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Research Article

Evaluation of Toll-Like Receptor Expression with Clinicopathologic Variables in Endometrium Cancer

Berat Berrin Gençoğlu Bakbak,[1] Tolgay Tuyan Ilhan,[2] Aybike Pekin,[3] Özlem Seçilmiş Kerimoğlu,[4] Setenay Arzu Yılmaz,[4] Ayşegül Kebapçılar,[4] Nasuh Utku Doğan,[5] Pınar Karabağlı,[6] Çetin Çelik[4]

[1]Department of Obstetrics and Gynecology, Health Sciences University Meram Training and Research Hospital, Konya, Turkey
[2]Department of Obstetrics and Gynecology, Mersin Maternity and Women's Health Hospital, Mersin, Turkey
[3]Department of Obstetrics and Gynecology, Necmettin Erbakan University Faculty of Medicine, Konya, Turkey
[4]Department of Obstetrics and Gynecology, Selcuk University Faculty of Medicine, Konya, Turkey
[5]Department of Obstetrics and Gynecology, Akdeniz University Faculty of Medicine, Antalya, Turkey
[6]Department of Pathology, Selcuk University Faculty of Medicine, Konya, Turkey

Abstract

Objectives: Clinical evidence supports the association of toll-like receptor (TLR) with abnormal cell proliferation and cancer. In this study, we investigated the expression of TLRs 2, 4, 5, and 6 in healthy endometrium and endometrium cancer to study the relationship of these receptors' expression with carcinogenesis.

Methods: Patients who had undergone a hysterectomy owing to endometrium cancer (group 1, 66 patients), endometrial hyperplasia (group 2, 14 patients), and other reasons besides endometrium cancer (group 3, 20 patients as controls) were included. The cases in the first group were classified by histological type of the cancer, stage, grade, and size of the tumor. In all the cases, expressions of TLRs 2, 4, 5, and 6 were assessed, and the relationship of these receptors with clinicopathologic signs was analyzed. For immunohistochemical staining, nuclear and cytoplasmic stainings were considered positive. A Chi-squared test was used to assess the correlation of the groups. A $p<0.05$ was considered significant.

Results: The mean ages of patients in groups 1, 2, and 3 were 59.8 (range 33–83), 48.3 (range 40–59), and 53.4 (range 38–84) years, respectively. All types of TLRs were highly expressed in both types of endometrium cancer (groups 1 and 2). TLR expression was observed with a ratio of 87.9% in group 1, 100% in group 2, and 35% in group 3. There was a statistically significant association of TLR 2 among the three groups ($p=0.000$). TLR 6 expression in both group 1 and group 2 was significantly higher than that in the control group ($p=0.000$, $p=0.000$, respectively). In addition, TLR 6 was higher in cases with late-stage cancer ($p=0.033$). Regarding tumor grade and the size of the tumor, no association was found between TLR 2 and TLR 6.

Conclusion: TLR 2 and TLR 6 were significantly more expressed in cases with endometrium cancer and endometrial hyperplasia. In addition, the presence of TLR 6 may indicate the presence of late-stage endometrial cancer.

Keywords: Endometrium; endometrial cancer; toll-like receptor; tumor microenvironment.

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The defense mechanism of the human body is divided into two general types of immune responses: the innate immune response and the adaptive immune response. The toll-like receptor (TLR) is a specific protein that plays an important role in innate immune response. TLRs that are secreted from inflammatory cells activate cytokines, extracellular matrix proteases, growth factors, and angiogenesis factors by activating the TLR signaling pathway in precan-

Address for correspondence: Berat Berrin Gençoğlu Bakbak, MD. Department of Obstetrics and Gynecology, Health Sciences University Meram Training and Research Hospital, Konya, Turkey
Phone: +90 332 322 78 66 **E-mail:** beratberring@yahoo.com

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cerous cells. TLRs also have adverse effect on the activity of cytotoxic T cells' immune response. These provide a specific microenvironment that supports cancer development and progression.[1] As a result of the activation of NF-kappaB by TLRs, anti-apoptotic protein levels increase and pro-apoptotic protein levels decrease.[2, 3] The relationship between cancer and TLR expression has been demonstrated in prostate, stomach, lung, breast, and cervical cancers.[4-9] The endometrium consists of hormonally active tissue. It is the first defense against inflammation. Modugno has suggested that inflammation could be an important part of endometrial cancer development.[10] This theory is supported by the idea that the menstrual cycle is an inflammatory process.

Our study aimed to evaluate the relationships of TLRs 2, 4, 5, and 6 with endometrial cancer cells and healthy endometrial cells using immunohistochemistry.

