Immunohistochemical Expression of Myeloid Differentiation Adaptor Protein-88(MYD-88)and Toll like Receptor (TLR-4) in Human Leiomyomas

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Abstract

Leiomyomas are the most common benign tumor of the female reproductive tract. The aim of this study was to explore the role of TLR4/ MYD-88signaling pathway in the pathogenesis of leiomyomas. Benign nature and smooth muscle origination of leiomyomas was easily documented via histological examination, in this cross-sectional study of 20 conventional leiomyomas cases TLR4/ MYD-88 antibody status was positive in 90% for TLR-4 and in 95% for MYD-88 of leiomyomas. Leiomyomas were stained with TLR4/ MYD-88 marker more than those in normal myometrium cases ($P<0.001$). In conclusion leiomyomas growth may be associated with TLR4 and MyD88 expression

***Key words**: Leiomyomas, TLR4, MYD-88, Myometrium,Immunohistochemistry*

Introduction

Uterine fibroids or leiomyomas of the uterus that arise from connective tissue and smooth muscle overgrowth of in uterus are benign tumors . Histologically, a monoclonal proliferation in smooth muscle cells occurs, it can develop wherever smooth muscle is present[1].

Women at reproductive age are mostly affected, incidence of fibroids is about 70% in women at 50 years age [2,3,4],most women develop uterine fibroid tumors before menopause[5,6] and are the leading indication for hysterectomy. When MRI examination revealed an abnormal increase in the size of a known uterine *leiomyoma* of the uterine wall. A treatment consisted of cyclophosphamide, adriamycin, and cisplatin with poor outcome [7], some women have painful, heavy periods while most women have no symptoms [8.] If fibroid become large, may push the bladder causing a need to urinate[8] . Female can have few or many uterine fibroid these make it difficult to pregnant, but this is uncommon[8]. Obesity and dietary components [8,] with hormone levels and genetic predisposition to leiomyoma growth often exists [9].

Estrogen and progesterone have a mitogenic effect on leiomyoma cells by influencing cytokines, hormones and a number of growth and apoptotic factors[10], estrogen promotes growth by increasing Insulin-like growth factor-1IGF-1, T*ransforming growth factor beta 1*,3 and decreasing p53. Progesterone promote the growth and survival of leiomyoma by up-regulating *Epidermal growth factor* EGF, B-cell lymphoma- 2 (Bcl-2) expression and down-regulating TNF-alpha[10] .

Five different fibroids classifications : 1-Intramural fibroids in the wall of the uterus the most common type.2- Subserosal fibroids in outer wall of the uterus can often grow to be larger and form a "stalk" on which the mass is attached (pedunculated fibroids). 3-Submucosal fibroids in muscle beneath the lining of the uterine wall (endometrium). 4-Intracavity fibroids in the cavity of the uterus. 5-Cervical fibroids form in the cervix of uterus,[11].

In humans the transmembrane protein Toll-like receptor 4 is a member of pattern recognition receptor (PRR) family encoded by the TLR4 gene. Its activation leads to an intracellular signaling pathway Nuclear factorNF-κB and inflammatory cytokine production which is responsible for activating the innate

immunity [12]. It is recognizing lipopolysaccharide (LPS) of Gram-negative bacteria as in Neisseria and Gram-positive bacteria also some proteins viral and a different endogenous proteins such heat shock protein [13]. TLR4 can detect on many tumor cells. It is capable of activating mitogen-activated protein kinase and pathways of NF-κB increased metastasis and carcinogenesis of tumor, inhibition apoptosis [14].On the other hand, TLR4 in inflammatory and immune cells of tumor microenvironment may lead to production of (Interleukin-6IL-6, tumor necrosis factorTNF, Interleukin 1 betaIL-1β, and IL-18) immunosuppressive cytokines, proinflammatory cytokines, (IL-10, TGF-β, etc.) and mediators angiogenic (Vascular endothelial growth factor VEGF, EGF, TGF-β), [14]. Myeloid differentiation primary response 88 (MYD88) is a protein that, in humans, is encoded by the MYD88gene[15]. The gene was originally discovered and cloned by Barbara Hoffman and Dan Liebermann in mice model[16], TLRs used an adapter proteinMYD88 to activate the transcription factor NF-κB., (TIR Domain Containing Adaptor ProteinTIRAP is necessary to recruit Myd88 to TLR 2,4, and MyD88 then signals by (interleukin-1 receptor-associated kinaseIRAK[17]. MyD88 mutant can act as a driver [18], promote tumor growth by its overexpression [19].

