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EXPRESSION OF TLRS IN BLABBER CANCER

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ABSTRACT
Toll like receptors (TLRs), also called pattern recognition receptors, it is part of innate arm of immune system have the ability to recognize foreign molecules and pathogens, TLRs are expressed by immune cells involved in immune function and normal epithelial cell, On the other hand, TLRs are also expressed by cancer cells that contribute to carcinogenesis. Aim of this Study is to evaluate the role of TLR2 and TLR4 in pathogenesis and prognosis of bladder cancer. Expression TLR2, 4 was evaluated in the tumor of 57 urinary bladder biopsy and 40 normal autopsies in immunohistochemical study. Increased expression of TLR2, 4 was significantly higher in tumor compared with normal samples. and according to grade and muscle invasion, TLR2 showed significant over expression in high grade than low grade(40.7%*vs*36.7%), and in muscle invasion than non muscle invasion (44.5%*vs*33.3%), while TLR4 was with no correlation to grade and muscle invasion. As well as no correlation between TLR2, 4 and tumor recurrence, but Schistosoma associated bladder cancer patients was statistically significant highly expressed TLR2. Our result suggest that TLRs play a critical role in inflammation against infections and were often active malignancies

KEY WORDS: immunohistochemistry, muscle invasion bladder cancer, schistosomiasis, carcinogenesis, epithelial cells and inflammation.

INTRODUCTION

Bladder cancer is the common malignancy involving the urinary system (Anastasiadis and deReijke, 2012). Incidence of bladder cancer in Iraq on constant rise, (80%) were males and (20%) were female patients with Average age for males were (66,07 years) and for the females were (67.82 years) (AL-Shwani, 2013), and is associated with an infection of the bladder called *Schistomiasis* (Botelho *et al*., 2013). On the other hand this cancer is a smoking- and occupational exposure-related disease with a substantial genetic component (Burger *et al*., 2013), so it is more common in industrial countries than in developing countries **(**Eagle**,** 2012).There is increasing evidence that TLR signaling pathway is involved in tumorigenesis and chemo resistance in different cancer types (kim *et al*., 2012). Normally, the expression of TLRs vary among tissues and cell types but, generally they are predominantly expressed in tissues involved in immune function (spleen and peripheral blood leukocytes) (Nishiya & DeFranco, 2004). As TLRs are expressed not only by immune cells but also by normal epithelial cells, these that were expressed in epithelial cells lining an organ (first line of defense against invasion of microorganisms) have a crucial role in regulation of proliferation and apoptosis (Rakoff-Nahoum and Medzhitov, 2009). On the other hand, TLRs are also expressed by cancer cells resulting in up-regulation of NF-κB cascade and produce anti-apoptotic proteins that contribute to carcinogenesis and cancer cell proliferation, In addition, they can mediate cytokines and chemokines release from cancer cell, so recruit optimized immune cells to enhance immunity in the tumor microenvironment, then resulting in further release of further proinflammatory cytokines, proangiogenic factors and growth factors, which impair the anti-tumor function (Sato *et al*., 2009). For example TLR-4recognize the lipopolysaccharide (LPS) found on Gram-negative bacteria, the mechanism for this response is via a TLR on macrophages that recognizes LPS then elicits a variety of molecules in the inflammatory response with recruitment and activation of macrophages, both natural killer (NKcells) and dendritic cells (key agents in the presentation of antigen to T cells) (Judith *et al.*, 2013). Besides these, TLRs may also be activated by endogenous ligands including cellular debris derived from cancer progression (O'Neill *et al*., 2009). Ayari *et al.*, 2011, showed the development of strong inflammatory response in normal urothelial as well as in bladder cancer cultured cells that were treated with TLR 2 & 3 agonist. Kauffman *et al*., 2010 found that TLR2 and 4 are present in >70% of bladder cancer cells with no significant correlation to cancer stage or grade. Result were obtained by (Stopiglia *et al*., 2015) demonstrated that TLR2 and TLR4 immune re-activities were significantly lower in low-grade, non-muscle invasive and muscle invasive bladder carcinoma than normal, and this may contribute to the high tumor relapse and progression rates.

MATERIALS & METHODS

This prospective study consisted of 97 individuals**,** of which tissue biopsy were collected from 57 bladder cancer patients with average age (63± 9.3), this sub-divided into (28 newly diagnosed bladder tumor patients included 25men, 3women and 29recurrent tumor patients (relapse) after received intravesical

chemotherapy and /or radiotherapy, 25men, 4women). Biopsy was collected fromAl- Yarmouk and Baghdad Teaching Hospital. In addition (40) apparently normal bladder autopsies **(**33men and 7women) with average age (51± 13.7**)** were collected from the Forensic Medicine Institute Baghdad/ Iraq. From each patient, a full medical history for diseases and previous laboratory finding was obtained, besides a cystoscopic examination by which transurethral resection (TUR) biopsies were taken from the apparent lesion, processed by standard oncological procedures, The tumor grade and muscle invasion was defined by a specialist pathologist according to(WHO/ISUP) and American Joint Committee on Cancer (AJCC, 7th ed., 2010).