Methods

This study was conducted at Selcuk University Hospital, and the study protocol was approved by the Ethics Committee of the University Hospital (Approval Number: 2012/86). This study included patients who had undergone hysterectomy procedures. The indications were endometrial cancer for 66 patients (group 1), endometrial hyperplasia for 14 patients (group 2), and benign gynecologic reasons (adenomyosis and uterine leiomyomas) for 20 patients (group 3). All patients in group 1 had undergone a total abdominal hysterectomy, bilateral salpingo-oophorectomy, total omentectomy, and pelvic-paraaortic lymph node dissection. Stage 1 and 2 disease is considered early stage, and stage 3 and 4 disease is considered advanced disease. In group 1, tumor diameter and grade were noted. The tumor diameter was classified as below 2 cm or above 2 cm. In all tissue samples, expressions of TLR 2, 4, 5, and 6 were evaluated with immunohistochemical staining. The staining pattern for each TLR type was compared between study groups. The pattern was also compared between early-stage and advanced-stage endometrial cancer.

Immunohistochemical Staining

Serial sections of diameter 3 microns were taken from the paraffin-embedded tissue samples of endometrial cancer, hyperplasia, and benign endometrium. One of these sections was stained with hematoxylin-eosin to confirm the diagnosis. Immunohistochemicals were applied to the tissue samples by an automated IHC/ISH stainer (Leica BOND-MAXTM, New Castle, UK). An anti-TLR2 antibody (anti-TLR2 antibody ab24192, ABCAM, Cambridge, MA, USA), an anti-TLR4 antibody (anti-TLR4 antibody [76B357.1] ab22048, ABCAM, Cambridge, MA, USA), an anti-TLR5 antibody (anti-TLR5 antibody [19D759.2] ab13876, ABCAM, Cambridge, MA, USA), and an anti-TLR6 antibody (anti-TLR6 antibody ab59920, ABCAM Cambridge, MA, USA) were used.

Immunohistochemical evaluation was performed in a semi-quantitative manner by the same pathologist. The distribution and intensity of the TLR 2, 4, 5, and 6 staining of the cells were evaluated and compared with the background tumor cells. Staining of the nucleus and/or cytoplasm was considered a positive result. According to the distribution and intensity of the immunohistochemical staining, the samples were subjectively divided into four groups: group 0: no staining, group 1: mild staining, group 2: moderate staining, and group 3: severe staining. The SPSS 16.0 (Chicago, IL) package program was used for statistical analysis, and $p<0.05$ was considered significant.

Results

The clinical characteristics of the patients with endometrial cancer are shown in Table 1. The distribution of TLR 2, 4, 5, and 6 staining for clinicopathologic variables (stage, grade, and tumor diameter) is shown in Table 2. Table 3 shows the rate of staining in the different groups.

The TLR staining was significantly higher in group 1 (87.9%) when compared with that in group 3 (35%, p=0.000). The rate of TLR positivity was 87.9% in group 1, 100% in group 2, and 35% in group 3; the difference was significant (p=0.000; see Table 2). When a paired comparison was made in terms of the TLR 2 expression rates, the differences between group 1 and group 3 (p=0.000), and between group 2 and group 3 (p=0.000) were significant. However, the difference between group 1 and group 2 (p=0.170) was not significant.

TLR 6 was positive in 87.9% of group 1, 92.9% of group 2, and 40% of group 3; the difference was significant (p=0.000; see Table 3). When a paired comparison was made in terms of the TLR 6 expression rate, the differences between group 1 and group 3 (p=0.000) and between group 2 and group 3 (p=0.002) were significant. The rates of TLR 4 and TLR 5 positivity were not significantly different (p=0.133 and p=0.120, respectively).

The intensity of TLR 2 and TLR 6 staining was also evaluated in the patients with endometrial cancer (Fig. 1). Severe TLR 2 staining was observed in only 4.5% of the TLR 2-positive patients, and severe TLR 6 staining was observed in 16.6% of TLR 6-positive patients.

