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Assessment of Toll like receptors (TLR-8 and TLR-9) in group of patients with different types of tumor in Babylon Provence of Iraq

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Abstract : Background: Toll-Like Receptors (TLRs) play a critical role in the early innate immune response to invading pathogens. Significant progress has been made over the past years in the understanding of TLR function. TLR agonists are currently under investigation in anticancer therapies for their ability to activate immune cells and promote inflammation.
Objectives: Assessment the state of Toll-Like Receptors (TLR-8 and TLR-9) in patients with different type of tumors and their relation to patient's demographic and clinical features.
Patients, material and methods: Case control study among all newly diagnosed patients with different types of tumor registered in the malignant registry center of Babylon clinically diagnosed and confirmed by consultant physician including 210 patients conducted from January until August 2016. Among All patients are eligible for study chosing (100) patients and exclusion ther others because of either they are start medication for tumor, have more severe cases and some refuse participation in the study , was there in associated with 100 control groups recruited from same geographical area of the patients, mainly are patient relatives, age and sex matched, selected randomly. Consent was taken from all patient and control in addition to the center permission for this study. Serum level of TLR-8 and TLR-9 was assess by ELISA in the laboratory department of Merjan medical city/ immunology unit using specific available kits.
Results: Mean age of patients was 49.9 years mostlyat 40s age group, no significant statistical difference according to the gender of patient and control groups. More patient and control group are from urban area. Majority of patients have breast malignancy (52%) and the other sites represented different sites of uterine, colonic, liver, ovarian, thyroid, stomach, and gall bladder and prostatic malignancies. Ten percent of patient have family history of malignancy and chronic disease associated with malignancy are present in 44% of patient.
Conclusion: Significant low concentration of TLR8 and high concentration of TLR9 was found in different types of tumors in initial their diagnosis with obvious significant correlation of high TLR9 concentration with demographic and clinical features of the patients while not to TLR8 level. Assessment of such types of receptors may play a role in anti-tumor therapy and research of related aspect.
Keywords: Toll-Like Receptors, TLR, Babylon, Iraq.

Introduction

Toll-Like Receptors (TLRs) play a critical role in the early innate immune response to invading pathogens by sensing microorganism and are involved in sensing endogenous danger signals. TLRs recognize highly conserved structural motifs known as pathogen-associated microbial patterns (PAMPs), which are

exclusively expressed by microbial pathogens, or danger-associated molecular patterns (DAMPs) that are endogenous molecules released from necrotic or dying cells. PAMPs include various bacterial cell wall components such as lipopolysaccharide (LPS), peptidoglycan (PGN) and lipopeptides, as well as flagellin, bacterial DNA and viral double-stranded RNA. DAMPs include intracellular proteins such as heat shock proteins as well as protein fragments from the extracellular matrix. Signaling by TLRs result in a variety of cellular responses including the production of interferons (IFNs), pro-inflammatory cytokines and effector cytokines that direct the adaptive immune response[1].

TLR3, TLR7, TLR8 and TLR9 recognize viral nucleic acids and induce type I IFNs. The signaling mechanisms leading to the induction of type I IFNs differ depending on the TLR activated. They involve the interferon regulatory factors, IRFs, a family of transcription factors known to play a critical role in antiviral defense, cell growth and immune regulation. Three IRFs (IRF3, IRF5 and IRF7) function as direct transducers of virus-mediated TLR signaling. TLR3 and TLR4 activate IRF3 and IRF7 [2].

Significant progress has been made over the past years in the understanding of TLR function. TLRs are essential receptors in host defense against pathogens by activating the innate immune system, a prerequisite to the induction of adaptive immune responses. Although TLR-mediated signaling is paramount in eradicating microbial infections and promoting tissue repair, the regulation must be tight. TLRs are implicated in a number of inflammatory and immune disorders and play a role in cancer by act as double-edged swords either promoting or inhibiting disease progression. TLRs, also used to prevent or treat human inflammatory and autoimmune diseases as well as cancer [3].

The immune system has acquired increasing importance as a key player in cancer maintenance and growth. Thus, modulating anti-tumor immune mediators has become an attractive strategy for cancer treatment. Toll-like receptors (TLRs) have gradually emerged as potential targets of newer immunotherapies. TLR-9 is preferentially expressed on endosome membranes of B-cells and plasmacytoid dendritic cells (pDC) and is known for its ability to stimulate specific immune reactions through the activation of inflammation-like innate responses. TLR-9 agonists have been also tested in several clinical trials in patients with solid tumors. These agents showed good tolerability and usually met activity endpoints in early phase trials. More recently, this view is changing as TLR-9 is considered more in general a receptor for non-self-nucleic acids found in the endosomes [4].

