflects the immunological status of the tumor microenvironment particularly immune tolerance and tumor immune escape and understanding their role in urinary bladder tumor future disease prediction and prognosis, this grants these receptors their vital role in assessing urinary bladder cancer patients that can act as a predictor for disease progression and therapeutic response like immune checkpoints inhibitors particularly anti-PD-1 and anti-TIM-3 since this immune therapy as they are found to be highly effective, particularly in advanced stages they also highly expensive.

Conflict of Interest

The author hereby declares no conflict of interest.

Consent for publication

The author declares that the work has consent for publication

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