The relationship between endometrial cancer prognostic factors and different TLR types was evaluated. The positive TLR 2 staining rate between early-stage and advanced-stage disease (p=0.128) was not significant. However, the positive TLR 6 staining rate was significantly higher

Table 1. The clinical characteristics of the patients and TLR staining scores

	TLR2 staining score				TLR4 staining score				TLR5 staining score				TLR6 staining score				n
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	
Group 1																	
Endometrioid type																	
Early stage	7	16	14	3		16	18	6	5	10	22	3	6	18	8	8	40
Late stage		10	6			5	9	2		9	5	2		8	6	2	16
Serous type																	
Early stage		4				1	3			3	1		2	1		1	4
Late stage	1	2	3			1	4	1	1	1	3	1		3	3		6
Grade																	
1	6	14	11	2		13	16	4	2	10	19	2	61	10	9	8	33
2	1	9	6	1		7	7	3	3	8	5	1	2	1	14		17
3	1	8	7			3	11	2	1	6	7	2	4	6	3	3	16
Tumor size																	
≤2 cm	4	20	13	1		11	19	8	4	13	17	4	7	19	7	5	38
>2 cm	4	12	10	2		12	15	1	2	11	13	2	6	10	7	5	28
Group 2																	
Endometrial hyperplasia		5	7	2		5	3	6	4	3	3	4	1	4	8	1	14
Group 3																	
Benign gynecologic disorders	13	4	2	1	1	7	8	4	2	3	12	3	12	6	1	1	20

n=Patient number.

Table 2. The distribution of TLR 2, 4, 5, and 6 staining for clinicopathologic variables (stage, grade, and tumor diameter)

	TLR2 (+)	TLR4 (+)	TLR5 (+)	TLR6 (+)
Group 1				
Endometrium cancer (%)	87.9[1]	100	90.9	87.9[2]
Stage				
Early	84.1	100	86.6	81.8[3]
Late	95.4	100	95.4	100[3]
Grade (%)				
1	81.8	100	93.9	81.8
2	94.1	100	82.3	88.2
3	93.7	100	93.7	81.2
Tumor size (%)				
≤2 cm	89.5	100	89.5	81.6
>2 cm	85.7	100	92.8	78.6
Group 2				
Endometrial hyperplasia (%)	100[1]	100	71.4	92.9[2]
Group 3				
Benign gynecologic disorders (%)	35[1]	95	90	40[2]

1: p=0.000; 2: p=0.000; 3: p=0.033.

in the patients with advanced-stage endometrial cancer (p=0.033; see Table 2). The difference in the positive staining rates of TLR 2 and TLR 6 was not significant according to grade (p=0.320 and p=616, respectively) and tumor diameter (p=0.644 and p=0.761, respectively).

Table 3. The rate of staining in the different groups

	TLR2 (+) n (%)	TLR4 (+) n (%)	TLR5 (+) n (%)	TLR6 (+) n (%)
Group 1 Endometrium cancer	58 (87.9)	66 (100)	60 (90.9)	58 (87.9)
Group 2 Endometrial hyperplasia	14 (100)	14 (100)	10 (71.4)	13 (92.9)
Group 3 Benign gynecologic disorders	7 (35)	19 (95)	18 (90)	8 (40)
p	p=0.000*	p=0.133	p=0.120	p=0.000*

*= p<0.05.

Discussion

Ten different types of TLRs have been observed in humans. They are structured as integral transmembrane glycoproteins, and are members of the natural immune response. The expression of TLRs can be found in the immune cells, epithelial cells, and cancer cells. By activatıng TLRs, pro-inflammatory and anti-inflammatory cytokines are released. The role of chronic inflammation has been understood in colorectal, hepatocellular, stomach, and cervical cancers. The adverse effect of this chronic inflammation on endometrial tissue was first pointed out by Modugno in 2005.[10] Due to the exposure to cyclic inflammation under physiologic cir-

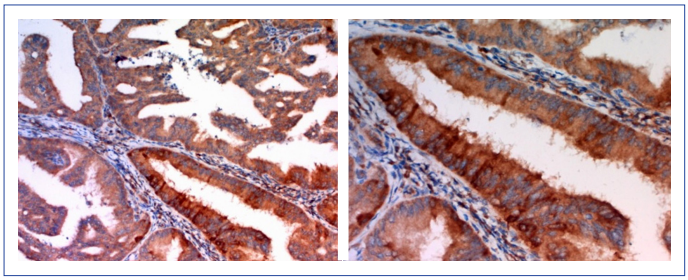

Figure 1. Severe cytoplasmic (Left, ×10) and glandular (Right, ×20) staining in Grade 1A endometrioid adenocancer.

cumstances, endometrial tissue is unique. In this context, TLRs can be used as diagnostic and prognostic markers for endometrial cancer. In our study, the TLR staining rate of group 1 was significantly higher than that of the other groups. These data support the role of inflammation in endometrial cancer.

Young et al.[11] show that TLRs 1–6 can be found in vaginal, cervical, endometrial, and fallopian tube epithelium. Schaefer et al.[12] have demonstrated the TLR 7–9 expression in addition to TLR 1–6 expression. In our study, we evaluated TLRs 2, 4, 5, and 6, which cause chemokine and cytokine secretion in the endometrial microenvironment.