Materials and Methods

This retrospective study included 20 tissue biopsies of leiomyomas and 20 apparently normal myometrium biopsies collected from Al- Yarmouk Hospital in Baghdad. A specialist pathologist defined leiomyomas according to FIGO classification system [11].

Tissue preparation for Immunohistochemical diagnosis of TLR4 and MYD88 in section of paraffin-embedded.

Formalin10% were used to fix Biopsies, then biopsies dehydrated in ethanol and cleared with xylenes and finally embedding in paraffin [20].

· Immunohistochemical staining was performed using:-

a-Mouse monoclonal primary antibodies TLR-4(Santa cruz USA) Isotype: IgG

b- mouse monoclonal primary antibodies MYD88(Santa cruz,USA)Isotype: IgG,

c-PolyExcel HRP/DAB Detection System Secondary antibody, kit for Mouse and Rabbit Primary Antibodies (Pathnsitu, USA), which contain:-

1-Polyexcel HRP: Goat anti-rabbit Conjugate with HRP,50 ml (Pathnsitu, USA).

2-Polyexcel target binder :50 ml of rabbit anti-mouse (Pathnsitu, USA).

Procedure

· Four µm Sections of paraffin embedded leiomyomas specimens were cut and mounted on poly-lysine coated charge slides.

· Tissue slides deparaffinized with xylene, rehydrated in ethanol 95, 80 and 70% ,after that sections were subjected to antigen retrieval of citrate buffer (pH 9.0) by boiling in microwave oven for 10 min. Then endogenous peroxidase activity blocking with hydrogen peroxide. Following that for 1 h the sections were incubated with ready to use primary TLR-4 and MYD88 antibodies in a humidified chamber. The sections then incubated with polyExcel rabbit anti- mouse secondary antibody (Pathnsitu,USA) for 10 minutes then with PolyExcel HRP of Goat anti-rabbit Conjugate with HRP (Pathnsitu, USA) for 10 minutes then protein were detected with 3,3′-diaminobenzidine(DAB) substrate –chromogen that result in brown colored at the antigen site, finally section was stained with Mayer's hematoxylin for 1 min at room tempreture, rinsed with water, dehydrated in graded ethanol and xylene, covered with appropriate mounting media of DPX and cover slip.

Slides were examined by light microscope .The number of positive stained cells was evaluated as follows:

0, less than 5%;

1, 5% to less than 25%;

2, 25% to less than 50%;

3,50% to less than 75%;

4,more than 75%.

The intensity immunostaining was evaluated as: no staining 0; weak -light yellow1; moderate-yellow2; intense-brown yellow3. The total score was obtained

by multiplying the percentage and intensity score of stained cells. The final score less than 6 represented low expression and that of greater or equal to 6 represented high expression[21].

The prevalence rate was evaluated by the TLR4 and MYD88 index, known as the percentage and intensity of positive TLR4 and MYD88 cells. This indicator was determined by calculating TLR4 and MYD88 positive cells in at least 1,000 cells in different randomly selected areas at 400 magnifications.

Statistical Analysis

Chi-square was used as test to compare significant between percentage (0.05 and 0.01 probability) in this study.

The Results

A. The scoring of TLR-4 in leiomyomas and normal myometrium tissues

The percentage and staining intensity of positive stained cells in patients and controls were in (Table-1). The multiply of both percentage and staining intensity gave the final scores of TLR-4 in sample. The total score less than 6 represented low level expression and that greater or equal to 6 represented high level expression(20),(Table-2), it was found that TLR-4 was 90% highly expressed in leiomyomas tissue while 85% normal myometrium (biopsies) showed low/negative expression, with significant difference($P \leq 0.01$) between these groups (Figure 1). The cytoplasmic expression of TLR-4 was found in uterine smooth muscle wall of *leiomyoma* group.

Table-1: The percentage and intensity staining of positive TLR-4 cells of in leiomyomas, normal myometrium biopsies.

