



ISSN NO. 2320-5407

Journal homepage: <http://www.journalijar.com>

INTERNATIONAL JOURNAL  
OF ADVANCED RESEARCH

## RESEARCH ARTICLE

## Evaluation of mitomycin C (MMC) and Bacillus Calmette-Guerin (BCG) therapies in association with vitamin D levels in enhancement of anti bladder cancer immune response.

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### Manuscript Info

#### Manuscript History:

Received: 14 September 2015

Final Accepted: 22 October 2015

Published Online: November 2015

#### Key words:

BC, NMIBC, MMC, BCG, VD, TNF- $\beta$ , TNF- $\alpha$ , IFN- $\gamma$ , Chemotherapy, Immunotherapy.

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### Abstract

**Back ground:** Bladder cancer (BC) is a complex and multifactorial malignancy. It is the second most common genitourinary cancer.

**Aim of study:** To investigate the influence of vitamin D in association with intravesical immune or chemotherapy in enhancement of antitumor immunity in bladder cancer patients (BC),

**Material and methods:** 89 subjects (59 cases and 30 controls) were enrolled in current study. Patients were categorized into three groups based on their treatment: patient control (PC) those who were newly diagnosed untreated patients, those treated with mitomycine C (MMC) intravesical chemotherapy, and those treated with (BCG) intravesical immunotherapy. Serum levels of VD, TNF- $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  were measured using ELISA technique in each of studied groups.

**Results:** Serum levels of VD showed insignificant elevations in each of PC group, treated patients with MMC group, and treated BCG group compared with HC group. Serum levels of TNF- $\beta$ , statistical analysis have shown significant dropping in each of PC, MMC, and BCG groups compared with HC group. Significant dropping in serum levels of TNF- $\alpha$  in PC group and insignificant elevation in MMCgroup were recorded, while there was insignificant dropping in BCG group compared with HC group. Serum levels of IFN- $\gamma$  revealed insignificant dropping in PC group and insignificant elevation in each of MMC and BCG groups compared with HC group.

**Conclusion:** serum levels of VD in both of cases and controls were within insufficient status, so cannot evaluate the association of VD status with BC incidence. Immunotherapy (BCG) was more efficient than chemotherapy (MMC) in enhancement of antitumor immune response.

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## INTRODUCTION

Bladder cancer is a prevalent disease with considerable correlated morbidity and mortality, it is the 9th (7th in men and 17th in women) most widespread cause of malignancy linked death globally (Jemal et al., 2011; Chung, 2013; Kobeissi et al., 2013; Vlaanderen et al. 2014).

Nearly 75% of all bladder cancer are diagnosed as non muscle invasive bladder cancer (NMIBC), 50% of these cases are recurring next transurethral resection of the bladder tumor (TURBT) (Witjes et al., 2015). Due to such high recurrence ratio presuppose repeat surveillance and intervention is recommended (Gakis et al., 2015). Intravesical chemotherapy (IVC) such as that with mitomycin C (MMC) and intravesical immunotherapy (IVI) such as Bacillus

Calmette Guerin (BCG) are extremely used to minimize the expectation of disease recurrence (Burger et al., 2013). The innate immune response involves the activation of toll-like receptors (TLRs) in monocytes, polymorphonuclear cells (PMN), and number of epithelial cells including those of intestine, Vagina, lung, and bladder (Liu et al., 2007). Activation of TLRs leading to enhancement of antimicrobial peptides (AMPs) and reactive oxygen species (ROS), that kill the infectious agent. Among these AMPs is cathelicidin which has important roles in the innate immunity (Schauber and Gallo, 2008).

The production of AMPs is enhanced by  $1,25(\text{OH})_2\text{D}$  in myeloid and epithelial cells. Furthermore  $1,25(\text{OH})_2\text{D}$  stimulates TLR-2 and coreceptor CD14 in keratinocytes (Schauber et al., 2007). Enhancement of TLR-2 leading to increment of CYP27B1 expression that induce the expression of cathelicidin (Liu et al., 2006).

