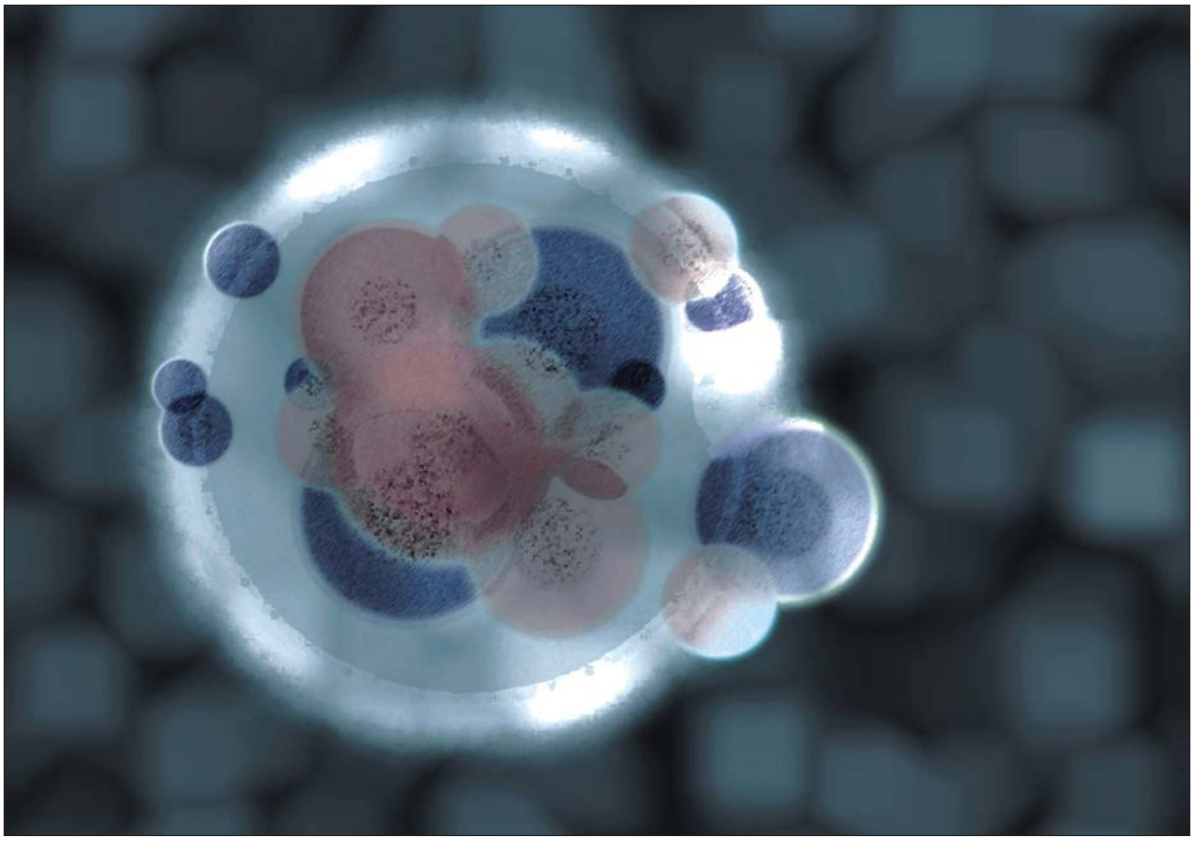

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Research Article
CD14 Expression and Microbial Infection in Bladder Tumours

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Abstract

Background and Objective: CD14 is a molecule involved in non-specific immunity that plays important roles in immune cell activation and signaling in response to bacterial cell wall derived components. The current study aimed to determine the diversity of bacterial infection in urinary bladder tumours and studying the immunoexpression of CD14 in bladder tissues associated with bacterial infection. **Materials and Methods:** In total, 65 patients with bladder tumours with urine and paraffin embedded tissue samples were enrolled in the study, among these patients, 50 had bladder carcinoma (BC) and 15 had benign tumours (BT). Specific culture media and biochemical tests were used to isolate and identify bacterial species and immunohistochemistry (IHC) was used to detect CD14 tissue expression in both groups. Chi-square test was used to compare the difference between percentages of this study by using SAS. **Results:** The results showed that 37 out of 50 BC patients were positive for gram-negative bacteria, 3 out of 50 patients were positive for gram-positive bacteria and 10 out of 50 patients were negative growth. All BT patients showed presence of gram-negative bacteria. IHC staining showed that $CD14^+$ was significantly over expressed in BC patients compared with BT patients (82% vs. 46.7%, $p \leq 0.01$). CD14 expression was also high in gram-negative bacteria infected bladder tissues in patients with both BC and BT and its expression was significantly correlated with this type of bacterial infection ($p \leq 0.01$). **Conclusion:** A significant association between CD14 expression and bacterial infected tissues was found and concluded that CD14 activation driven by microbial inflammation probably contributes to bladder cancer.

