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Original Article

Expression of Toll-like receptor 4 in ovarian serous adenocarcinoma and correlation with clinical stage and pathological grade

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Abstract: Toll-like receptor 4 (TLR4) plays an essential role in adaptive and innate immunity, and its expression has been described in various tumors. This study aimed to examine the expression of TLR4 in serous tumors and to evaluate its correlation to clinicopathological parameters. The expression of TLR4 was immunohistochemically examined in 63 species of normal ovarian epithelia and 336 species of serous epithelial lesions. Moreover, the association between TLR4 expression and various clinicopathologic features was assessed. The expression intensity of TLR4 in benign and borderline to malignant ovarian tumours showed a gradual rising trend. We identified positive correlations between TLR4 expression levels and both FIGO stage and pathological stage. In serous adenocarcinoma, TLR4 expression levels were significantly associated with chemoresistance. There was no relationship between the expression of TLR4 and the patient's age or pretreatment serum CA125 levels. Our data suggest that TLR4 might stimulate serous ovarian carcinoma initiation and progression. TLR4 expression is correlated with poor chemoresponse, which has important implications for the development of new therapeutic strategies for drug-resistant ovarian cancer.

Keywords: Serous ovarian carcinoma, toll-like receptor 4, immunohistochemistry

Introduction

Ovarian cancer is the most common cancer affecting women, and the fifth leading cause of cancer-related deaths in females worldwide [1]. Despite advances in surgical and medical therapy, the overall mortality of ovarian cancer has remained largely unchanged over the past decades [2]. Ovarian cancer has a high recurrence rate and is chemo-resistant, leading to high patient mortality rates [3]. Therefore, a refined investigation of the mechanisms of tumourigenesis and chemoresistance of this disease are essential to improve survival rate as well as treatment and prevention. Serous ovarian tumors, which represent the most frequently encountered clinical histotype, are derived from the ovarian surface epithelium. Their tumourigenesis has been associated with ovulation-associated wound repair and/or inflammation [4].

Toll-like receptors (TLRs) are a family of transmembrane receptors that regulate the activation of both innate and adaptive immunity [5]. There are 12 TLRs, and 10 human isoforms of TLRs (TLR-1 to 10) have been identified. TLR4, the first one described, recognizes the bacterial lipopolysaccharide (LPS), which has been shown to play a critical role during infection in the regulation of tissue renewal and repair [6]. The relationship between inflammation and tumourigenesis and progression is widely accepted [4]. Based on their important role in inflammation and tissue regeneration, TLRs are likely candidates to mediate the tumourigenesis of ovarian tumors.

TLR4 expression was detected in a subgroup of patients with ovarian tumors, supporting the notion that in the ovary, malignant progression is accompanied by activation of TLR4 [7]. However, the relationship between TLR4 expression