Although the expression of TLR has been studied in various cancers, the studies of gynecologic malignancies are limited to ovarian, cervical, and endometrial cancers.[8, 13-15] Allhorn et al.[15] compared the levels of TLR 3 and 4 in postmenopausal healthy women and endometrial cancer and endometrial hyperplasia samples by using immunohistochemistry and PCR analysis. They found that the endometrial cancer and endometrial hyperplasia had lower TLR expressions than what the healthy endometrial samples had.

In our study, a positive staining rate was significant for the TLRs in endometrial cancer when compared with that for the TLRs in the healthy endometrial samples. In addition, a high positive staining of the microenvironment was observed in the endometrial hyperplasia and endometrial cancer samples when compared with that in the healthy endometrial samples. We therefore conclude that high positive staining of TLR 2 and 6 in endometrial cancer and endometrial hyperplasia suggests inflammatory cytokine-mediated tumor progression and carcinogenesis.

The TLR 2 expression rates of the three groups were statistically significant (p=0.000). When comparıng the TLR 2 expression of the endometrial cancer and endometrial hyperplasia groups, both groups had high positive TLR 2 staining, and the difference was not significant. group 1 and group 2 had higher TLR 2 staining than what the control group had. These results support that TLR 2 could be used as a screening method for endometrial cancer, and as a prognostic parameter in endometrial hyperplasia.

Ng et al. [16] investigated the relationshıp between TLR 2 and oral squamous cell cancer.[16] They stated that the TLR 2 expression was significantly higher in the inflammatory cells that were close to the cancer cells. Therefore, the TLR 2 expression was increased on the epithelial cells that were exposed to inflammation. We believe that this change is mainly due to the structural change or change of the chromosomal proteins.

Guo et al. [17] discovered that TLR 2 has an effect on the invasion and migration of colorectal cancer cells, and that TLR 2 has an effect like oncogenes. Pandey et al. [18] conducted a study using DNA extraction of peripheral blood samples. They found a significant relationshıp between cervical cancer and TLR 2 and 4 expressions. In our study, similar to oral squamous cell, cervical, and colorectal cancers, TLR 2 expression in the endometrial cancer and endometrial hyperplasia groups was significantly higher than that in the control group. These data suggest a similarity between invasive cancer development from pre-invasive disease.

Allhorn et al. [15] have proposed that TLR 3 and 4 expression plays a role in pathological changes in the endometrium. In addition, high staining rates of TLR 3 and 4 were observed in normal endometrial tissues. They stated that this finding is the result of TLR defense mechanism of the uterus, which is used during menstruation against ascending microorganisms. In the same study, lower receptor levels were observed in endometrial cancer and endometrial hyperplasia group than those in control group. The lowest TLR expression was observed in poorly differentiated endometrial cancer.[15] Papez et al. [19] demonstrated the relationship between TLR 4 expression with high-grade and advanced-stage endometrial cancer. In our study, however, we did not observe the relationship between endometrial cancer and TLR 4 or TLR 5.

In group 1 and group 2, TLR 6 was significantly higher than that in the control group, and the difference was not significant. When compared to that in early-stage endometrial cancer, the TLR 6 level was significantly higher in advanced-stage endometrial cancer. We therefore hypothesize that TLR 6 expression is a poor prognostic factor for endometrial cancer. When the relationship between TLR types with histological grade and tumor diameter was evaluated, the relationship for each variable was not significant. These data show that TLR 6 could be used as a biomarker for endometrial cancer and endometrial hyperplasia.

In the endometrial cancer group, the expressıon of TLR 2 and 6 was significantly higher than that in the control group. The staining intensity of the endometrial cancer samples was evaluated for TLR 2 and TLR 6, and severe

staining was observed in only 4.5% of the samples for TLR 2 and 6.6% of the samples for TLR 6. These results show that TLR 2 and TLR 6 could be used in endometrial cancer and endometrial hyperplasia regardless of staining intensity.

This study shows that TLR 2- and TLR 6-mediated pathways have a significant effect on the development of endometrial cancer. Different staining patterns of different tissues suggest that TLR has tissue specificity, as suggested in the literature. Because of the significant difference of TLR 6 expression between advanced-stage and early-stage endometrial cancer, TLR 6 could be used as a prognostic factor. Comprehensive studies to understand the relationship between TLR and survival from cancer are necessary to support our results.

Disclosures

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Ethics Committee Approval: This study was conducted at Selcuk University Hospital, and the study protocol was approved by the Ethics Committee of the University Hospital (Approval Number: 2012/86).

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