VD effect, wide range of cells in adaptive immune response.  $1,25(\text{OH})_2\text{D}$  is able to repress T cells proliferation and modification of cytokines expression by these cells with shifting of immune response from T helper-1 (Th1) to T helper-2 (Th2) (Hewison, 2012). Regarding monocytes, VD could suppress the pro-inflammatory cytokines production (IL-1, IL-6, IL-8, IL-12, and TNF- $\alpha$ ) by these cells (Almerighi et al., 2009). Interestingly, VD have been shown to inhibits B cell proliferation and block B cells differentiation and immunoglobulin secretion. Dendritic cells (DCs) are able to express special cytokines group that can deviate T lymphocytes toward divergent function. VD inhibit maturation, differentiation and survival of DCs leading to inhibition of antigen presentation and T cell proliferation and hyporesponsiveness (White, 2008; Wong and Tsao, 2006). The function of T regulatory (Treg) cells is maintenance of self tolerance.  $1,25(\text{OH})_2\text{D}$  have been shown to increase the expression of Treg receptor CD25 heterodimeric partner (IL-2 receptor  $\beta$ ) (Mahon et al., 2003). Tumor necrosis factor (TNF) family of cytokines contains of more than 15 proteins include TNF $\alpha$ , TNF $\beta$ , CD40 (CD40L), Fas ligand (FasL), TNF related apoptosis inducing ligand (TRAIL). Each member of this family can enhance cell death over binding to particular transmembran receptors (Eriksson and Vandenabeele, 2011; Chu, 2013). TNF- $\alpha$  (also known a endotoxin-induced factor in serum differentiations factor, and cachetin) and TNF- $\beta$  [lymphotoxin(LT)] are the most prominent factors in this family (Bouillet and O'Reilly, 2009). TNF- $\alpha$  is a significant risk factor tumorigenesis, tumor invasion, progression, and metastasis. Moreover, it's a key factor in cancer-associated chronic inflammation (Carbone et al., 2012). Interferon gamma IFN- $\gamma$  is an extraordinarily pleotropic cytokine, which can promote both the innate and adaptive immune response against pathogens and tumors, in addition to maintain immune homeostasis (Lin and Young, 2013). The major IFN- $\gamma$  secretary cells are T helper1(Th1) cells, natural killer (NK) cells, and cytotoxic T lymphocytes (Young, 1996; Bach et al., 1997). IFN- $\gamma$  is a Th1 proinflammatory cytokine that has ability to induce both pro-and anti-inflammatory responses which is critical for balanced immune response (Gayoso et al, 2011). IFN- $\gamma$  signaling enhances Th1 development through triggering expression of T-bet and repression of GATA3 expression, a protein which derives the differentiation of Th2 (Lin and Young, 2012).

## Material and methods:

This study included 59 volunteers cases (49 males, 10 females) with histopathologically confirmed BC during the period from December 2013 to August 2014 from the Hospital of Gazi AL-Harey in medical city of Baghdad, and Al-Emamain Al-Khadhemain Teaching hospital. Family unrelated, apparently healthy 30 (males 22, females 8) individuals from Al-Emamain Al-Khadhemain Teaching hospital and College of Medicine/ Al-Nahrain University were selected to represent the control group. According to treatment and type of intravesical chemo or immunotherapy, BC patients were sub divided into three groups, these are : 1) Patients Control (PC) group (20 cases), these are newly diagnosed (untreated patients), 2) Treated patients with Mitomycin C chemotherapy (MMC) group (22 cases), 3) Treated patients with immunotherapy (BCG) group (17 cases). The studied factors in these groups were compared with healthy control (HC) group (30 subjects) in addition to comparison among the cases groups mentioned.