Key words: Bacterial infection, CD14, innate immunity, bladder tumor, inflammation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Microbial infection is associated with the development of many different types of cancers[1]. Studies have shown a link between bacterial infection and cancer and indicated that persistent infection leads to chronic infection[2]. The transcription factor NF-κB is considered one of the key molecules connecting chronic inflammation and cancer[3]. Infection with gram-negative bacilli like *Escherichia coli* might play a role in bladder cancer development and this effect may be mediated by activation of the NF-κB pathway. NF-κB activation enhances the expression of more than 200 genes that have been reported to suppress apoptosis and encourage cell transformation, proliferation, invasion, metastasis and resistance to chemotherapy, radio-therapy and/or inflammation[4]. Bladder epithelial cells can be activated by lipopolysaccharide (LPS) which is a major component of the outer membrane of gram-negative bacteria. Bladder epithelial cells use pathogen recognition receptors such as Toll-Like receptor 4(TLR-4) and CD14 for LPS responses and LPS induced signaling. CD14 is a LPS-binding receptor[5,6]. Gram-positive bacterial cells employ CD14 for immune recognition. Peptidoglycan (PGN) and lipoteichoic acid have been demonstrated to activate macrophages in a CD14 dependent manner[7,8]. Considering these results, this study aimed to identify the diversity of bacterial infection in bladder tissues of Iraqi patients and study the relation between bacterial infection and CD14 protein expression in bladder tumour tissues.

MATERIALS AND METHODS

Urine and tissue samples were collected from 65 patients with urinary bladder tumours at AL-Yarmook Teaching Hospital and Gazii AL-Harriri Hospital for specialist surgeries within the period from January-June 2012, out of these, 50 patients had bladder cancer and 15 had benign bladder tumours. Urine samples were collected prior to laparoscopy or surgery. The diversity of bacterial infections in bladder tumors was investigated by culturing the patient urine samples. Enriched and differential media along with biochemical tests were used to isolate and identify the bacterial species. After overnight incubation, bacterial colonies were thoroughly examined. The bacterial identification was confirmed by sub-culturing the colonies from MacConkey's and nutrient agar (Himedia Co., India) plates to the API 20 microtubes system (BioMerieux, France). Tissue biopsies from cases of bladder cancer and benign tumors as controls were fixed in 10% buffered formalin, embedded in paraffin wax and stained with haematoxylin and eosin. Immunohistochemistry was used to detect CD14 expression in the tissues biopsies from both groups. Novocastra™ Novolink™ Polymer Detection System (Leica Microsystems, Newcastle,UK) was used to detect the presence of CD14 using antibodies specific to CD14. Tissue sections were deparaffinised in xylene before boiling in a water bath containing Tris-EDTA buffer (BDH, Poole, UK) at pH 9 for antigen retrieval. Endogenous peroxidase activity was neutralised using Novocastra™ Peroxidase Block (3% hydrogen peroxide). Novocastra™ Protein Block was then applied to avoid diffuse, non-specific background staining. The sections were then incubated with mouse monoclonal primary antibody to CD14 (dilution1:100, Leica,UK) for 1 h at room temperature after which the post-primary block was applied for 30 min. The slides were then incubated with the secondary antibody, Novolink™ polymer (anti-mouse IgG). Peroxidase activity was developed using a DAB substrate solution to produce a visible brown precipitate at the antigen site. Biopsies were then washed and counter stained with haematoxylin.

Statistical analysis: Chi-square test was used to compare the difference between percentages of this study by using Statistical Analysis System-SAS (2010). Data was considered highly significant at $p<0.01$.

