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Original Article

Systemic Interleukin-6 Response after Intravesical Instillation of Bacillus Calmette-Guérin and Mitomycin C in Superficial Bladder Cancer

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Abstract

Interleukin-6 (IL-6) is proposed to play a significant role in pathogenesis of urinary bladder cancer (UBC). This role may be influenced by chemotherapy (mitomycin C; MMC) or immunotherapy (Bacillus Calmette-Guérin; BCG). A case-control study was conducted to determine IL-6 levels in serum of newly diagnosed cases (NDC) of superficial UBC, as well as in patients treated with MMC or BCG intravesical instillation. A total sample of 111 patients (36 NDC, 45 MMC and 30 BCG) was included, as well as a control group of 107 healthy controls (HC). IL-6 was detected by enzyme-linked immunosorbent assay. Results revealed that median levels of IL-6 were significantly elevated in NDC group (15.8 pg/mL; $P<0.001$) compared to MMC and BCG groups (7.5 and 5.3 pg/mL, respectively) or HC (4.4 pg/mL); while, there were no significant differences between the latter three groups (MMC, BCG and HC). Receiver operating characteristic curve analysis revealed that IL-6 is a very good predictor of UBC in NDC group *versus* HC (area under the curve=0.885; 95% confidence interval=0.828-0.942; $P<0.001$; cut-off value=10.5 pg/mL; Youden index=0.62; sensitivity=80.6%; specificity=81.3%). Logistic regression analysis confirmed this significance and IL-6 was associated with a higher risk of UBC (odds ratio=1.18; 95% confidence interval=1.11-1.26; $P<0.001$). In conclusion, this study indicated that IL-6 level was upregulated in serum of NDC of UBC. Further, IL-6 level was restored to normal levels after intravesical instillation of MMC or BCG.

Keywords: Urinary bladder cancer; Interleukin-6; Mitomycin C; Bacillus Calmette-Guérin

1. Introduction

Urinary bladder cancer (UBC) is one of the ten most common and aggressive types of malignancy, with global estimates of 573,278 new cases and 212,536 deaths in 2020. It is four times more likely to affect males than females. In Iraq, UBC ranked seventh among other cancers with estimates of 1,359 new cases and 690 deaths. It is also four times more common in males than in females (1). Initiation of UB carcinogenesis is suggested due to interactions between environmental factors and genetic susceptibility (2). Cigarette-smoking, aromatic amines, ionizing radiation, dietary nitrites/nitrates, chlorinated hydrocarbons, polycyclic aromatic hydrocarbons, alkylating agents and *Schistosoma haematobium* are the most important exogenous risk factors included in UBC (3). These agents, in addition to their carcinogenic effects, can also disrupt the immune functions, which in turn may act as co-factors in carcinogenesis (4). In this respect, it has been indicated that exposure to environmental carcinogens is associated with an imbalance between pro-inflammatory and anti-inflammatory responses.

Thus, inflammation has been proposed as an additional risk factor for UBC (5).

Inflammation is a protective response mediated by immune and non-immune cells and can be triggered by a variety of stimuli, including pathogens, toxic compounds, damaged cells or irradiation, to promote tissue repair and recovery (6). Inflammation can become chronic if the causative agent persists or the mechanisms responsible for controlling it are broken down. In this case, genetic mutations and cell proliferation can occur, which often creates an environment favorable for the development of cancer (7). Available data indicate that that inflammation predisposes to the development of different cancers, including UBC, and promotes all stages of carcinogenesis (initiation, invasion and metastasis) (8). Additional data describe that the inflammatory response is bidirectional. On the one hand, it mediates immune response against UBC, while on the other hand, carcinogenesis can be promoted due to a prolonged inflammatory response (9). Locally and systematically produced pro-inflammatory cytokines are important mediators of these responses, and studies have described their pivotal role in induction, development and invasion of UBC (10). Interleukin (IL)-6 is one of the pro-inflammatory cytokines proposed to have a role in tumorigenic regulation of UBC (11).