The Percentage score/TLR-4	TLR-4 intensity score						Total No.
	NO/scor0	Weak/score1	Moderate/score 2		Intense/score 3		
	Patients Num.(%)	Control Num.(%)	Patients Num.(%)	Control Num.(%)	Patients Num.%)	Control Num.(%).	
Score zero **<5%**	0	17	0	0	0	0	17
score1 5-≤25%	0	0	0	0	0	0	0
score2 25-≤50%	0	0	2	0	0	0	0
Score3 50-≤ 75%	0	0	0	3	12	0	15
score4 **≥ 75%**	0	0	0	0	6	0	6
Total No.	0	17	2	3	18	0	40

Table-2: Final TLR-4 score in leiomyomas , normal myometrium biopsies.

expression of TLR-4	Patients Num.(%)	Normal myometrium Num. (%)	Total Num. (%)	Chi-Square (χ2)
Highly expression (≥6)	18 (90.00)	3(15.00)	21	14.52 **
Low/negative expression (<6)	2 (10.00)	17(85.00)	19	14.52 **
Total No. (%)	20	20	40 (100%)	
Chi-Square (χ2)	14.10 **	12.94 **	---	---
** (P≤0.01).				

B-Scoring of MYD-88 in leiomyomas and normal myometrium tissues

The percentage and intensity of positive stained cells in leiomyomas and normal biopsies (Table -3), multiplied 0–12 then divided to: High expression (≥6) and Low/negative expression (<6).

It was found that MYD-88 was highly expressed in 95% of leiomyoma tissue and in 5% of normal myometrium biopsies, with significant difference between two groups was noted p<0.001 (Table-4 , Figure2). The cytoplasmic or membrane expression of M YD-88 was found in uterine muscle wall of *leiomyoma* group.

Table-3: percentage and intensity of MYD-88 positive cells in leiomyomas, normal myometrium biopsies.

Percentage score of MYD-88	Intensity score of MYD-88						Total No.
	No/ Score 0, weak/ score1		Moderate/Score 2		Intense/Score 3		
	Patients Num. (%)	Control Num.(%)	Patients Num.(%)	Control Num.(%)	Patients Num.(%)	Control Num.(%)	
Score 0 <5%	0	19	0	0	0	0	19
Score1 5-≤25%	0	0	0	0	0	0	0
Score2 25-≤50%	0	0	1	0	0	0	1
Score 3 50-≤ 75%	0	0	0	1	11	0	12
Score4 ≥ 75%	0	0	0	0	8	0	8
Total No.	0	19	1	1	19	0	40

Table -4: Final score of MYD-88 expression in leiomyomas patients and normal myometrium biopsies.

MYD-88 expression	Patients Num.(%)	Normal myometrium Num.(%)	Total Num.(%)	Chi-Square (χ2)
High expression (≥6)	19 (95.00)	1(5.00)	20	14.63 **
Low/negative expression (<6)	1 (5.00)	19 (95.00)	20	14.63 **
Total No. (%)	20	20	40(100)	---
Chi-Square (χ2)	14.63 **	14.63 **	---	---
** (P≤0.01).				