RESULTS

The results of urine culture from the patient groups showed that the incidence ratio of positive urine cultures was 55/65 (84.6%) (Table 1), whereas that of negative urine cultures was 10/65 (15.4%). Positive urine cultures were detected in 40/50 (80%) of BC samples and in 15(100%) BT samples.

The most frequent causative microbial agents of urinary tract infections (UTIs) were gram-negative bacilli, with greater prevalence in bladder cancer patients than UTIs caused by gram-positive bacteria.

Escherichia coli was the most common microorganism isolated from the study subjects with UTIs. Of all the bacterial isolates, 26(40%) *E. coli* isolates were obtained from the patient groups (Table 1).

The gram-positive bacteria comprised two isolates of *Staphylococcus aureus* with a ratio of 3.07%. Table 1 showed the kinds and percentages of bacteria isolated from the patient groups.

Immunohistochemical evaluation of tumour tissues: The tissue expression of CD14 was evaluated in patients with BC

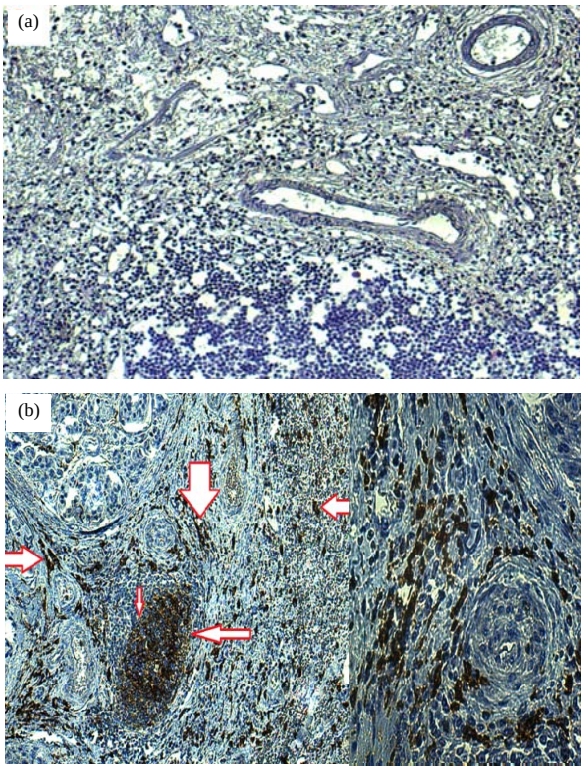

Fig. 1(a-b): Invasive transitional cell carcinoma, poorly differentiated (Grade II), (a) Showing no detectable CD14 immunostaining in bladder tissues (score 0 negative) (20X) and (b) Showing positive CD14 immunostaining (Score +++, brown) (arrows) (10X, 40X)

Table 1: Urine culture results in the investigated groups

	Isolated numbers			
Culture results	BC	BT	Total	%
E. coli	20	6	26	40.00
K. pneumonia	7	3	10	15.40
P. mirabilis	6	4	10	15.40
P. aeruginosa	4	2	6	9.23
Staphylococcus aureus	2	0	2	3.07
Streptococcus faecalis	1	0	1	1.50
No growth	10	0	10	15.40
Total number	50	15	65	100.00

Table 2: CD14 expression in bladder patient groups

		CD14 expression			p-value
Study groups		Positive	Negative	Total	χ^2-value
BC	No.	41.0	9.0	50	
	%	82.0	18.0	100	
BT	No.	7.0	8.0	15	
	%	46.7	53.3	100	
Total	No.	48.0	17.0	65	0.0062
	%	73.8	26.2	100	9.344**

**(p<0.01): Highly significant

Table 3: Frequency of CD14$^+$ cell scores in bladder patient groups

	BC		BT		Total	
CD14 score	No.	%	No.	%	No.	%
+++	22	53.6	0	0.0	22	45.8
++	12	29.3	0	0.0	12	25.0
+	5	12.2	5	71.4	10	20.8
Scatter≤10	2	4.9	2	28.6	4	8.3
Total	41	85.4	7	14.5	48	100
p-value	-	0.0024	-	0.0018	-	0.0036
χ2 -value	-	8.784**	-	10.036**		8.591**

**(p<0.01): Highly significant

Table 4: Association between CD14$^+$ expression and tumour grade

	CD14 expression			p-value
Parameter	Positive	Negative	Total	χ^2 -value
Grade of BC				
Low grade (G≤1)	11 (61.1%)	7 (38.9%)	18	0.0026
High grade (G≥2)	30 (93.8%)	2 (6.2%)	32	
Total	41 (82%)	9 (18%)	50	7.205**

**(p≤0.01): Highly significant

and BT (controls). In this study, positive immunohistochemical expression of CD14 protein was significantly higher in BC patients compared with that in BT patients (82% vs. 46.7%, p≤0.01) (Table 2). CD14 expression was identified by a positive anti-CD14 reaction, demonstrated in the lower part of Fig. 1(a-b).