Immunohistochemical detection of TLR2, TLR4 proteins in Paraffin Embedded Sections

Biopsies were processed by(neutral buffered formalin10%, dehydrated through a graded series of ethanols, cleared in xylenes then embedded in paraffin and were stained with routine hematoxylin and eosin stains, as well as immunohistochemically, following the procedure described in the texts of (Suvarna *etal*.,2013). Hematoxylin and eosin section were examined for histological grading and muscle invasion examination.

by using: primary antibody of Rabbit anti-human TLR2 (Abcam , UK): polyclonal, Isotyp: IgG; Mouse anti-human TLR4 (Abcam,UK): clone: 76B357.1, Isotype: IgG2b and secondary antibody of ab80436-expose mouse and rabbit specific HRP/DABdetection IHC kit immunohistochemistry was carried out.

Immunohistochemistry Procedures

- Tissue Sectioning: Sections with 4 - 12µm were Prepared on the microtome and placed on clean, positively – charged microscope slides
- Slide baking: the slides of patients and the positive controls were Placed in jar slide holder in tissue-drying oven for 45 minutes at 60°C
- Deparaffinization: Slides wash 3 times for 5 minutes in xylene.
- Rehydration:

a) slides Washed one time for 5 minutes in 100% alcohol.

b) slides Washed one time for 3 minutes in 90% alcohol

c) slides Washed one time for 3 minutes in 70% alcohol.

d) slides Rinsed for 5 minutes in distilled water then in PBS for 3 mintutes.

- Peroxidase Block : after draining and carefully blotting around the specimen to remove any remaining liquid and to keep the reagents within the prescribed area, sections were covered with enough drops of hydrogen peroxide (H_2O_2) block and then incubated in humid chamber for 10 minutes. Slides Washed 2 times in diluted washing buffer .
- Antigen Retrieval Buffer : slides were placed in a Cooplin jar containing 1x target Retrieval Buffer of 100X citrate buffer, covered with a vented plastic wrap and placed the jar in microwave and set high power to boil and set low power to keep it boiling for 10 minutes the sections let to cool in the microwave for at least 20 minutes, Washed 3 times in diluted buffer.
- Sections were covered with enough drops of protein block of (Goat Serum and Rabbit Serum) and slides incubated in humid chamber for 10 minutes at room temperature to block nonspecific background staining. After that the slides had been drained around the sections.
- optimally (100ml) of diluted primary antibody applied on the sections and the slides placed in the humid chamber and incubated one hour at room temperature. the slides were then washed gently 3times in diluted washing buffer.
- The secondary antibody : enough drops of complement (rabbit anti mouse secondary antibody) were applied on the sections, then the slides were placed in the humid chamber and incubated for 10 minutes at room temperature. The slides were washed 2 times in buffer.
- Horseradish peroxidase (HRP conjugate) : drops of secondary antibody (Goat anti –rabbit HRP Conjugate) were applied covering the specimen and incubate in humid chamber for 15 minutes at room temperature. Slides then Rinsed 4 times in buffer.
- The DAB –chromogen : optimal drops of DAB chromogen-substrateo (3,3 diaminobenzidine solution-Imidazole-HCL buffer PH 7.5) Were applied using transfer pipette and covered Whole tissue, the slides were incubated in darkness at room temperature for 10 minutes. Slides rinsed 4 times in buffer.
- Counter- stain: Mayer s heamatoxylin stain was applied to the sections covering whole specimen for 1 minute, then rinsed in distilled water.
- Dehydration: The sections were dehydrate by immersing the slides sequentially in ethanol and xylene containing jar as fallow : a) slides washed in 70% alcohol, 1 minute . b) Slides washed in 90% alcohol, 1 minute. c) Slide washed in 100% alcohol, 1 minute d) slides washed in xylene 1 minute.
- Mounting media: one to two drops of Mounting media :DPX (Distyrene, Plsticizer, and xylene), DPX applied onto the xylene –wet sections , and the sections covered with cover slip and left to dry.
- Slides were examined and the stained cells were counted with assistance of an experienced histopathologist by light microscope at 40x magnification. Scoring oTLR2, TLR4 expression was carried out using a two tier scoring system. The first parameter corresponds to the percentage of immunoreactive of positive tumor cells (cytoplasmic and membranous staining) also known as the quantity score (QS) and evaluated by counting the number of stained tumor cells per 1000cells, more than 1000 cells in each section evaluated under 40x high power field and the percentage of positive cells was calculated, the second parameter is (staining intensity score) which is the degree of cytoplasmic and membranous tumor cells colour and scored as: 1 light brown yellow, 2 brown , 3dark brown.

- **Scoring For TLR4 (d'Adhemar *et al*., 2014),** a) the quantity score (QS) or immunopositivity(IP) was estimated as follow: Score 1: 1-10%, Score 2: >11-40%, Score 3: >41-70%, Score 4:>70
  b) staining intensity score or immunointensity (II) was estimated as follows:Score 0 : no staining, Score 1 : weak staining, Score 2 : moderate staining, Score 3: strong staining. The product of the quantity and the staining intensity scores Represents the total IHC score that ranges from 0 to 12 . a cut-off value of >4 determined IHC positivity .
- **Scoring for TLR2 as (yuan *etal*., 2013)**

a)the quantity score(QS) was estimated as follows : Score 0: no staining Score 1: <10% Score 2: 11 - 33% Score 3: 34 - 66% Score 4: >67%

b)staining intensity score was estimated as follows: Score 0 : no staining Score 1 : weak staining Score 2 : moderate staining Score 3: intense staining The intensity and the percentage of positive cell scores were multiplied (0–12) and classified into three groups: weak (0–4), moderate (5–8) and strong (9–12).