IL-6 is described as a pleotropic cytokine that influences various cellular phenomena in immune and inflammatory responses, such as proliferation, differentiation, survival, and trafficking (12). Low circulatory levels of IL-6 have been reported during the normal physiological state, but these levels may increase 100-1000 times in pathological conditions, particularly during inflammation and tumorigenesis (13). IL-6 is mainly produced by monocytes and macrophages, but other immune and non-immune cells can also produce IL-6, including, T and B lymphocytes, dendritic cells, fibroblasts, keratinocytes, vascular endothelial cells and mesangial cells (14). Besides, IL-6 can be produced by malignant cells in the tumor microenvironment, and play an important role in tumor cell expansion and differentiation (15). Increased levels of IL-6 in serum and tumor site have been reported in several types of cancer (16). This increase has been correlated with a poor prognosis and decreased survival rates in UBC patients due to its effect on various aspects of tumorigenesis, including proliferation, apoptosis, angiogenesis and metastasis (17). However, the systemic levels of IL-6 may be affected by the type of therapy that was administrated. In this context, the significance of IL-6 released by bladder cancer cells following chemotherapy (mitomycin C; MMC) or immunotherapy (Bacillus Calmette-Guérin; BCG) has not been well defined (18). Therefore, this study sought to evaluate the predictive significance and role of IL-6 in UBC progression by determining the circulatory levels of this cytokine in newly diagnosed UBC patients and healthy controls (HC). Further, UBC patients who received intravesical instillation therapy (MMC or BCG) were also included in the evaluation to predict the effect of administered therapy on serum level of IL-6.

2. Materials and Methods

2.1. Participants

After obtaining the approval of the Ethics Committee at the Iraqi Ministry of Health and Environment, a case-control study was conducted from June to December 2019 on UBC patients who were admitted to the Urology Outpatient Clinic at Baghdad Teaching Hospital (Baghdad, Iraq). A cohort of 111 patients was included in the study. The median age of patients was 62 years, and there were 83 males (74.8%) and 28 females (25.2%). The disease was diagnosed by consultants and histopathologists. Included patients were older than 18 years and had histologically confirmed superficial UBC. Pregnant women and those who did not provide written consent were excluded. Depending on provided therapy, the patients were classified into three groups. The first (45 patients) received an induction course of at least six cycles of intravesical MMC (40 mg per week); the second (30

patients) was treated in a similar manner but with BCG vaccine (80 mg/ per week); the third included newly diagnosed cases (NDC; 36 patients) who did not receive therapy. Data regarding age, gender, cigarette-smoking, alcohol use, and family history of cancer (sibs, parents and grandparents) were recorded for each patient. A group of 107 healthy controls (HC) was also included in the study. They were blood donors and health personnel who had no evidence of hematuria, malignancy and infectious diseases.

2.2. Determination of IL-6 Serum Level

Five milliliters of blood were obtained from the patients during their clinic visit and at least one week after the last dose of MMC or BCG. The blood was processed to collect serum, which was kept frozen at -20 ºC until determination of IL-6 level. The human IL-6 Quantikine enzyme-linked immunosorbent assay (ELISA) kit was used to quantitate IL-6 level in serum, and instructions of manufacturer were followed (R and D Systems, Inc., USA).

2.3. Statistical Analysis

Categorical variables were given as mean and percentage frequency, and significant difference was assessed using Pearson Chi-square test. Continuous variables (age and IL-6) were tested for normality (Kolmogorov-Smirnov and Shapiro-Wilk tests). Both variables were not normally distributed and thus given as median and interquartile range (IQR: 25-75%). Significant difference between medians was assessed using Mann-Whitney *U* test. Receiver operating characteristic (ROC) curve analysis was performed to assess the predictive significance of IL-6 in UBC. Through this analysis, the area under the curve (AUC), 95% CI, cut-off value, sensitivity and specificity were estimated. The Youden index was used to optimize the cut-off value. Multinomial logistic regression analysis was applied to determine odds ratio (OR) and 95% confidence interval (CI). The analysis was either unadjusted (Model I), adjusted for age (Model II), adjusted for age and gender (Model III), adjusted for age, gender and cigarette-smoking (Model IV) or adjusted for age, gender, cigarette-smoking and alcohol use (Model V). A probability $P \leq 0.05$ was taken statistically significant. IBM SPSS Statistics 25.0 (Armonk, NY: IBM Corp) and GraphPad Prism version 8.0.0 (San Diego, California USA) were employed to performed the statistical analysis.