The ages of patients ranged from 24 to 83years (females: 26 to 72years, males: 38 to 83years) and ages of controls ranged from 51 to 80years ( females: 54 to 70years, males: 51 to 80 years) . Informed consents from patients as well as control were taken which included age, previous and current occupation, smoking, drinking, residence, and first relative family history of Bladder cancer as well as body BMI and SSE. Three ml of venous blood was collected from each participant in plan tubes. The latter was undergone centrifugation where the serum was obtained and preserved at  $-20^{\circ}\text{C}$  until used. ELISA technique was used to estimate serum levels of VD (MyBiosource / USA), INF- $\gamma$  (Biosource Europe S. A. Belgium), TNF- $\beta$  (USBiological / USA), and TNF- $\alpha$  (USBiological / USA). Ethical permission was obtained from all volunteer to collect samples and conduct this study. Selections of cases were accomplished with the assistance of urologists within such hospitals. The study was conducted in the medical research unit at College of Medicine-Al-Nahrain University.

## Results:

The statistical analysis for results of serum levels of VD showed insignificant elevations in each of PC group ( $23.23 \pm 1.97$ ,  $P=0.18$ ), treated patients with MMC group ( $23.71 \pm 1.56$ ,  $P=0.11$ ), and treated with BCG ( $21.24 \pm 1.04$ ,  $P=0.67$ ) group compared with HC group ( $20.25 \pm 1.55$ ). Regarding groups of patients there is no differences between PC and MMC groups, while there was insignificant dropping ( $P=0.434$ ) in BCG group compared with PC group. Moreover, there was insignificant dropping ( $P=0.322$ ) in BCG group compared with MMC group.

Serum levels of TNF- $\beta$  showed significant dropping in each of PC ( $P=0.012$ ), MMC ( $P=0.005$ ), and BCG ( $P=0.027$ ) groups compared with HC group, and serum levels were  $7.79 \pm 2.11$ ,  $6.13 \pm 0.61$ ,  $9.29 \pm 2.65$ , and  $27.96 \pm 8.27$  respectively. In comparison with PC group, there was insignificant elevation in each of MMC ( $P=0.845$ ) and BCG ( $P=0.868$ ) groups respectively. While showed insignificant elevation ( $P=0.721$ ) in BCG group compared with MMC group.

Tumor necrosis factor alpha showed significant dropping in serum levels in PC ( $P=0.023$ ) group and insignificant elevation in MMC ( $P=0.53$ ) group, while there was insignificant dropping in BCG ( $P=0.86$ ) group compared with HC group, and serum levels were  $107.43 \pm 13.17$ ,  $175.02 \pm 12.33$ ,  $156.74 \pm 16.08$  respectively. Regarding the groups of patients, there was significant elevation ( $P=0.008$ ) in MMC group and insignificant elevation ( $P=0.067$ ) in BCG group compared with levels of PC group. Moreover, BCG group showed insignificant dropping ( $P=0.484$ ) compared with MMC group.

Serum levels of INF- $\gamma$  revealed insignificant dropping in PC ( $P=0.19$ ) group and insignificant elevation in each of MMC ( $P=0.74$ ) and BCG ( $P=0.18$ ) groups compared with HC group, and serum levels were  $343.15 \pm 28.96$ ,  $378.05 \pm 28.96$ ,  $439.12 \pm 30.49$ , and  $389.33 \pm 19.42$  respectively. Regarding groups of patients, it has shown insignificant elevation ( $P=0.359$ ) in MMC group and significant elevation ( $P=0.020$ ) in BCG compared with PC group. Furthermore, BCG group showed insignificant elevation ( $P=0.127$ ) compared with MMC group.

serum levels of studied parameters of studied groups.