Statistical Analysis: The statistical significance of difference between mean of a normally distributed qualitative (discrete) variables of two groups was assessed using the chi – square test or fisher exact test.

RESULTS

Immunohistochemistry of TLR2

Scoring of TLR2 in bladder tissue of bladder cancer patients and normal autopsies

The percentage of positively(PR) stained cells, Staining intensity of positively stained cells (SI) in bladder cancer tissue and normal autopsies were summarized in (Table 1) and were multiplied (0–12) and classified into three groups: weak (0–4), moderate (5–8) and strong (9–12). It was found that TLR2 was strongly expressed in all 57 transitional cell carcinoma tissue and weakly expressed in 14normal urothelium (autopsies), with Chi–square showed significant difference between the two groups p= 0.000 (Table 2).

TABLE 1: The percentage of positively (PR) stained cells, staining intensity of positively stained cells (SI) of TLR2 in bladder tissue of bladder cancer patients and normal autopsies

Percentage of positively stained cells of TLR2	Staining Intensity of TLR2						Total No.
	No (Score 0)	weak (score1)	Moderate (Score 2)		Strong (Score 3)		
	Patients No.	Controls No.	Patients No.	Controls No.	Patients No.	Controls No.	
(Score 0) No staining	0, 0	26, 0	0	0	0	0	26
(Score 1) <10%	0	0	0	0	0	0	0
(Score 2) 11 - 33%	3	0	0	0	0	0	3
(Score 3) 34 - 66%	10	2	2	0	0	0	14
(Score 4) >67%	6	12	14	0	22	0	54
Total no.	19	40	16	0	22	0	97

TABLE 2: Final score of TLR2 expression in bladder tissue of bladder cancer patients and normal urothelium

TLR2 expression	Patients No. (%)	Normal urothelium No. (%)	Total No. (%)
Weak (0–4)	19 (33.33)	40(100)	59(60.8)
Moderate (5–8)	16 (28.07)	0	16(16.5)
Strong (9–12)	22 (38.5)	0	22(22.6)
Total No. (%)	57(58.7)	40(41.2)	97(100)
Chi-square	61.2 6		
P value	0.000 (highly significant)		

Scoring of TLR-2 IHC in bladder tissue of newly diagnosed and recurrent cases of bladder cancer:

Table 3 and 4 shows that, weak, moderate and strong expression of TLR2 were detected in both newly diagnosed cases as well as recurrent cases of bladder cancer with 32.1%, 32.1%, and 35.8% respectively (in newly diagnosed) and 34.4%, 24.1% and 41.1% respectively (in recurrent cases), with no significant difference detected between them (P=0.605).

Scoring of TLR-2 in relation to tumor grade

Regarding tumor grade of transitional cell carcinoma, weak, moderate and strong expression of TLR-2 were detected in 8(26.7%), 11(36.6%),and 11(36.6%) of low grade cases, while weak, moderate and strong expression in high grade were 11(40.7%), 5(18.6%) and 11(40.7%) respectively and it showed significant difference between tumor grade and TLR-2 score.(Table 5 and 6), (Fig 1, 2 and 3)

Scoring of TLR-2 in relation to tumor muscle invasion

Strong expression of TLR2 was detected in 10 (33.3%) of 30 non-invasive TCC cases and in 12 (44.5%) of 27 invasive cancer, with statistical analysis revealed significant difference of their expression (P =0.022) as shown in (Table7 and Fig 4).

TABLE 3: The percentage of positively (PR) stained cells, Stainingintensity of positively stained cells (SI) in bladder tissue of newly diagnosed and recurrent bladder cancer cases

Percentage of positively stained cells of TLR2	Staining Intensity score						Total No.
	No (Score 0)	Weak (Score1)	Moderate (score 2)		Strong (Score 3)		
	Newly No.	Recurrence No.	Newly No.	Recurrence No.	Newly No.	Recurrence No.	
(Score 0) no staining	0 , 0	0 , 0	0	0	0	0	0
(Score 1) <10%	0	0	0	0	0	0	0
9Score 2) 11 - 33%	1	2	0	0	0	0	3
(Score 3) 34 - 66%	6	4	1	1	0	0	12
(Score 4) >67%	2	4	8	6	10	12	42
Total	9	10	9	7	10	12	57

TABLE 4: Final score of TLR-2 in newly diagnosed and recurrent bladder cancer patients

TLR2 expression	Newly diagnosed No. (%)	Recurrence No. (%)	Total No. (%)
Weak (0–4)	9 (32.1%)	10 (34.4%)	19(33.33)
Moderate (5–8)	9 (32.1%)	7 (24.1%)	16(28)
Strong (9–12)	10 (35.8%)	12 (41.5%)	22(38.6)
Total No. (%)	28(49.1)	29(50.9)	57(100)
Chi-square	1.00		
P- value	0.605 (not significant)		

TABLE 5: The percentage of positively(PR) stained cells, Staining intensity of positively stained cells (SI)of TLR-2 in low and high grade bladder cancer