3. Results

3.1. Baseline Characteristic Data

As shown in table 1, the three groups of UBC (NDC, MMC and BCG) showed a median age of over 50 years (65.0 [IQR: 43.5-73.5], 62.0 [IQR: 52.0-73.0] and 57.5 [IQR: 45.0-70.0] years, respectively). These medians were significantly higher compared to HC (40.0 [IQR: 29.0-47.0] years; $P<0.001$). In fact, most patients were classified under the age group ≥ 50 years (72.2, 82.2 and 66.7%, respectively) and the difference was significant compared to HC ($P<0.001$). UBC was more common in males than in females in the three patient groups; NDC (88.9 *vs*. 11.1%), MMC (64.4 *vs*. 35.6%) and BCG (73.3 *vs*. 26.7%). Most UBC patients were current cigarette-smokers (73.0%); they had smoked at least 10 cigarettes per day for the past two years, and a proportion of patients drank alcoholic beverages (27.0%). In both cases, the difference was significant compared to HC (35.5 and 8.4%, respectively; $P<0.001$). A positive family history of UBC or other types of cancer was observed in 18.9% of patients (33.3% in NDC, 11.1% in MMC and 13.3% in BCG).

3.2. Serum Level of IL-6

Median levels of IL-6 were significantly elevated in NDC group (15.8 [IQR: 11.4-31.8] pg/mL; $P<0.001$) compared to MMC and BCG groups (7.5 [IQR: 4.0-9.6] and 5.3 [IQR: 2.9-6.8] pg/mL, respectively) or HC (4.4 [IQR: 2.3-7.7] pg/mL), while, there were no significant differences between the latter three groups (MMC, BCG and HC) (Figure 1). When the three groups of patients were stratified by characteristics in table 1, there were no significant differences in each stratum. However, among NDC group, there was a tendency for IL-6 to show elevated levels in patients

older than 50 years, females, cigarette-smokers, and patients with no family history of cancer (Table 2). ROC curve analysis revealed that IL-6 is a very good predictor of UBC in NDC group *versus* HC (AUC=0.885; 95% CI=0.828 - 0.942; *P*<0.001; cut-off value=10.5 pg/mL; Youden index=0.62; sensitivity=80.6%; specificity=81.3%) (Figure 2). Multinomial logistic regression analysis confirmed the significance of IL-6 in increasing the risk of UBC. Under the five models of analysis, the OR value exceeded 1.0 in the NDC group *versus* HC. The highest OR was recorded in models IV (OR=2.20; 95% CI=1.42-3.42; *P*<0.001) and V (OR=2.18; 95% CI=1.40- 3.41; *P*=0.001) (Table 3).

Table 1. Demographic and clinical characteristics of urinary bladder cancer patients and controls

Characteristic†		UBC therapy group			HC; N = 107	*p*
		NDC; N = 36	MMC; N = 45	BCG; N = 30		
Median age; year		**65.0 (43.5-73.5)**	**62.0 (52.0-73.0)**	**57.5 (45.0-70.0)**	**40.0 (29.0-47.0)**	**< 0.001**
Age group; year	< 50	10 (27.8)	8 (17.8)	10 (33.3)	84 (78.5)	< 0.001
	≥ 50	26 (72.2)	37 (82.2)	20 (66.7)	23 (21.5)	
Gender	Male	32 (88.9)	29 (64.4)	22 (73.3)	51 (47.7)	< 0.001
	Female	4 (11.1)	16 (35.6)	8 (26.7)	56 (52.3)	
Cigarette-smoking	Smoker	25 (69.4)	36 (80.0)	20 (66.7)	38 (35.5)	< 0.001
	Non-smoker	11 (30.6)	9 (20.0)	10 (33.3)	69 (64.5)	
Alcohol use	Yes	11 (30.6)	8 (17.8)	11 (36.7)	9 (8.4)	0.001
	No	25 (69.4)	37 (82.2)	19 (63.3)	98 (91.6)	
Family history of cancer	Yes	12 (33.3)	5 (11.1)	4 (13.3)	NA	0.026
	No	24 (66.7)	40 (88.9)	26 (86.7)	NA	