Endosomal toll-like receptors (TLR-7, TLR-9) activate innate immune responses by signaling damage-associated molecular patterns (DAMP) from decaying tumor cells. This has led to the development of DNA-based TLR-9 agonists, which induce antitumor activity through innate and subsequent adaptive immune responses. During tumor celldeath, a variety of molecules are dispersed that act as signals to the innate immune system warning of damage ordanger. They are recognized by a variety of receptors, including the toll-like receptors (TLR) found on innateimmune cells, resulting in activation of a cancer-associated inflammatory response [5].

TLR agonists are currently under investigation as vaccine adjuvants in anticancer therapies for their ability to activate immune cells and promote inflammation. In humans, although TLRs have been detected on many cell types, most TLRs are expressed primarily on monocytes, mature macrophages, and DCs. However, there is a potential for TLR signaling to occur on cells other than professional APCs that could negate antitumor responses or even worse, promote tumor growth. TLR signaling within different T cell subsets and cancer cells can potentially impact the generation of antitumor responses. A growing body of evidence indicates that TLRs are expressed or can be induced on various cell types, including T cells and tumor cells[6]. **Objectives:** Assessment the state of TLR8 and TLR-9 in patients with different type of tumors and their relation to patient's demographic and clinical features.

Patients, materials and methods

Patient data base: We collected all data available about the patients registered in the malignant registry center of Babylon with different types of tumors, including 100 adult patients are clinically diagnosed and confirmed by consultant physician in the specialist center.

Study design and setting: This is a case control study among group of patients with different types of tumors registered in malignant registry center of Babylon living in the community of middle Euphrates of Iraq of different areas from Babylon, Najaf, Karbalaa, Diwania, Simawa and Kut cities. This study carried out from January until August 2016 to evaluate the status of Toll Like Receptors (TLR-8 and TLR-9) in those group of patients, conducted in associated with medical laboratory department of that center.

Cases definition and ascertainment:

Inclusion criteria: A total of 210 newly diagnosed patients with different type of tumors were registered in the malignant registry center of Babylon, and 100 of the patients are eligible for study aim those are newly diagnosed without medication and agree to participate in study.

Exclusion criteria: We areexclude110 patients from the study either because they are start medication for tumor, have more severe casesa nd some refuse participation in the study.

Permission from patients: The study protocol was done under permission from the center, in addition to verbal consent to the laboratory studies was obtained from each individual participating in this study.

Assessment of exposure: After assessment the criteria required to the patients to be eligible for the study, the status of Toll Like Receptors (TLR-8 and TLR-9) of each patient was assess.

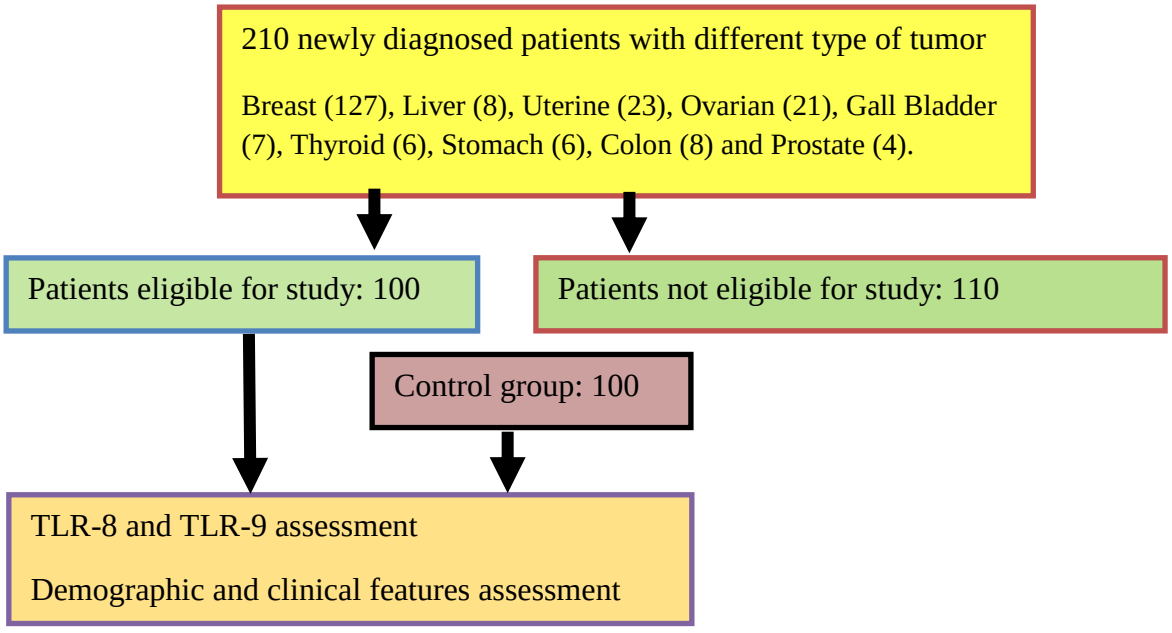
Potential confounders: Chronic disease like hypertension, DM, allergy are consider as confounder factors that may related affect the concentration of TLR-8 and 9.