The percentage of positive stained cells of TLR-2	Staining Intensity score						Total No.
	No Score 0	Weak Score 1	Moderate Score 2		Strong Score 3		
	Low Grade No.	High Grade No.	Low Grade No.	High Grade No.	Low Grade No.	High Grade No.	
(Score 0) no staining	0	0	0	0	0	0	0
(Score1) <10%	0	0	0	0	0	0	0
(Score 2) 11 - 33%	1	2	0	0	0	0	3
(Score 3) 34 - 66%	5	5	2	0	0	0	12
(Score 4) > 67%	2	4	9	5	11	11	42
Total	8	11	11	5	11	11	57

TABLE 6: Final score of TLR-2 in low and high grade bladder cancer patients

TLR-2 expression	Low grade No. (%)	High grade No. (%)	Total No. (%)
Weak (0–4)	8(26.7)	11(40.7)	19(33.3)
Moderate (5–8)	11(36.7)	5(18.5)	16(28)
Strong(9–12)	11(36.7)	11(40.7)	22(38.6)
Total No.(%)	30(52.4)	27(47.7)	57(100)
Chi-square	8.02		
Pvalue	0.018 (significant)		

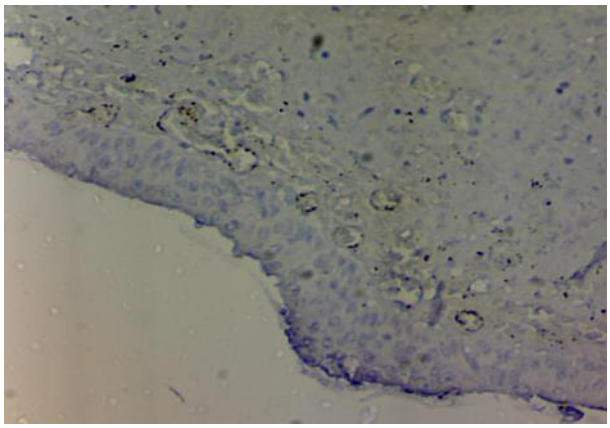

FIGURE1: Normal bladder tissue **(**biopsy) showing loss TLR2 expression, (immunohistochemicalstaining for TLR2, X100)

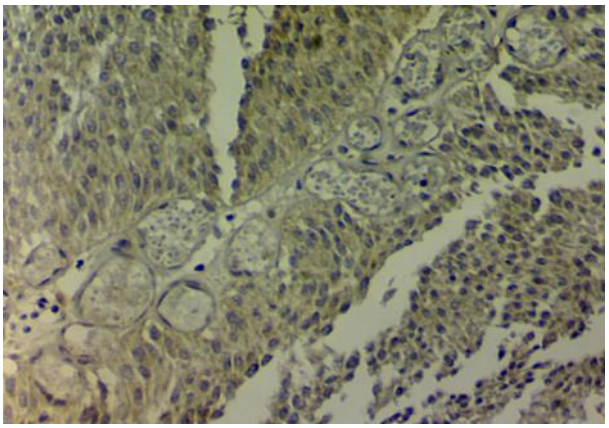

FIGURE 2: TCC Low grade, showing moderate cytoplasmic staining (immunohistochemical staining for TLR2, X400)

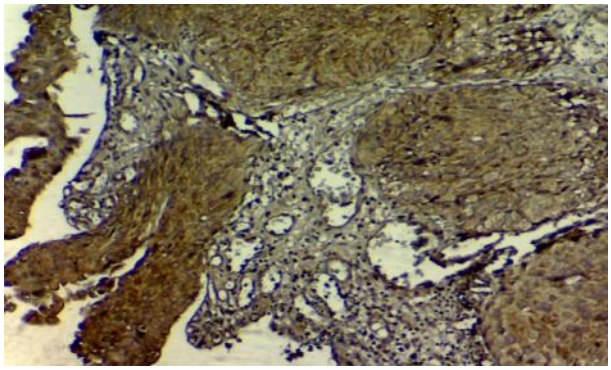

FIGURE 3: TCC, high grade, showing strong cytoplasmic staining of TLR2. (immunohistochemical staining for TLR2,X100)

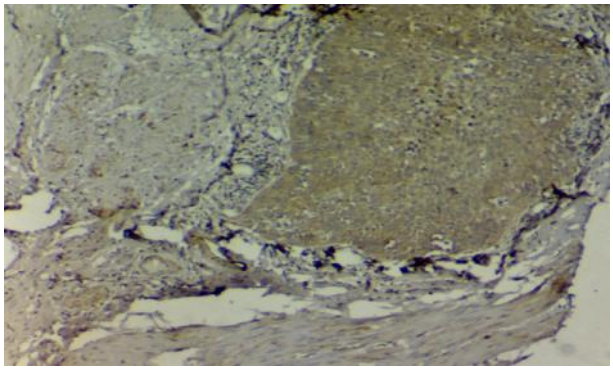

FIGURE 4: TCC ,muscle invasive, high grade showing moderate cytoplasmic reaction (immunohistochemical staining for TLR2, X100)

TABLE 7: Scoring of TLR-2 in invasive and non- invasive TCC

TLR-2 expression	Invasive tu. No. (%)	Non –invasive tu. No. (%)	Total No. (%)
Weak (0–4)	10 (37.0)	9 (30.0)	19(33.3)
Moderate (5–8)	5 (18.5)	11(36.7)	16(28.07)
Strong (9–12)	12 (44.5)	10 (33.3)	22(38.6)
Total No.(%)	27(47.7)	30(52.4)	57(100)
Chi-square	7.63		
p value	0.022 (significant)		