†Values were given as median and interquartile range (non-parametric variable) or number and percentage frequency (categorical variable). UBC: Urinary bladder cancer; HC: Healthy controls; NDC: Newly diagnosed cases; MMC: Mitomycin C; BCG: Bacillus Calmette–Guérin; NA: Not applicable; *p*: Probability of Kruskal-Wallis test (to compare non-parametric variables) or Pearson Chi-square test (to compare categorical variables)

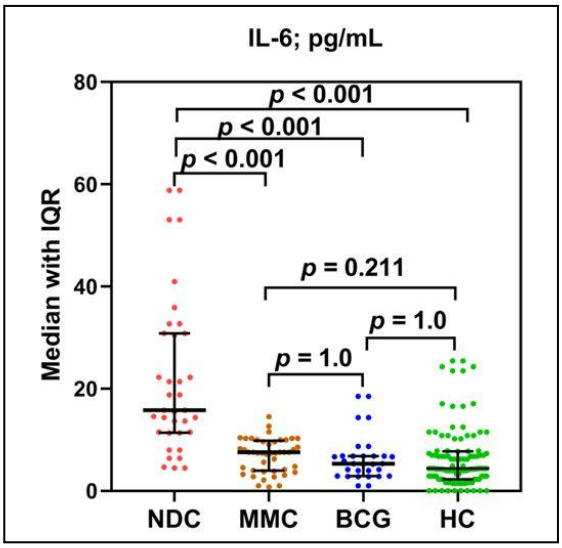

Figure 1. Scatter dot plot showing median with IQR (interquartile range) of IL-6 levels in serum of NDC (newly diagnosed cases), MMC (mitomycin C) and BCG (Bacillus Calmette–Guérin) groups of urinary bladder cancer patients and HC (healthy controls). The level was significantly increased in NDC group compared to MMC, BCG and HC groups, while there were no significant differences between MMC, BCG and HC groups. The comparisons were made using Mann-Whitney *U* test

Table 2. Median levels of IL-6 stratified by characteristics of urinary bladder cancer patients and controls

Characteristic		IL-6 median level (IQR); pg/mL		
		NDC; N = 36	**MMC; N = 45**	**BCG; N = 30**
Age group; year	< 50	12.9 (8.0-21.4)	4.5 (3.9-8.0)	6.8 (5.2-6.8)
	≥ 50	18.8 (13.7-32.7)	7.5 (4.6-10.0)	4.7 (2.8-6.8)
	P	0.558	0.427	0.47
Gender	Male	14.6 (11.3-22.2)	4.7 (3.2-8.4)	5.2 (2.9-6.7)
	Female	35.9 (30.5-53.0)	7.5 (7.5 10.1)	6.8 (2.8-14.3)
	P	0.525	0.201	0.344
Cigarette-smoking	Smoker	30.5 (14.3-35.9)	7.5 (4.4-10.0)	5.6 (2.8-8.7)
	Non-smoker	15.8 (11.3-22.2)	7.4 (3.1-7.4)	5.2 (4.2-6.8)
	P	0.543	0.521	0.795
Alcohol use	Yes	15.8 (11.5-21.4)	6.7 (4.7-8.8)	6.7 (5.2-8.7)
	No	15.5 (11.3-32.7)	7.5 (4.0-10.0)	4.2 (2.8-6.8)
	P (*pc*)	0.675	0.968	0.132
Family history of cancer	Yes	12.9 (8.0-30.8)	6.2 (1.0-9.1)	7.7 (6.7-8.7)
	No	18.8 (13.7-33.2)	7.5 (4.1-9.8)	5.2 (2.9-6.8)
	P (*pc*)	0.267	0.542	0.327