Validation of the result toTLR-8 and TLR-9 level was not done due to our limitation in the cost, time and technical facility.

Power calculation: Assuming that the patients have such status of TLR-8 and 9 in review similar articles, a sample size of 100 patients and control group of 100 individual had a power of 80% at a type 1 error rate of 5%. Statistical analysis were done by using Chi –square, ANOVA, Odd ratio and T–Test of SPSS program.

Control group: 100 individuals relative to patients with age and sex matching of same recruitment of the patients are consider as control group selected randomly with role of one every three sequence.

Flowchart of individuals included in this study



Materials and methods:

Venous blood was taken from all cases and control groups in sterile venipuncture. Four mls were collected in plain tube for serum separation. Small aliquot was achieved for deep freezing until all samples was analyzed using ELISA technique of special kits provided by Elab- Seince Company. This analysis was done in the laboratory department of Merjan medical city/ immunology unitusing semi-automated ELISA according to corresponding associated kit leaflet of Bioelisa Company.

Results:

Demographic features of patients and control groups are demonstrated in the table number 1, that showing mean age of patients 49.9 years and control 50.1 years with no significant statistical difference. Most of the patients rank in the age group of 40s while the control group at 50s decade. No significant statistical difference according to the gender of patient and control groups but with prominent female to male occurrence of malignancy. More patient and control group are from urban area (68%) than rural one (32%) with significant statistical difference (P value 0.031).

Majority of patients have breast malignancy (52%) and the other sites represented 8% for each uterine and colonic malignancies, 6 % for liver, ovarian, thyroid and stomach malignancies, and 4% for gall bladder and prostatic malignancies. Ten percent of patient have family history of malignancy. Presence of chronic disease associated with malignancy are present in 44% of patient.

Table 1. Demographic features of patients and control groups

Characteristic		Patient No. 100	Control No. 100	P. value
Age	Mean (Range) yrs	49.9 (27 -74)	50.1 (28 -74)	0.183
	Groups			
	21 -30 Years	6.0	5.0	
	31 -40 Years	14.0	11.0	
	41 - 50 Years	30.0	28.0	
	51 -60 Years	26.0	29.0	
	> 60 Years	24.0	27.0	
Gender				
	Male	22.0	26.0	
	Female	78.0	74.0	0.097
Address				
	Urban	68.0	62.0	
	Rural	32.0	38.0	0.031
Type of malignancy: No. (%)				
	Breast malignancy	52.0 (52%)		
	Liver malignancy	6.0 (6%)		
	Uterine malignancy	8.0 (8%)		
	Ovarian malignancy	6.0 (6%)		
	Gall Bladder malignancy	4.0 (4%)		
	Thyroid malignancy	6.0 (6%)		
	Stomach malignancy	6.0 (6%)		
	Colon malignancy	8.0 (8%)		
	Prostate malignancy	4.0 (4%)		
Family history of malignancy				
	Presence	10.0		
	Absence	90.0		
Chronic disease (Hypertension, DM, allergy)				
	Presence	44.0		
	Absence	56.0		

Table 2 showing the highly statistical significant low concentration of TLR-8 in patients (2.25 ± 0.44) than control groups (5.43 ± 0.43) while the TLR-9 concentration present in highly significant high concentration (6.69 ± 0.67) than control group (2.49 ± 0.32).