TABLE 8: Scoring of TLR-2 in bladder cancer patients according to gender

TLR-2 expression	Male No. (%)	Female No. (%)	Total No. (%)
Weak (0–4)	16 (32.0)	3 (42.8)	19(33.3)
Moderate (5–8)	14(28.0)	2 (28.6)	16(28.07)
Strong (9–12)	20 (40.0)	2 (28.6)	22(38.6)
Total No. (%)	50(87.7)	7(12.2)	57(100)
Chi-square	37.0		
P-value	0.000		

TABLE 9: Scoring of TLR2 for bladder cancer patients in relation to risk factors

	TLR2 IHC scores				
Risk factors	Weak (0-4)	Moderate (5-8)	Strong (9-12)	Total No. (%)	Chi-square P value
Smoking					3.65
Yes No. (%)	11 (32.3)	10 (29.4)	13 (38.1)	34(59.64)	0.161
No No. (%)	8 (34.8)	6 (26.0)	9 (39.2)	23(40.3)	(not significant)
S heamatobium					378.
Yes No. (%)	1 (11.1)	2 (22.2)	6 (66.7)	9(15.78)	0.000
No No. (%)	18 (37.5)	14 (29.1)	16 (33.4)	48(84.21)	(highly significant)
UTI					9.87
Yes No. (%)	10 (34.4)	5 (17.2)	14 (48.4)	29(50.8)	0.007
No No. (%)	9 (32.1%)	11(39.4)	8 (28.5)	28(49.1)	(highly significant)
Stones					452
Yes No. (%)	3 (50.0)	1 (16.6)	2 (33.4)	6(10.5)	0.000
No No. (%)	16(31.3)	15(29.4)	20(39.2)	51(89.5)	(significant)
Family History					290
Yes No. (%)	3 (42.8)16	2 (28.6)	2 (28.6)	7(12.28)	0.000
No No. (%)	16(32.0)	14 (28.0)	20 (40.0)	50(87.7)	(highly significant)
Total	19(33.3)	16(28.07)	22(38.6)	57(100)	

Scoring of TLR-2 in bladder cancer patients according to gender

According to gender, weak, moderate and strong expression of TLR2 in male were 16(32%), 14(28%) and 20(40%) respectively, while the expression were decreased in female to 3 (42.8%), 2 (28.6%) and 2 (28.6%) respectively, with highly significant difference (P =0.000) were noted as seen in (Table 8).

Scoring of TLR-2 in TCC of bladder in relation to risk factors

According to risk factors of bladder cancer, out of 57 bladder cancer patients, 34(59.64%), 9(15.78%), 29(50.87%), 6(10.52%), 7(12.28%) were (smoking, with a history of Schistosomiasis, UTI, stones and family history of cancer) respectively, this risk factors may inducing in urotheli Significant correlation (P= 0.000) was found between TLR2 over expression and bladder cancer group with history of *schistosomiasis*, in which

TLR2 moderate and strong expression was in 2 (22.2%) and 6 (66.7%) respectively, while 48 non- schistosomal bladder cancer cases showed moderate and strong expression in 14 (29.1%) and 16 (33.4%) respectively, also significant difference was found in TLR2 expression between cancer patients with and without UTI with 14 (48.4%) out of 29 and 8 (28.5%) of 28 respectively (P=0.007) ,while significant correlation was found as well in TLR2 over expression and patients without history of stone of which it was (39.1%) in patients without stones and (33.4%)in patients with stones (P= 0.00),in addition, patients with negative family history of cancer showed a highly significant over expression in TLR2 scoring (P =0.00) in which it was 20 (40.0%) out of 50 ,in comparison with cases having bladder cancer with family history over expression was seen in 2 (28.6%) of 7, on the other hand, non-significant difference (P= 0.161) were demonstrated between TLR-2 expression and smoking, with 34 cases out of 57 cancer patients were smoke, with moderate and strong expression was seen in 10 (29.4%) and 13 (38.1%) of them, while moderate and strong expression in non-smoker cancer patients was 6 (26.0%) and 9 (39.2%) of 23 cases (Table 9).

Immunohistochemistry of TLR-4

Scoring of TLR-4 in bladder tissue of bladder cancer patients and normal autopsies

The percentage of positively(PR) stained cells, Staining intensity of positively stained cells (SI) in bladder cancer tissue and normal autopsies, then the product of PR and staining intensity scores represents the total IHC score that ranges from 0 – 12, with cut-off value of >4 determined IHC positivity, these were summarized in (Table10, Table11). Out of 57 bladder cancer cases, 55 (95.8%)showed over expression of TLR-4, while only 4 of 40 normal urothelium (10%)showed this over expression, from these Chi –square test showed highly significant difference in TLR4 expression of these two group (P= 0.000).