NDC: Newly diagnosed cases; MMC: Mitomycin C; BCG: Bacillus Calmette–Guérin; *P*: Probability of Mann-Whitney *U* test

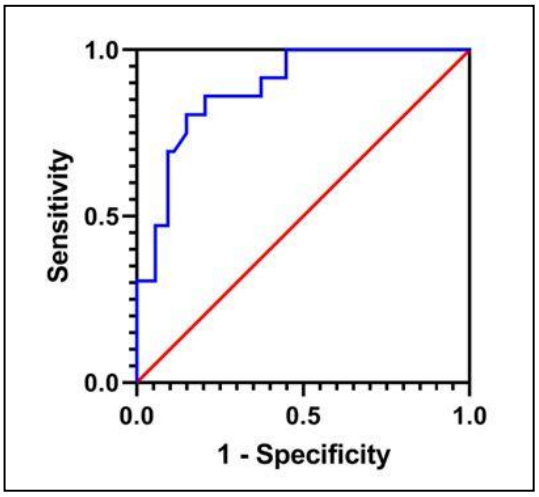

Figure 2. Receiver operating characteristic (ROC) curve analysis of IL-6 in newly diagnosed cases of urinary bladder cancer (UBC). The analysis revealed that IL-6 was a very good predictor in discriminating between UBC patients and healthy controls (area under the curve=0.885; 95% confidence interval=0.828 - 0.942; *P*<0.001; cut-off value = 10.5 pg/mL; Youden index=0.62; sensitivity=80.6%; specificity=81.3%)

Table 3. Logistic regression analysis of IL-6 in newly diagnosed patients of urinary bladder cancer

Logistic regression analysis†	Odds ratio	95% Confidence interval	*P*
Model I	1.18	1.11 - 1.26	< 0.001
Model II	1.13	1.06 - 1.21	< 0.001
Model III	2.21	1.42 - 3.43	< 0.001
Model IV	2.20	1.42 - 3.42	< 0.001
Model V	2.18	1.40 - 3.41	0.001

†: Healthy controls were the reference category; Model I (unadjusted); Model II: Adjusted for age; Model III: Adjusted for age and gender; Model IV: Adjusted for age, gender and cigarette-smoking; Model V: Adjusted for age, gender, cigarette-smoking and alcohol use

4. Discussion

UBC is a heterogeneous disease with a relatively high incidence rate and requires the search for early detection markers. Cytokines, especially pro-inflammatory cytokines, have been proposed as important biomarkers involved in promoting different types of malignancies including UBC (10). One of these cytokines is IL-6, and investigations have revealed that increased systemic levels of this cytokine are associated with a poor prognosis and low overall survival in UBC and gastrointestinal cancer (16, 17). However, factors that may influence IL-6 level in UBC patients have not been well investigated, particularly those related to therapy. Therefore, this study targeted this point and included three groups of UBC patients, namely NDC, MMC and BCG groups, and serum level of IL-6 was examined in the these groups in, as well as HC.

In the NDC group, the IL-6 levels were markedly increased, and the logistic regression analysis estimated an OR of 1.18 Model I). ROC curve analysis confirmed this significance, and an AUC of 0.885 was revealed. In this context, the diagnostic sensitivity and specificity of IL-6 were almost 80.0%. As early as 1997, it was demonstrated that IL-6 may function as autocrine growth factor for UBC cells, and its role in promoting inflammatory-related carcinogenesis and tumor growth was indicated (19). Subsequent investigations have shown that elevated levels of IL-6 may play an important role in carcinogenesis and development of UBC. Besides, IL-6 may serve as a promising predictor of disease (17, 20). Further, a link between IL-6, angiogenesis, and promotion of UBC in tumor-bearing mice was also found, and IL-6 was positively correlated with the activation of STAT3 (signal transducer and activator of transcription 3) (11). IL-6 can also provide a suitable microenvironment for the induction of the cancer stem cell surface marker CD44 (21). However, other investigations have reported that IL-6 may have anti-tumor effects. In mouse model of bladder carcinoma, it was found that recombinant IL-6 could inhibit tumor growth in a dose-dependent manner (22). In addition, xenograft animal studies revealed that overexpression of IL-6 was associated with a down-regulation of tumorigenesis in bladder cells and knocking-down IL-6 reversed this effect (23).