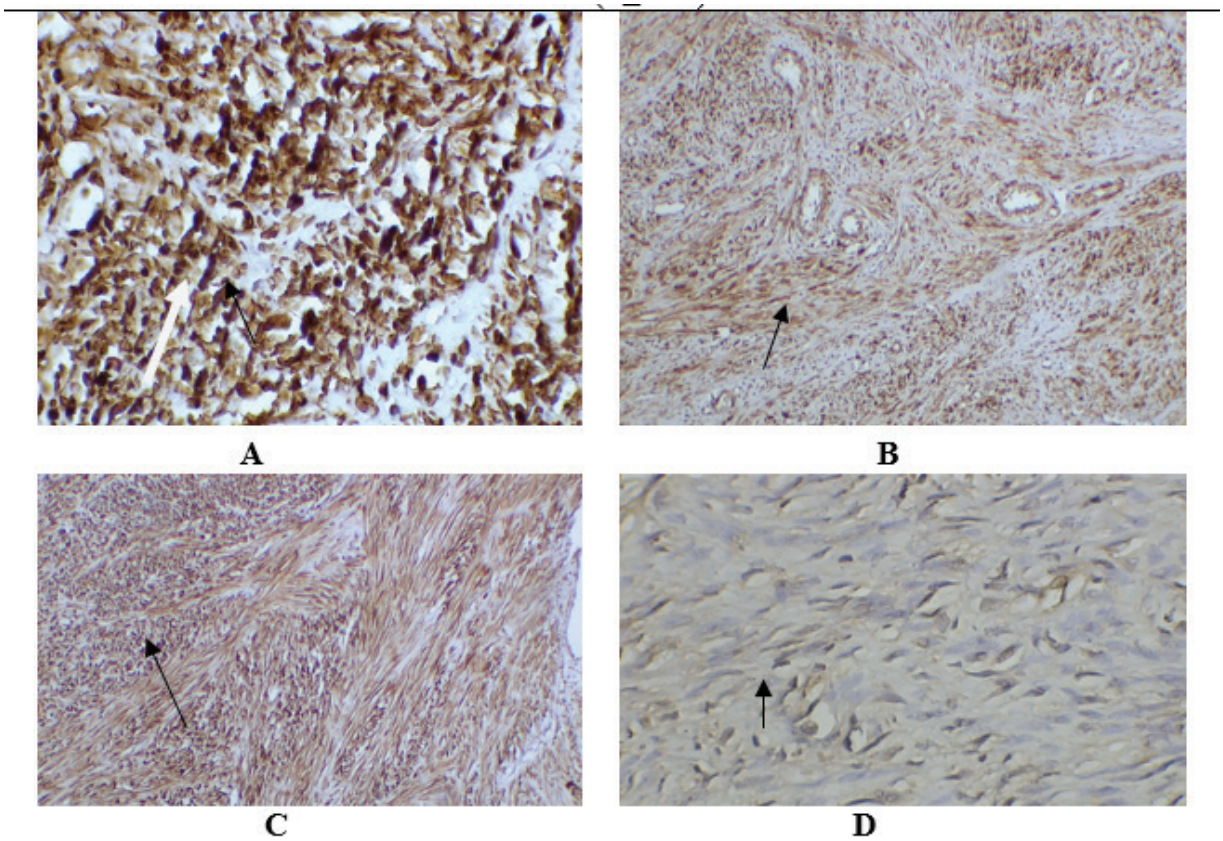

Figure (1): Immunoreactivity for TLR-4 in leiomyomas of uterine muscles, brown discoloration of cytoplasm,(A)high expression X 200;(B,C) high expression X 100;(D) negative expression X 200.