TABLE 10: The percentage of positively(PR) stained cells, staining intensity of positively stained cells (SI) of TLR-4 in bladder tissue of bladder cancer patients and normal autopsies

Percentage of positively stained cells of TLR4	Staining Intensity of TLR4						Total No.
	0 (Score 0)		Weak (Score1)		Moderate (Score 2)	Strong (Score 3)	
	Patients No.	Controls No.	Patients No.	Controls No.	Patients No.	Controls No.	
(Score1) 1 -10%	0 , 0	24, 0	0	0	0	0	24
(Score 2) >11-40%	0	0	0	0	0	0	0
(Score 3) >41-70%	1	0	4	0	6	4	15
(Score 4) >70-%	1	12	5	0	40	0	58
Total No.	2	36	9	0	46	4	97

TABLE 11: Final score of TLR-4 expression in bladder tissue of bladder cancer patients and normal urothelium

TLR4 expression	Patients No. (%)	Normal urothelium No. (%)	Total No. (%)
Positive>4	55 (95.8)	4(10)	59(60.8)
Negative<4	2 (4.2)	36(90)	38(39.1)
Total No (%)	57(58.76)	40(41.2)	97
Chi-square	625.		
P-value	0.000 (highly significant)		

TABLE 12: The percentage of positively(PR) stained cells and Staining intensity of positively stained cells (SI) of TLR-4 in bladder tissue of newly diagnosed and recurrent bladder cancer cases

Percentage of positively stained cells TLR4	Staining Intensity of TLR4						Total No.
	No (Score 0)	Weak (Score1)		Moderate (Score 2)		Strong (Score 3)	
	Newly No.	Recurrence No.	Newly No.	Recurrence No.	Newly No.	Recurrence No.	
(Score1) 1 -10%	0 , 0	0 , 0	0	0	0	0	0
(Score 2) >11-40%	0	0	0	0	0	0	0
(Score 3) >41-70%	0	1	3	1	2	4	11
(Score 4) >70-	1	0	1	4	21	19	46
Total No.	1	1	4	5	23	23	57

TABLE 13: Final score of TLR-4 in newly diagnosed and recurrent bladder cancer patients

TLR4 expression	Newly diagnosed No. (%)	Recurrence No. (%)	Total No. (%)
Positive>4	27 (96.5)	28 (96.6)	55(95.8)
Negative<4	1 (3.5)	1 (3.4)	2(4.2)
Total No (%)	28 (49.1)	29 (50.9)	57 (100)
Chi-square	0.357		
P-value	0.850 (not significant)		

Scoring of TLR-4 IHC in bladder tissue of newly diagnosed and recurrent cases of bladder cancer

Table12 shows that TLR-4 over expression were detected in 27 of 28(96.5%) of newly diagnosed cases, as well as from 28 out of 29 (96.6%) recurrent cancer patients, with Chi –square showed no significant difference in expression of this marker between these two groups(Table13).

Scoring of TLR-4 in relation to bladder tumor grade

Regarding tumor grade, TLR-4 was detected as positive in all 30 low grade TCC cases (100%) and decreased to 25 out of 27 (92.6%) of high grade tumor with non-significant difference between them (P= 0.22) as seen in (Table14 and 15) (Fig 5, 6 and 7). It was noted that both cytoplasmic and membranous staining was presented in tumor cells.

TABLE 14: The percentage of positively(PR) stained cells, Staining intensity of positively stained cells (SI) of TLR-4 in low and high grade TCC

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Percentage of positively of TLR4	Staining Intensity of TLR4						Total No.
	0 , Weak (score 0), (score1)		Moderate (score 2)		Strong (score 3)		
	Low grade	High grade	Low grade	High Grade	Low Grade	High Grade	
(Score1) 1 -10%	0 , 0	0 , 0	0	0	0	0	0
(Score 2) >11-40%	0	0	0	0	0	0	0
(Score 3) >41-70%	0	1	1	3	2	4	11
(Score 4) >70-	0	1	2	3	25	15	46
Total NO.	0	2	3	6	27	19	57

TABLE 15: Final score of TLR-4 expression in low and high grade bladder cancer tissue

TLR-4 expression	Low grade No. (%)	High grade No. (%)	Total No. (%)
Positive>4	30 (100)	25 (92.6)	55(95.8)
Negative<4	0	2 (7.4%)	2(4.2)
Total No.(%)	30(52.6)	27(47.4)	57
Fisher exact	*P*= 0.220		

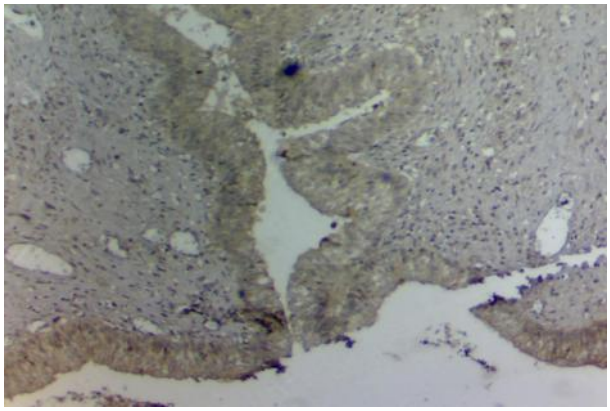

FIGURE 5: faint staining of TLR4 in normal bladder surface epithelium, immunohistochemical staining for TLR4 (X100)