In terms of scores, BC patients with the score +++ represented the highest frequency (53.6%), whereas, the score + represented the highest frequency in BT patients (71.4%). Table 3 shows the frequency distribution of CD14 IHC scores in the group subjects. Chi-square test showed and demonstrated a significant statistical difference in CD14 IHC scores in tissue samples (p = 0.0036) and between urinary bladder carcinoma and benign bladder tumours.

Regarding the tumour grade of BC, CD14 was detected in 11 out of 18 of Grade I,18 out of 19 of Grade II and 12 out of 13 of Grade III cases.

There was a highly significant association between the grade of BC and CD14 immunohistochemical expression (p≤0.01). In the present study, most of the high grade tumours showed positive immunohistochemical CD14 expression 30/32 (93.8%), whereas only 11/18 cases (61.1%) of low grade tumours showed positive immunohistochemical CD14 expression (Table 4).

CD14 expression in relation to bacterial infection: Of 50 BC patients, 37 presented with gram-negative bacterial infection, of which 32 (64%) patients were positive for CD14 immunohistochemical staining (Table 5), with an intense reaction (+++) being the most frequent score (54.1%). All BC

Table 5: Number of patients expressing CD14 in relation to the bacterial infection in bladder cancer patients

	- Ve		+Ve		NG		Total	
CD14								p-value
expression	$CD14^+$	$CD14^-$	$CD14^+$	$CD14^-$	$CD14^+$	$CD14^-$	CD14	χ^2-value
BC	32/50	5/50	3/50	0/50	6/50	4/50	50/65	0.0016
%	64	10	6	0.0	12	8	76.9	9.963**
BT	7/15	8/15	0/15	0/15	0/15	0/15	15/65	0.0072
%	46.7	53.3	0.0	0.0	0.0	0.0	23.1	8.108**
Total	39/65	13/65	3/65	0/65	6/65	4/65	65/65	0.0029
%	60	20	4.6	0	9.2	6.2	100	9.867**

**($p \leq 0.01$): Highly significant

Table 6: Percentage of CD14 expression in relation to the bacterial infection in BC patients

	N.G		-VE		+VE		Total	
CD14 score	No.	%	No.	%	No.	%	No.	%
Total no. (%)	4	40	5	13.5	0	0	9	18
Scatter≤10	1	10	1	2.7	0	0	2	4
+10-30%	2	20	3	8.1	0	0	5	10
30-50% ++	2	20	8	21.6	2	66.7	12	24
+++	1	10	20	54.1	1	33.3	22	44
Total	10	20	37	74.0	3	6	50	100

patients with gram-positive bacteria showed positive immunohistochemical staining for CD14 and a moderate reaction (++) was the most frequent score among them (66.7%) (Table 6).

DISCUSSION

In this study, the diversity of bacterial infection in bladder tumour tissues (benign and carcinoma) was found to present different types of bacteria (gram-positive and gram-negative) in urine sample cultures. The incidence ratio of positive urine cultures was higher than that of negative cultures in both groups. In urinary bladders, factors like calculi can agitate the urothelium and lead to localized chronic inflammation. Prolonged use of a urinary catheter increases the chances of carrying bacteria along the urinary tract, as well as obstructive urinary stasis, which is conducive to bacterial infection of the bladder. These factors predispose the urothelium to a state of sustained chronic inflammation and appear to increase the risk of bladder cancer[9].

The most frequent microorganisms determined as the causative agents of UTI were gram-negative bacilli, with a greater prevalence in bladder cancer patients than that of gram-positive bacteria. This was expected because most UTIs are known to be of an ascending type and are usually caused by flora from the faecal reservoir entering through the urethra into the bladder, particularly in patients with intermittent or indwelling catheters.