Table 3 reveal that TLR-8 concentration abnormality have no significant association with patient demographic features like gender, address, type of malignancy, family history of malignancy and even presence or absence of chronic disease, while concentration abnormality of TLR-9 showing significant association with patient features of gender, address, type of malignancy, family history of malignancy as showing in table 4.

Table 2. Status of TLR-8 and TLR-9 concentration in patients and control groups:

TLR	Patient No. 100 (M ±SD)	Control No. 100 (M ±SD)	P. value
TLR-8 concentration (pg/ml)	2.25 ± 0.44	5.43 ± 0.43	0.000
TLR-9 concentration (pg/ml)	6.69 ± 0.67	2.49 ± 0.32	0.000

Table 3. Association of TLR-8 state with patient parameters

Characteristic		No. Patient with normal concentration of TLR-8	No. Patient with abnormal concentration of TLR-8	P. value
Gender	Male	12.0%	10.0%	
	Female	50.0%	28.0%	0.12
Address	Urban	32.0%	30.0%	
	Rural	30.0%	8.0%	0.36
Type of malignancy	Breast Ca.	36.0%	22.0%	
	Liver Ca.	4.0%	2.0%	
	Uterine Ca.	4.0%	4.0%	
	Ovarian Ca.	4.0%	2.0%	0.086
	Gall Bladder	2.0%	0.0%	
	Thyroid Ca.	2.0%	4.0%	
	Stomach Ca.	4.0%	2.0%	
	Colon Ca.	6.0%	0.0%	
	Prostate Ca.	0.0%	2.0%	
Family history of malignancy				
	Presence	6.0%	4.0%	
	Absence	56.0%	34%	1.25
Chronic disease (Hypertension, DM, allergy)				
	Presence	16 .0%	28.0%	
	Absence	24.0%	32.0%	0.93

Table 4.Association of TLR-9 state with patient parameters

Characteristic		No. Patient with normal concentration of TLR-9	No. Patient with abnormal concentration of TLR-9	P. value
Gender	Male	4.0%	18.0 %	
	Female	4.0%	74.0 %	0.012
Address	Urban	6.0 %	56.0 %	
	Rural	2.0 %	36.0 %	0.023
Type of malignancy	Breast Ca.	4.0%	54.0%	
	Liver Ca.	0.0%	6.0 %	
	Uterine Ca.	0.0%	8.0%	
	Ovarian Ca.	2.0%	4.0%	0.020
	Gall Bladder	0.0%	2.0%	
	Thyroid Ca.	0.0%	6.0%	
	Stomach Ca.	0.0%	6.0%	
	Colon Ca.	0.0%	6.0%	
	Prostate Ca.	2.0%	0.0%	
Family history of malignancy				
	Presence	2.0%	20.0 %	
	Absence	6.0%	72.0 %	0.041
Chronic disease (Hypertension, DM, allergy)				
	Presence	3 .0%	28.0%	
	Absence	5.0%	64.0%	0.58

Discussion:

Demographic features:

Mean age of our patients in this study come close to the age of control group with no significant statistical difference due to our selection of control group matched in age and gender to the patients in attempt to reduce bias of some confounders like age. Most of the patients rank in the age group of 40s decade mostly to predominant evidence of breast malignancy in the patient group and certainly the breast cancer is one of the most common malignancy in our society. No significant statistical difference according to the gender of patient and control groups due to the same explanation of control selection criteria and their matching to patients group. More patient and control group are from urban area with significant statistical difference and this may explained by hypeine level and life style such as women who use oral birth control pills for most of their life have a slightly higher chance of developing breast cancer. Also those who live a sedentary lifestyle are at an increased risk of breast cancer[7]. Majority of the patients have breast malignancy (52%) and this percentage consistent with the percentage of Babylon center of malignancy as 60.47 % for patients at time of study. Also this result is consistent with the study done by Ruddy, 2013[8]who stated that, breast cancer is the second most common cancer among women in the world. Breast cancer occurs almost entirely in women, but men are able to get it, too. Maukayed, 2013[9] mentioned that the geographical variation in breast cancer incidence or mortality rates is compared statistically with solar UVB amounts. Such studies in Australia, China, France, Nordic countries, Spain and the United States have found lower breast cancer rates in regions of higher solar UVB.