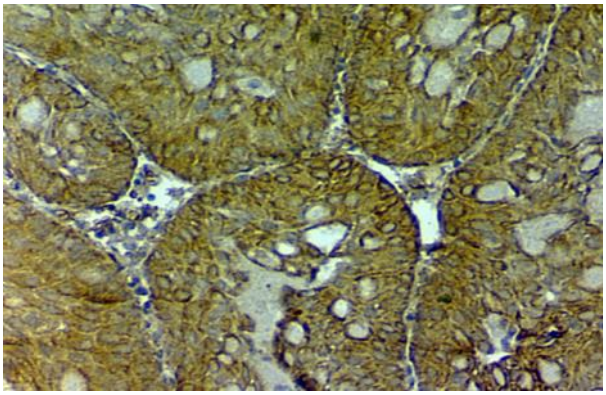

FIGURE 6 : TCC, Low grade showing intense membrane staining of TLR4 (positive expression), immunohistochemical staining for TLR4, X100)

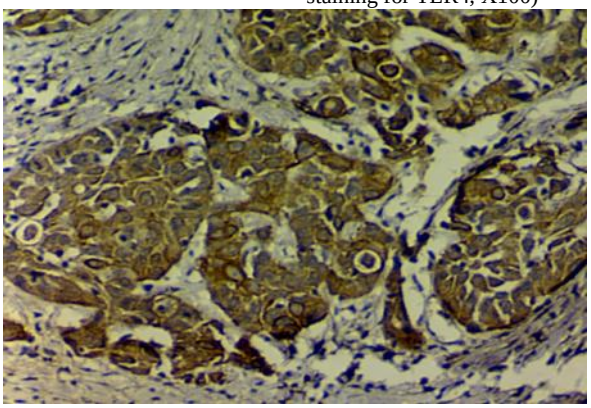

FIGURE 7 : Strong cytoplasmic, membranous staining of TLR4 in TCC, high grade (immunohistochemical staining for TLR4, X100)

Scoring of TLR-4 in relation to tumor muscle invasion

A positive TLR-4 was detected in 25of 27(92.6%) invasive cancer patients, as well as in all 30 (100%)non-invasive cases (Table16), with statistically no significant difference demonstrated (P= 0.22).

Scoring of TLR-4 in bladder cancer patients according to gender

The positive expression of TLR-4 was demonstrated in all 50 male patients (100%), while its expression was decreased in female (71.5%), with significant statistical difference (P =0.013) estimated between them(Table17).

The relation of risk factors with TLR-4 expression in bladder tumor tissue

Table18 shows that, a statistically high significant correlation of TLR-4 expression was noted only between it and non - Schistosoma infection (P= 0.000), with a positive expression was detected in 47 of 48 (97.92%) non- Schistosomal bladder cancer cases, while 8 out of 9 (88.9%) Schistosomal bladder cancer patients showed this positive expression. On the other hand, its expression were non-significant with smoking (P= 0.056), UTI (P =0.237), history of stone (P =1.00), and family history of cancer (P= 1.00)

TABLE16: Expression of TLR-4 in bladder cancer biopsy in relation to tumor muscle invasion

TLR-4 expression	Invasive No. (%)	Non- invasive No. (%)	Total No. (%)
Positive>4	25 (92.6)	30 (100)	55 (95.8)
Negative<4	2 (7.4)	0	2(4.2)
Total No.(%)	27(47.4)	30(52.6)	57
Fisher exact	p = 0.220		

TABLE 17: Final score of TLR-4expression in male and female bladder cancer patients

TLR-4 expression	Male No. (%)	Female No. (%)	Total No. (%)
Positivity >4	50 (100)	5 (71.5)	55(96.5)
Negative<4	0	2 (28.5)	2(3.5)
Total No.(%)	50(87.71)	7(12.28)	57(100)
Fisher exact	0.013		

Table18: Scoring of TLR-4 for bladder cancer patients in relation to risk factors

	TLR4 IHC scores			
Risk factors	Negative (<4) No. (%)	Positive (>4) No. (%)	Total No. (%)	Statistical analysis
Smoking				
Yes	1 (2.9)	33 (97.1)	34(59.64)	Chi-square 3.67
No	1 (4.3)	22 (95.7)	23(40.35)	0.056 (non-sig)
Schistosomiasis				
Yes	1 (11.1)	8 (88.9)	9 (15.78)	Chi-square 190.
No	1 (2.08)	47 (97.92)	48(84.21)	0.000(highly sig)
UTI				
Yes	0	29 (100)	29(50.87)	Fisher's exact
No	2(7.1)	26 (92.9)	28(49.12)	P = 0.237
Stones				
Yes	0	6 (100)	6(10.52)	Fisher's exact
No	2 (3.9)	49(94.1)	51(89.47)	p = 1.000
FamilyHistory				
Yes	0	7 (100)	7(12.28)	Fisher's exact
No	2 (4.0)	48(96.0)	50(87.71)	P = 1.000
Total	2(3.5)	55(96.4)	57(100)	