Escherichia coli was the most common microorganism isolated from the study subjects with UTIs. The majority of UTIs are caused by *E. coli*, which are normally present in the colon but can pass through the urethra and into the bladder, thus causing an infection. The high incidence of *E. coli* in BC patients is also compatible with the fact that *E coli* releases a cytotoxic necrotizing factor that activates Rho family signaling. This leads to activation of COX2, which is involved in several stages of tumour development, like apoptosis inhibition[2]. E*. coli* infection might thus play a role in the development of bladder cancer and this effect might be mediated by activation of the NF-κB pathway, resulting in apoptosis inhibition and increased inflammation.

This finding is in concurrence with the Iraqi data reported by Al-Mahdi[10] in which the percentage incidence of this microorganism was 42.9% and with data reported from Western countries by Ghiro *et al.*[11], however, lower incidence percentages were reported by Ashoor[12] and Al-Wadi[13] (22.3 and 28.6%, respectively).

The gram-positive bacteria *Staphylococcus aureus* was isolated in this study. *S. aureus* is a major cause of urinary tract infection among patients with urinary tract catheterization. Most of the isolates are methicillin resistant *S. aureus*. *S. aureus* bacteriuria can lead to subsequent invasive[13]. The current results correspond to those reported by Ashoor[12] (3%).

Immunohistochemical expression of CD14 was significantly higher in BC patients compared with that in BT patients (82% vs. 46.7%, $p \leq 0.01$) (Table 2). The difference in CD14 expression levels among individuals should correlate with variation in the ability to raise an inflammatory reaction[14]. These results concur with the result of Backhed *et al.*[15], who

reported CD14 expression on a bladder carcinoma cell line (T24), by confirming IHC results of CD14 expression by three different techniques: RT-PCR, Western blotting and flow cytometry. Ashoor[12], characterized the immunoexpression of CD14 in bladder tissues from both cancer and non-cancer patients, without any significant difference. However, such a result is purely dependent on the number of receptors present on the membrane as well as the quality of the antibodies and staining reagents used and can consequently be misguided.

The majority of high grade tumour cases showed positive immunohistochemical CD14 expression in comparison to low grade tumours. This is consistent with the fact that CD14 high cancer cells express higher levels of numerous inflammatory mediators and show increased tumour growth, forming larger tumours compared with those of CD14 low cells in bladder cancer[16].

The results are compatible with those of Kinouchi *et al.*[17], who found that $CD14^+$ macrophages are distributed predominantly at the invasive front of colorectal cancer tissues, rather than in normal tissues. Our results are also in agreement with the report by Krol *et al.*[18], indicating that CD14 is associated with malignancy and metastatic potential.

This study explored the association between CD14 IHC expression and bacterial infected bladder tissues. Showed that high CD14 expression was found in gram-negative bacterial infected bladder tissues of both cancerous and benign patients and that CD14expression was significantly associated with this kind of bacterial infection (Table 5). All patients with gram-positive bacterial infected bladder tissues also showed positive CD14 expression (Table 5). These results are compatible with the fact that the CD14 molecule largely contributes to the differences in the inflammatory responses activated by bacterial components like gram-negative bacterial lipopolysaccharides (LPS) and gram-positive bacterial peptidoglycans (PGN), in bladder cancer cell lines[14].

Toll-like receptors (TLRs) have been shown to play important roles in the recognition of microbial components and in the cellular signal transduction pathways that result in inflammatoryreactions[19-21]. The LPS receptor TLR4 requires other molecules including CD14 and MD-2 to recognize LPS and promote signal transduction[22]. The results of this study are in agreement with those of Li *et al.*[23], who provided novel insight regarding the mediation of *H. pylori* infection via CD14 in gastric cancer induction in humans.

CONCLUSION

CD14 is a receptor for pathogen associated molecular patterns and its presence on the surface of macrophages in tissues allows cell activation leading to inflammatory reactions, thus suggesting that CD14 activation driven by microbial inflammation probably contributes to BC.

SIGNIFICANCE STATEMENTS

This study explores the crucial role of urinary tract infections mediated via CD14 in the development and progression of bladder cancer in an Iraqi population. Thus, the early detection of urinary infection and its treatment represent a strategy targeting reduced numbers of bladder cancer cases in the future.

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