Studied groups	VD ng/ml (M $\pm$ SE)	TNF- $\beta$ pg/ml (M $\pm$ SE)	TNF- $\alpha$ pg/ml (M $\pm$ SE)	IFN- $\gamma$ pg/ml (M $\pm$ SE)
HC	20.25 $\pm$ 1.55 <sup>a</sup>	27.96 $\pm$ 8.27 <sup>a</sup>	161.02 $\pm$ 19.86 <sup>a</sup>	389.33 $\pm$ 19.42 <sup>ab</sup>
PC	23.23 $\pm$ 1.97 <sup>a</sup>	7.79 $\pm$ 2.11 <sup>b</sup>	107.43 $\pm$ 13.17 <sup>b</sup>	343.15 $\pm$ 28.96 <sup>b</sup>
MMC	23.71 $\pm$ 1.56 <sup>a</sup>	6.13 $\pm$ 0.61 <sup>b</sup>	175.02 $\pm$ 12.33 <sup>a</sup>	378.05 $\pm$ 28.96 <sup>ab</sup>
BCG	21.24 $\pm$ 1.04 <sup>a</sup>	9.29 $\pm$ 2.65 <sup>b</sup>	156.74 $\pm$ 16.08 <sup>ab</sup>	439.12 $\pm$ 30.49 <sup>a</sup>

HC: healthy control, PC: patients control, MMC: mitomycin C, BCG: Bacillus Calmette-Guerin.

Different small letters indicate a significant differences.

## Discussion

Statistical analysis of current study did not shown any significant differences in VD levels between each of studied groups of patients and control group. Scientific literatures suggested that all VD levels in studied groups occurred within insufficiency status.

Of note, over VDR, VD regulates large number of genes, including those involved in the immune function, inflammation, angiogenesis, and cellular proliferation, differentiation and apoptosis (Holick, 2004; Giovannucci, 2005).

Baeke et al. reported that VDR expressed in normal, immune, and malignant cells (Baeke et al., 2007).

Current study showed efficient action of MMC intravesical chemotherapy over enhancement of antitumor response. Elevated levels of TNF- $\alpha$  and TNF- $\beta$  following such treatment revealed a significant role of MMC in augmentation of anticancer immunity. Of note, chemotherapy can stimulate killing of malignant cells primarily over necrosis, a proinflammatory form of cell death (Grivennikov et al., 2010).

In fact, chemotherapy may promote progression of tumor over enhancing of inflammatory microenvironment (Vakkila and Lotze, 2004). On the other hand, chemotherapy can induce antigen presentation by tumor-infiltrating dendritic cells (DCs) and stimulate of cytokines secretion, which leads to induced adaptive antitumor immunity (Zitvogel et al., 2008; Deaglio et al., 2007). Cheung et al. proposed that BCG-induced inflammation may boost the permeability of bladder urothelium, allowing MMC to reach the aim tissue more facilely and exert its antitumor influence (Cheung et al., 2013). Wang et al. reported that MMC treatment increased death of cells as well as unregulated Fas and Fas-Associated Death Domain (FADD) expression (Wang et al., 2007).

To date, only, a few has been known on how chemotherapy induced inflammation effects treatment response of BC. Many of the implied mechanisms remain to be illustrated in further studies.

Also present study showed the efficiency of the BCG immunotherapy to stimulate antitumor immunity over elevation of TNF- $\beta$ , TNF- $\alpha$ , and INF- $\gamma$  levels.

In the management of NMIBC, BCG is the most successful intravesical immunotherapy used for treatment of urothelial BC (Herr and Morales, 2008)[403], and INF- $\gamma$  most important cytokine in such treatment due to its crucial role in immunotherapeutic of BCG therapy (Fiorentino et al., 1989; Fiorentino et al., 1991).

Dysregulation of the Th1/Th2 balance is often found in several diseases particularly in malignancy conditions (Romagnani, 1997). Romagnani et al. and Lebel-Binay et al. reported that naive Th0 cells differentiation into Th1 or Th2 cells is affected by several factors such as the type of antigen-presenting cells (APC), the nature of antigen, and type of cytokines present in the local microenvironment.