Cancer statistics are hard to come by, since only 50 % of the healthcare in Iraq is public, the other half of our healthcare is provided by the private sector, and that sector is deficient in their reporting of statistics. Hence, all of our statistics in Iraq must be multiplied by two. Any official numbers are likely only half of the real number.

In Babylon Province, cancer rates have been escalating at alarming rates that due to using of depleted uranium weapons by US forces during and following the 2003 invasion. Breast was by far the most common site of cancer accounted for 16% of all Iraqi patients. Lungs and the bronchi were the second most common site of cancer. Leukemia was the third most common cancer in Iraq accounting for 7% of all cancers. The four most

commonly diagnosed types of cancer among males were cancers of the lung and bronchus, bladder, leukemia and Non- Hodgkin lymphoma (NHL), accounting for about 37.7% of estimated cancer cases in males. The four most commonly diagnosed types of cancer among females were cancers of the breast, leukemia, uterus including cervix and corpus) and cancers of brain and CNS, accounting for about 47.5% of estimated cancer cases in females. Breast cancer alone is accounted for % (31%) of all new cancer cases among females[10].

The top 10 cancer incidence in Iraq was breast cancer followed by lung cancer, leukemia, bladder cancer, brain and CNS, non-Hodgkin's lymphoma, colo-rectal cancer, stomach cancer, skin cancer excluding melanoma, larynx cancer. Cancer incidence rate significantly increases after 2000 in comparison with the period before 2000.Cancer incidence in Iraq is relatively high and trends are upgoing in terms of quantity and variables related like age, sex, etc.[11].

Ten percent of patient have family history of malignancy and can't compare with control group those selected apparently healthy without personal or family history of malignancies. Presence of chronic disease like hypertension, DM, allergy associated with malignancy are present in 44% of patient and consider in our study as confounder factors that may alter the result.

Toll Like Receptors status:

Highly statistical significant low concentration of TLR-8 was found in patients than control groups but the normal and abnormal levels of such receptor showing no significant difference in the patients that explained by Sabina et al., 2013[6] in which concluded that, the antitumor responses are largely attributed to their aptitude to stimulate APCs such as DCs which in turn, activate tumor-specific T cell responses. However, there is a potential for TLR signaling to occur on cells other than professional APCs that could negate antitumor responses or even worse, promote tumor growth, the engagement of TLRs on different T cell subsets and different cancer types could promote tumor growth or conversely, contribute to antitumor responses. TLR signaling within different T cell subsets and cancer cells can potentially impact the generation of antitumor responses. Based on evidence from preclinical models and clinical trials. The underline cause of tumor might be predicted by using the TLR expression in most tissues and immunological cells. In consistent with Soe and Ouchi, 2010[12]they was stated that TLR7 and TLR8 bind viral SSRNA, whereas TLR9 interacts with unmethylated CpG DNA from bacteria and some viruses. For this reasons the patients have more exposure to viral or bacterial infection might be activating or expression of TLR than other with no viral infection, to confirm this fact more studies for patients with history of viral infection which recommended to investigation the TLR s expression. There was no significant association with patient demographic features. While the TLR-9 concentration present in highly significant high concentration than control group with big difference in normal and abnormal levels of such receptor that explained by Brignole, 2010[13]who stated that the TLR9 expression has been shown to exhibit anti-proliferative and pro-apoptotic effects, also been shown to decrease cell proliferation and increase caspase-dependent apoptosis, resulting in increased survival of tumor.

The engagement of specific TLRs on cancer cells can impact tumor growth by various mechanisms, including inducing apoptosis and potentiating the effects of chemotherapy, so this fact indicate that any future treatments intended to activate the immune system against cancer could benefit from the inclusion of TLR agonists[14].

This result might be indicated that the TLR 9 have unstable expression depending on the immunological state of patients, history of infection either bacterial or viral, enhancement of apoptosis in which that might be increased with tumor activity, so that showing significant association with patient features of gender, address, type of malignancy, family history of malignancy[15,16].

Conclusion:

Significant low concentration of TLR8 and high concentration of TLR9 was found in different types of tumors in initial their diagnosis with obvious significant correlation of high TLR9 concentration with demographic and clinical features of the patients while not to TLR8 level. Assessment of such types of receptors may play a role in anti-tumor therapy and research of related aspect.

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