DISCUSSION

Immunohistochemical study of Toll like receptors 2 and 4

In this study, (38.5%, 28.07%, and 33.33%) of bladder cancer patients showed strong, moderate and weak expression for TLR2 while, all of normal autopsies were in weak expression, statistically significant increase was found in TLR2 expression in bladder cancer tissue in comparison to normal autopsies (P=0.000). Analogous result was obtained with TLR4 in which TLR4 over expression was detected in 55 (95.8%) out of 57 bladder cancer while, only 4(10%) of normal autopsies were with high expression of TLR4 with significant difference (P= 0.000).Similar finding were recorded by (Ng *et al*., 2011) in their suggestion that *TLR2 over* expression by malignant keratinocytes may be indicative of resistance to apoptosis as a prosurvival mechanism and (Yuan *et al*., 2013) in their study of TLR4 and gastric carcinoma and as well, d'Adhemar *et al*., 2014 in pointed the role of TLR4 expression in reducing overall survival (d'Adhemar *et al*., 2014), yet different results were obtained by (Ayari *et al*., 2011; Wang *et al.*, 2014; Stopiglia *et al*., 2015) in decreasing TLR experssion in tumor cells. The outcome of these studies and ours in term of increase or decrease TLR2, 4 expression has been proposal to regard TLR2, 4 could leads to either up-regulation of cellular defense mechanisms through recruitment of leukocytes and increscent of vascular permeability, to be followed by lysis of tumor cell by both natural killer (NK) and cytotoxic T cell, or provide microenvironment that is necessary for tumor cells to proliferate and evade the immune response (Drexler and Foxwell, 2010). As TLR promotes carcinogenesis by up-regulation of *Nuclear factor kappa-B* (NF-κB) cascade as well as, by production of anti-

apoptotic proteins, which were explained by the difference in intensity and nature of the inflammatory response with chemokines expressed by tumor cells and host cells regulating the migration of different leukocyte type as macrophages, T cells, NK cells and dendritic cells (Rakoff Nahoum and Medzhitov, 2009), with the defense cell proportion within the tumor will determine the immune profile at the tumor site (Srikrishna and Freeze, 2009). These immune cells release further proinflammatory cytokines, proangiogenic factors and growth factors, which impair the anti-tumor function (Sato *et al*., 2009), also up-regulation of DNA repair genes and increased functional DNA repair (Srikrishna and Freeze 2009; Rakoff- Nahoum and Medzhitov 2009; Harberts and Gaspari, 2013). In current study, (35.8%) of newly diagnosed cancer patients have strong expression of TLR2 whereas (41.5%) of recurrence case were strong expression of TLR2 with non–significant difference between them, similar finding was reported with TLR4 and indicated no significant difference in TLR4 over expression in newly 27 (96.5%) and recurrent cases 28 (96.6%), this was agree with (Kim *et al*., 2012) in ovarian epithelial cancers (OEC) of TLR4. On regard to tumor grade and muscle invasion, strong correlation was found between TLR2 over expression with grade and muscle invasion, (p 0.02, 0.018). Another finding of the current study was the lack of significant correlation between TLR 4 over expression with grade and invasion, there was a complete agreement with (d'Adhemar *et al*., 2014) in no correlation between grade and TLR4 over expression in ovarian cancer, and with (Kim *etal*.,2012) in lack of correlation between grade and stage of OECs with TLR4 but different result were obtained by (Stopiglia *et al*., 2015) whom demonstrated that TLR2 and TLR4 immune reactivities were significantly lower in low-grade, non-muscle invasive and muscle invasive bladder carcinoma than normal, and this may contribute to the high tumor relapse and progression rates. Morever, (Fávaro *et al*., 2012) demonstrated significant decrease of TLR 2 and 4 protein levels in animal's model for non-muscle invasive bladder cancer in relation to normal animals. In contrast to (Yuan *et al.*, 2013) which detected strong positive immunohistochemistry (IHC) staining of TLR4 for advanced-stage gastric carcinoma whereas moderate or weak staining for early-stage tumors while, normal gastric epithelia and stroma were generally negative for TLR4.

The ccorrelation of TLR2, 4 with risk factors of bladder cancer, showed ssignificant associations between TLR2 over expression with schistosomaisis (P=0.000) and urinary tract infection(UTI) (P=0.007), which may be explained as the effect of chronic inflammation and irritation that could promote the proliferation of cells and thus, the development of cancer (Balkwill and Coussens, 2004) by stimulate *Nuclear factor kappa- B* (NF-κB) activation as a result of their interaction with TLR2, 4/(CD14) Cluster of Differentiation Antigen receptor complexes, led to suppression of apoptosis and reduced activation of Tumor protein53(p53) and its responsive genes (Gudkov *et al*., 2011) as well as activation of oncogenes cytokine Tumor necrosis factor- alpha (TNF-α), Interleukin-1 (IL-1), IL-6, IL-8, IL-10, IL-2 and Matrix metallopeptidase 9(MMP9), and induction of immunosuppression (sato *et al*., 2009; Ioannou and Voulgarelis, 2010).

CONCLUSION

Recent study confirmed that bladder cancer cells over expressed TLR2, 4 and this described by the involvement of TLR2,4 signaling in promoting tumor development that correlated with presence of TLR2,4 in low and high grade BC. Our findings demonstrate that expression of TLR2 is associated significantly with high grade and muscle invasion, while, there was no significant correlation between TLR4 expression and grade, stage recurrence of the disease.

RECOMMENDATION

Because genetic variations between Toll-like receptors this may lead in associated with cancer risk, Some reports have provided evidence that TLRs facilitates tumor progression and angiogenesis, whereas others suggest that TLRs signaling inhibits tumor progression, so large number of studies need to demonstrate a role for Toll-like receptors (TLRs) in carcinogenesis, and the therapeutic potential of TLRs in bladder cancer treatment using different way of diagnosis like real time polymerase chain reaction(RT PCR), enzyme linked immunosorbant assay (ELISA)test and experimental studies.

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