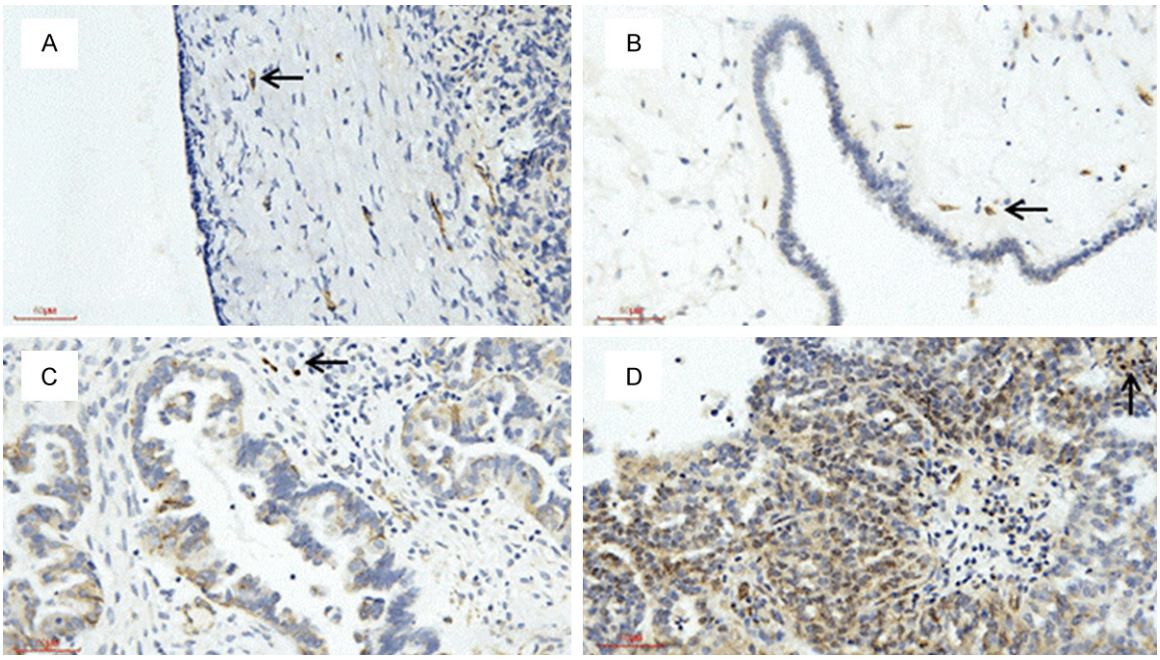

Figure 1. TLR4 expression profiles detected in normal ovarian epithelia and various epithelial ovarian lesions. TLR4 expression in neutrophilic granulocytes was used as a positive internal control for the immunohistochemical reaction (marked with arrows). TLR4 expression and localization in (A) normal ovarian epithelia, (B) benign serous cystadenoma, (C) borderline serous cystadenoma, and (D) serous adenocarcinoma. The distinct brown staining is located predominantly in the membrane and cytoplasm of TLR4-positive cells.

Table 1. Expression of TLR4 in different ovarian epithelial lesions

	n	-	+	++	+++	$\times^2$	P
						88.4263	< .0001[a]
Normal ovarian epithelia	63	32	21	8	2	0.2506	0.6166[b]
Cystadenoma	124	62	38	17	7	14.6871	0.0001[c]
Borderline adenoma	95	33	17	21	24	51.3000	< .0001[d]
						18.0570	< .0001[e]
Cystadenocarcinoma	117	17	16	35	49	68.5096	< .0001[f]
						14.3599	0 .0002[g]

[a]Comparison among the 4 groups; [b]Normal ovarian epithelia vs. cystadenoma; [c]Normal ovarian epithelia vs. borderline adenoma; [d]Normal ovarian epithelia vs. cystadenocarcinoma; [e]Serous cystadenoma vs. borderline adenoma; [f]Serous cystadenoma vs. adenocarcinoma; [g]Borderline adenoma vs. adenocarcinoma.

and the clinicopathological features of aggressive ovarian tumors has not been studied, and therefore the role of TLR4 in tumourigenesis remains unclear. In this study, we analyzed the expression of TLR4 with respect to several clinicopathological factors in a cohort of 117 ovarian cancers.

Materials and methods

Tissue specimens and patients

We studied tissue specimens (n = 235) obtained with written consent from patients in the Department of Pathology, Shanghai First Maternity and Infant Hospital, between April 2000 and July 2010. The samples consisted of 63 normal ovarian epithelia, 124 serous cystadenomas, 95 borderline serous adenomas, and 117 serous adenocarcinomas (including 6 stage I, 31 stage II, 58 stage III, and 22 stage IV carcinomas). None of the patients received radiation therapy or chemotherapy before surgery. Two gynecological oncology pathologists confirmed diagnoses, and tumors were graded and subtyped according to World Health Organization (WHO) criteria. The clinical stage of each carcinoma was determined according to the established International Federation of Gynecology and Obstetrics (FIGO) standards. All carcinoma patients had undergone cytoreductive surgery and a standardized postsurgical course of Taxol and platinum-based chemotherapy. The chemotherapy response was evaluated using WHO criteria. The study was approved by the Ethics Committee of Shanghai First Maternity and Infant Hospital.