Growing evidences revealed that T cells can extend both tumor-suppressive and -promoting effects in BC. Naoe et al. found that the BCG-pulsed DCs could activate the  $\gamma\delta$ T cells and NKT cells, which showed unspecific cytotoxic activity against a BC cell line (Naoe et al., 2007). Agarwal et al. found that patients of BC developed Th2 dominant condition with deficient development of Th1 immune response that showed predisposition to reversal such therapy. Satyam et al. also showed that in patients of BC obvious polarization towards of Th2 type cytokines expression while Th1 appears to suppressed.

Interaction of urothelial and bladder tumor cells with BCG leads to internalization of BCG, that increases of antigen-presenting molecules expression. This stimulates an immune response through cytokine liberation. Th1 cytokines (IFN- $\gamma$ , TNF, IL-12, and IL-2) and Th2 cytokines (IL-10, IL-4, IL-6, and IL-5) along with IL-17 and IL-8 are all involved. This sophisticated immune cascade enhances of antitumor influence mediated by many cells such as NK, CTL, DCs, macrophages, and neutrophils (Fuge et al., 2015; García-Cuesta et al., 2015; Redelman-Sidi et al., 2014). Killing of BC cells over direct cytotoxicity by such cells, via secretion of soluble factors like tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) (Redelman-Sidi et al., 2014).

In a robust immune system, in the bladder, macrophages take up the BCG antigens and display it to T helper cells, which triggers immune interaction cascade between each of MHC I, lymphocyte function antigen I, IL-1, IL-2, CD80, CD28. This leads to development of hypersensitivity reaction within mucosal surface of urinary bladder (Gonzalez et al., 2003; Nadasy et al., 2008). The cytokines released over such stimulation which considered significant for development antitumor immune response are predominantly those related to T helper 1 cells particularly TNF- $\alpha$  and INF- $\gamma$ , while BCG failure associated with T helper 2 cytokines particularly IL-10 (Kaklamanos et al., 2011; Chade et al., 2008).

Luo<sup>a</sup> et al. reported that intravesical BCG therapy can shift T helper 2 circumference toward T helper 1 milieu, leading to efficient antitumor immunity in majority of BC patients. Also, Luo<sup>b</sup> et al. suggested that blocking production of IL-10 and/ or activity may be useful for potent BCG immunotherapy of malignant bladder urothelium. Tumor necrosis factor appears one of the potential molecular relationships between chronic inflammation and development of cancer. Feng et al. found that elevated expression of TNF- $\alpha$  was noticed in cancer tissue compared with healthy urothelium. Besides, they found that change of TNF- $\alpha$  expression was associated with angiogenesis of BC, particularly in BC development. In human bladder cancer cells, TNF- $\alpha$  could induce the secretion of matrix metalloproteinase-9 (MMP9) that has been involved in tumor invasion and metastasis (Lee et al., 2008). However, TNF- $\alpha$  is a decisive factor in stimulating of antitumor immune response. Kowalczyk D et al. reported that Th1 response was characterized by an increased secretion of TNF- $\alpha$ , TNF- $\beta$ .

Müller et al. reported that efficient inhibition of tumor growth not only involve defined cell death and killing mechanisms by CTLs and NKs, but also the enhancement of tumor cell senescence by IFN- $\gamma$  and TNF producing Th1 cells. Agarwal et al. found that cytokines produced by BCG enhancement and considered significant for the antitumor response are predominately those related to Th1 cells such as IFN- $\gamma$  and TNF- $\alpha$ .

### **Conclusion:**

The elevation of TNF- $\alpha$ , TNF- $\beta$ , and IFN- $\gamma$  levels following intravesical BCG therapy is a clear evidence of the effectiveness of such immunotherapy in stimulating of cell mediated immune response and supporting of immunosurveillance.

### **Acknowledgments:**

My greatest appreciation and friendship go to my closest friend Dr. Qassim Al-Mayah head of medical research unit/College of Medicine/Al-Nahrain. Also I am grateful to all staff member of such unit for their help and cooperation.

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