The results revealed that intravesical instillation with MMC or BCG restored the IL-6 level in HC. Serum level of this cytokine showed no significant differences between therapy groups (MMC and BCG) and HC. This may suggest that both types of therapy were associated with a down-regulation of IL-6. MMC is an anti-tumor agent that reduces the risk of UBC recurrence in patients with superficial tumor, and MMC maintenance has been shown to be effective against transitional cell carcinoma recurrence (24). In relation to IL-6, a decreased level of this cytokine was found in the supernatant of rat pancreatic islet culture after treatment with MMC (25). Recently, *in vitro* evidence indicated that endothelial cells treated with MMC showed decreased mRNA expression of the *IL6* and *IL8* genes (26). Collectively, these data along the current study results indicate that MMC treatment is associated with a down-regulation of IL-6 and this may explain the therapeutic potential of MMC in UBC.

Besides MMC, BCG is also a preferred and successful intravesical therapy in superficial UBC with 70% response rate, but the underlying mechanism has not been fully elucidated. However, it has been indicated that BCG initiates a significant immune response in the UB, and induces the synthesis of various pro-inflammatory cytokines including IL-6 leading to the infiltration of the urinary epithelium by inflammatory immune cells (27). Other *in vitro* and *in vivo* studies confirmed that BCG instillation was associated with increased local and systemic levels of IL-6 and other inflammatory cytokines (28). This may indicate that local uptake of BCG (urothelial cells and antigen presenting cells) triggers the release of IL-6, which in turn, promotes rapid and heavy influx of neutrophils into the bladder within short time of BCG instillation (29). However, in the current study, the serum level of IL-6 in BCG-treated patients approached HC levels and was significantly lower than that of the

NDC group. These results point to the fact that the IL-6 systemic response to BCG instillation may be time-dependent. In the short time evaluation (hours) after BCG instillation, the IL-6 shows up-regulated levels, while in the long term evaluation (days or months), the level may fall to normal levels. In this context, Calais da Silva and colleagues analyzed the gene expression of several pro-inflammatory cytokines in the blood cells of UBC patients treated with BCG within 24 hours and after prolonged periods (months). It was found that BCG instillation increased the expression rapidly, while long-term evaluation showed lower expression of some of the cytokines examined (18).

The IL-6 level was also examined in UBC groups (NDC, MMC and BCG) stratified according some risk factors that are proposed to have a role in etiology of disease (age, gender, cigarette-smoking, alcohol-use and family history of cancer). The IL-6 levels appeared to be unaffected by these factors as there were no significant differences between the subgroups in each stratum.

This study indicated that IL-6 level was up-regulated in serum of newly diagnosed UBC cases. Further, IL-6 level was restored to normal levels after intravesical instillation of MMC or BCG. However, the sample size may limit these findings, and larger cohort studies are recommended to evaluate the usefulness of IL-6 as a biomarker in UBC patients before and after intravesical instillation with MMC or BCG.

Authors' Contribution

Study concept and design: A. H. A.
Acquisition of data: R. M. A.
Analysis and interpretation of data: R. M. A.
Drafting of the manuscript: T. H. M.
Critical revision of the manuscript for important intellectual content: A. H. A.
Statistical analysis: S. T. A.
Administrative, technical, and material support: R. M. A.

Ethics

This study was approved by the ethics committee of the University of Baghdad, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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