Table 2. Correlation analysis between clinicopathological characteristics and the expression of Nestin

	n	-	+	++	+++	*Rs* (95% CI)	*P*
FIGO Stage						0.4686 (0.3084, 0.6289)	< .0001
I	6	4	1	1	0		
II	31	9	7	11	4		
III	58	2	6	19	31		
IV	22	2	2	4	14		
Histological grade						0.5474 (0.4024, 0.6924)	< .0001
I	10	7	1	1	1		
II	39	8	10	14	7		
III	68	2	5	20	41		
Chemoresistance						0.5335 (0.3758, 0.6912)	< .0001
Yes	21	15	2	2	2		
No	96	2	14	33	47		
Maximum tumour diameter						0.0614 (-0.1194, 0.2422)	0.5142
≤ 10 cm	55	9	7	18	21		
> 10 cm	62	8	9	17	28		
Residual tumour diameter						0.0374 (-0.1433, 0.2180)	0.6993
≤ 2 cm	64	10	9	19	26		
> 2 cm	53	7	7	16	23		

FIGO: International Federation of Gynaecology and Obstetrics. CI: Confidence interval.

Slide preparation, immunohistochemistry and scoring

All tissues were fixed in 10% buffered formalin for 24 h, and then embedded in paraffin. Serial, 4-μm-thick coronal sections cut from paraffin blocks were stained with hematoxylin and eosin (HE). HE slides were reviewed in each case and histologic diagnosis was confirmed using accepted criteria. Immunohistochemical studies were carried out as described previously [8]. In brief, these were performed using the GTVision™ III Detection System (Including DAB)/Mo & RKit (Gene Tech Company Limited, Shanghai, China). According to the manufacturer's protocol, the polyclonal rabbit anti-human TLR4 antibody (Abcam, Cambridge, MA, USA) was used at a 1:50 dilution, and a negative control without primary antibody was run with each case. TLR4 expression was assessed by semiquantitative evaluation of the intensity and extent of cytoplasm staining.

The staining intensity was divided into four categories: 0 (no staining), 1 (faint yellow), 2 (brown-yellow) or 3 (dark yellow). The quantity of cells stained was scored as follows: 0 (less than 5% positive cells), 1 (5-25% positive cells), 2 (26-75% positive cells) or 3 (more than 76% positive cells). For each section, a final score of 0-6 was obtained by summing the stain intensity and positive cell scores. It was determined as - (0), + (1-2), ++ (3-4), and +++ (5-6). All the evaluations were made by two independent pathologists who were blinded to the clinicopathological data.

Statistical analysis

Statistical analysis was performed using SPSS software version 10.0. The Kruskal-Wallis H and the Mann-Whitney U tests were used to compare TLR4 expression between the different groups. The Spearman and Kendall tests were applied to analyze the association between TLR4 expression and clinicopathological parameters. A *p*-value of $P < 0.01$ was considered to be significant in all statistical analyses.

Results

TLR4 expression in normal ovarian epithelia, benign cystadenomas, borderline adenomas, and adenocarcinomas

TLR4 immunoreactivity was observed in the cancer cell membrane and cytoplasm. TLR4 expression was also detected in neutrophilic granulocytes in the tumour stroma. The blank controls were negative for TLR4 staining. TLR4 was moderately or strongly expressed in borderline cystadenomas or ovarian carcinomas, in contrast to adenomas and normal ovarian tissues, where it was only weakly expressed or absent. Representative results of staining intensity are shown in **Figure 1**. There was a significant difference between the normal ovarian tissues and the benign cystadenoma, borderline adenoma and adenocarcinoma groups, but there was no difference in TLR4 expression between adenoma and normal tissue (**Table 1**).

Table 3. Correlation analysis of TLR4 expression, patient age and pretreatment serum CA125 level

	N	Age			CA125		
		Median (P_{25}~P_{75})	r_s	P	Median (P_{25}~P_{75})	r_s	P
Normal ovarian epithelia	63	49 (43~54)	-0.1361	0.2875			
Serous cystadenoma	124	45.0 (36.0~54.5)	-0.1456	0.1066	30.8 (18.7~50.4)	-0.1482	0.1005
Borderline serous adenoma	95	42 (38~52)	0.0990	0.3400	90.9 (50.3~180.2)	-0.1072	0.3011
Serous adenocarcinoma	117	55 (49~61)	0.0866	0.3530	378.4 (155.7~773.5)	0.1838	0.0473

The expression of TLR4 increased from normal epithelium to benign and borderline serous adenoma to serous adenocarcinoma (**Figure 1** and **Table 1**).