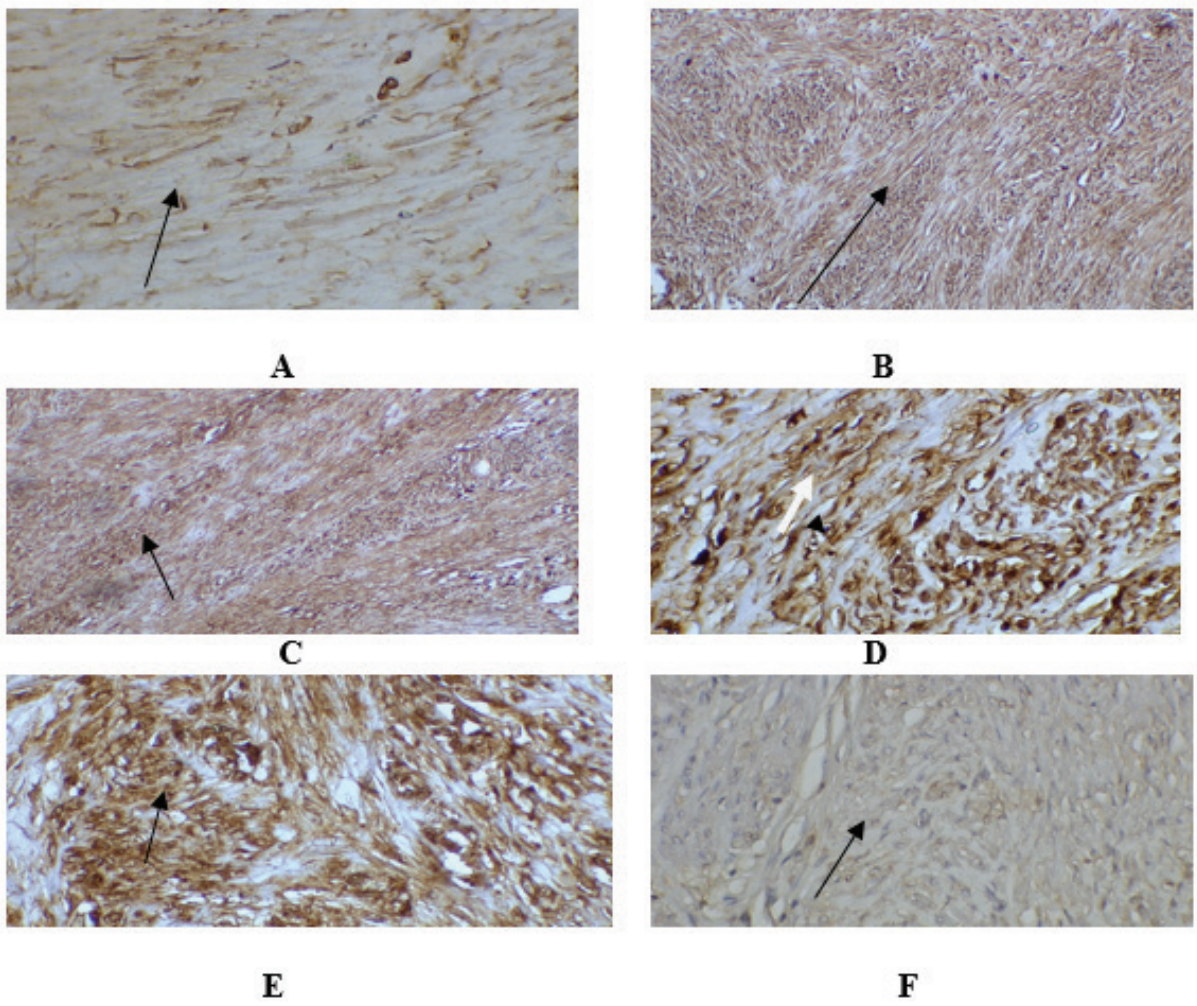

Figure (2):Immunoreactivity for MYD-884 in leiomyomas of uterine muscles, brown discoloration of cytoplasm or membrane, (A)low expression X 200; (B &C)high expression X 100; (D&E)high expression X 200; (F) negative expression X 200.

Discussion

Around the world most common neoplasms of the female genital tract arising from smooth muscle cells of uterus [22,23,] once leiomyomas are benign lesions of the uterus [24] Neoplasm of uterus constitutes 1% of uterine malignant smooth muscle tumours[25,26].

A distinguishable difference regarding expression of TLR-4; 90%cases of leiomyomas had highly cytoplasmic immunoreactivity, while15 % of normal myometrium was positive. This result is similar to what [27] reported and there was statistically significant difference was found.

The pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern (DAMPs) recognize by the toll-like receptor4, which expresses on immune and tumor cells, activation of TLR4 in tumor can boost immunity antitumor in addition to raise immune surveillance and tumor progression[28]. TLR4 can signal through TIR-domain-containing adapter-inducing interferon-β ,*TRIF* to induce type I IFN through *Interferon regulatory factor 3* IRF3 and through MyD88 to induce pro-inflammatory cytokines *via* nuclear factor, NFκB[29], these signaling events are separated, signaling from TRIF-related adaptor molecule/TRIF (TRAM/TRIF) begin from endosomes while TIR Domain Containing Adaptor Protein TIRAP/MyD88 signaling start from the surface[30].

Out of 20 leiomyomas cases, (95%) was with high expression of cytoplasmic and/or membranous MYD-

88, while only (5%) of normal myometrium was with high expression, test Chi –square highly significant difference (P= 0.0001) in MYD-88 level of two group .The adaptor protein MyD88 regulate innate immunity against bacterial and viral diseases in the body. MyD88 recruit to activate the MyD88-dependent pathway after Interleukin 1 receptors (IL-1R) with Toll-like receptors recognize pathogens, but MyD88 mutation associated with development of lymphoma, inflammation progression and carcinogenesis. The signaling by MyD88 played dual duty in cancer by enhancing and promoting of tumor and inflammation, flora imbalance of intestine so to induce invasion and cell self-renew of tumor, and anti-tumor function to maintain homeostasis in host and immune responses against cancer cells and tumor cell cycle arrest [31].

During inflammation TLR4/MyD88 signaling mostly occurs [32], Repressing TLR4/MyD88 signaling lowering cell viability, enhancing apoptosis and up the levels of inflammatory factors following infection in macrophages [33]. Strong correlation between the inflammatory microenvironment of tumor and progression, prognosis of a number of cancer types as ovarian, uterus, rectal and prostate cancer [34].

Conclusion

Significant highly expression in the TLR4 and MYD88 in leiomyomas female cases than control group which indicate a strong relation between their high expression and tumor progression and inflammatory signaling induction in.

Source of Funding: Self

Conflict of Interest: Non

Ethical Clearance: The study was performed under the guide lines supervision of Ethical Committee in the College of Veterinary Medicine , University of Baghdad.

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