Analysis of the relationship between clinicopathological parameters and TLR4 expression

The relationship between TLR4 expression and the clinicopathological features of the 117 ovarian carcinoma specimens is shown in **Table 2**. Overexpression of TLR4 was found more often in advanced stage disease. Increased TLR4 expression levels were significantly correlated with International Federation of Gynaecology and Obstetrics stage and tumor cell differentiation grade. When analysing the subgroups of platinum-resistant and platinum-sensitive patients, a significant association was observed in the clinical outcome of cases with negative versus positive TLR4 expression. We found no significant association between TLR4 expression and maximum or residual tumour diameter. Mean age at diagnosis was 49 years. The serum level of CA125 is an auxiliary marker for early diagnosis and recurrence of ovarian cancer. There was no correlation between TLR4 expression levels and age or pretreatment serum CA125 levels (**Table 3**).

Discussion

In the current study, TLR4 expression gradually increased from benign and borderline to malignant ovarian tumors, which indicated that TLR4 might be involved in the development and progression from ovarian normal tissue to cancer. Although no significant association between TLR4 expression and the patient's age at diagnosis or CA125 levels was identified, there were positive correlations between TLR4 expression and tumor clinical stage or pathological grade. In advanced tumor stages, TLR4 expression was elevated, which also confirmed that the protein plays an important role in the regulation of the tumor cell differentiation process. It is speculated that TLR4 expression in ovarian carcinoma cells may indicate a poor prognosis. Serous ovarian cancer accounts for approximately 70% of all cases of ovarian malignancy, representing the most common type of ovarian carcinoma. The pathogenesis of epithelial ovarian cancer remains largely unclear [9].

The persistence of chronic inflammation plays a critical role in initiating, sustaining, and advancing tumor growth. Chronic inflammation contributes to cancer development and can predispose to carcinogenesis. Quite a few cancers are reported to occur through chronic inflammation-related processes [10]. In the ovary, inflammation may underlie ovulatory events because an inflammatory reaction is induced during the process of ovulation. Ovarian cancer development might be the result of an aberrant form of tissue repair in which the control mechanisms have been lost [11]. The results of our study, however, show presence of TLR4 in the microenvironment of ovary carcinoma. It is of interest that TLRs tested were expressed not only on inflammatory infiltrate cells, but also on tumor cells. TLR4 has an important role in maintaining tissue homeostasis by regulating tissue repair and regeneration. TLR4 may be the connection between inflammation and cancer and could be involved in the triggering event that leads to the inflammatory response. Thus, increased TLR4 expression may play a role in the carcinogenesis and progression of epithelial ovarian carcinoma.

Ovarian cancer is highly lethal. Malignant epithelial tumors respond poorly to chemotherapy and drug resistance is a major cause of ovarian cancer recurrence [2, 3]. Recent studies have shown that TLR4 expression on tumor cells is associated with drug resistance, but the drug resistance of TLR4 in ovarian cancer research remained to be confirmed in larger cohorts [7].

Our study on 117 specimens from patients with epithelial ovarian carcinoma confirms the relationship between TLR4 and drug resistance. Although the underlying mechanism needs further study, we hypothesize that the TLR4 signaling pathway is involved.

In conclusion, our results show that the immunohistochemical assessment of TLR4 expression could provide information of potential clinical value for prediction of treatment response or prognosis in ovarian cancer patients. Further studies on the activation of the TLR4 signaling pathway in a tumor context may help to better understand the processes that link inflammation and cancer, as well as the biological and clinical importance of the interplay between tumor and stroma in ovarian cancer. These findings suggest that TLR4 holds promise as a therapeutic target for serous ovarian cancer.

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Disclosure of conflict of interest

None.

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