Original Article

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Evaluation of Toll-Like Receptor (TLR) 3, 4, 7, 8 and 9 in Mucinous and Serous Ovarian Cancer

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Abstract

Background: The aim of this study was to investigate the expression of TLR3, TLR4, TLR7, TLR8 and TLR9 in ovary tissue of ovarian cancer patients.

Methods: In the case control study, 122 paraffin-embedded tissue blocks of patients with ovarian cancer and control groups were collected. Immunohistochemistry technique was used for detecting the expression of TLR3, TLR4, TLR7, TLR8 and TLR 9 in ovary tissues.

Results: The mean Immunoreactivity Score (IS) of TLR3 in the case and the control groups was 0.6±0.99 and 0.16±0.1, respectively. The mean IS of TLR7 in the case and the control groups was 1.03±1.05 and 0.66±1.07, respectively. A significant difference was observed between the case and control groups in terms of IS of TLR3 ($p=0.001$) and TLR7 ($p=0.013$). However, no significant difference was observed between the case and control groups regarding IS of TLR 4, 8 and 9 ($p>0.05$).

Conclusion: High IS of TLR3 and TLR7 in these patients may confirm the likelihood of association of viral infection with ovarian cancer. Also, considering that TLR3 is one of the receptors that have viral and bacterial ligands, there is a possibility that a bacterial infection is also involved in the development of ovarian cancer. In addition, the expression of TLR4, TLR8 and TLR9 was not different in ovarian tissue of the case and control groups. It is proposed to conduct this study with RT-PCR technique on the paraffin block sample and compare the results with the above results in future studies.

Keywords: Ovarian neoplasms, Paraffin Embedding, Toll-like receptor 3, 7, 8, 9.

Introduction

Ovarian cancer is the most dangerous cancer (1-3) of the female reproductive system (2). It can metastasize through the abdomen before causing symptoms (4). Almost 80% of Ovarian Cancer (OC) at late stages is diagnosed, leading to a poor prognosis. Moreover, the most of patients due to recurrence or drug resistance die within 5 years of diagnosis (5). Significant efforts and treatment strategies have not yet improved overall survival (6). Ovarian cancer has various histological subtypes, distinct molecular features and different clinicopathological characteristics can be generally divided to type I and type II tumors. Among them, type II tumor is the most common histologic subtype of ovarian cancer, constituting three quarters of ovarian carcinoma6. Furthermore, Mucinous Ovarian Carcinoma (MOC) is a heterogeneous group of tumors and shows 10-15 % of all cases (7).

Toll-Like Receptors (TLRs) as a main family of pattern recognition receptors are expressed in immune and non-immune cells including fibroblasts and epithelial cells. They cause those immune cells identify pathogens and stimulate inflammatory reactions (8). These responses lead to the secretion of cytokines, which increase the stability of infected cells and the release of chemokines, which are absorbed by immune cells into necrotic cells (8). Inflammation can promote cancer through inhibiting apoptosis and motivating angiogenesis and cell proliferation. Sheyhidin *et al* reported a significant increase in TLR3, TLR4, TLR7 and TLR9 mRNA levels in esophageal squamous cell carcinoma samples (8). Another study showed that TLRs regulate biological responses, such as inflammatory and immune responses during cancer (9).

Benign conditions, epithelial tumors, and ovarian cancer cell lines express TLR2, TLR3, TLR4, and TLR5. Different expression of TLR6 and TLR8 is observed in benign and malignant epithelium of some patients, whereas the expression of TLR1, TLR7, and TLR9 is weak (10). Another study reported that TLRs are expressed on the surface of immune cells and tumor cells (11).

Given that few and contradictory studies have been conducted regarding the expression of TLR receptors in ovarian cancer, and there is no comprehensive study in this regard, especially in our country, the aim of this study was to investigate the expression of TLR3, TLR4, TLR7, TLR8 and TLR9 in patients with ovarian cancer.

Materials and Methods

Clinical samples

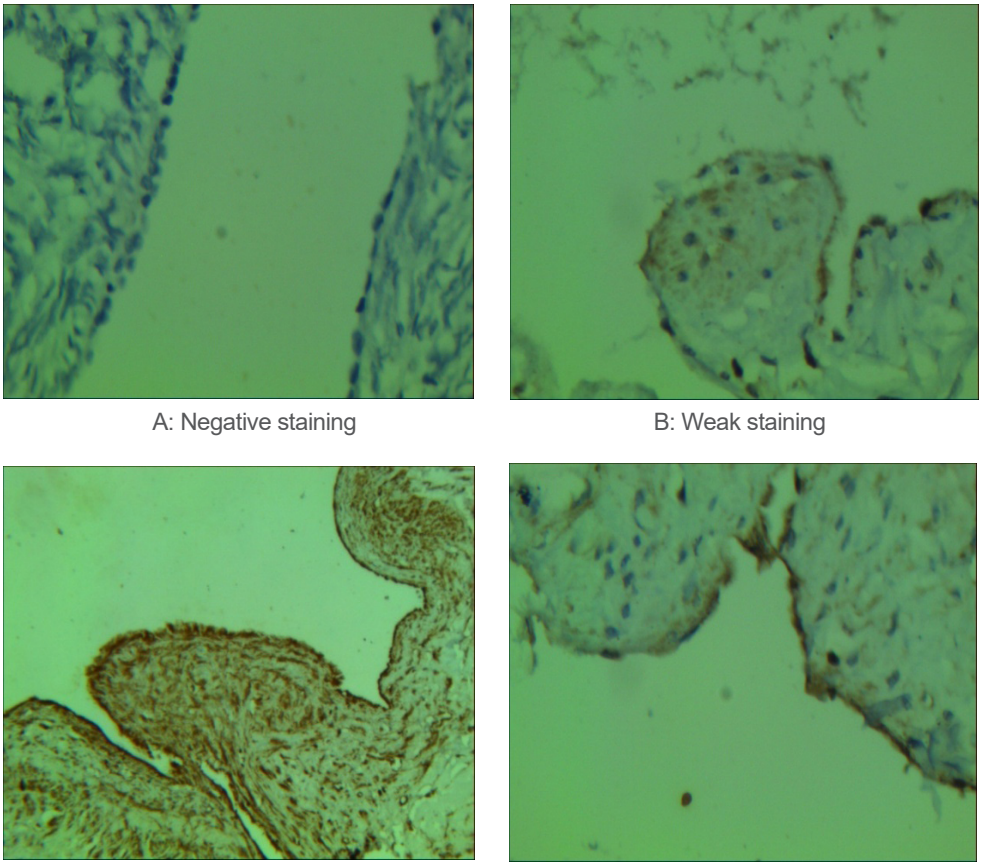
In a case control study, 122 paraffin-embedded tissue blocks of patients with ovarian cancer and control group were collected according to pathological results. Ovary tissues without any malignant, hyperplasia, metaplasia and necrosis were considered as control group. Paraffin blocks that were archived more than 5 years excluded from this study. Moreover, this study was approved by the Ethics Committee of Kerman University of Medical Sciences (IR.KMU.REC.1396.1567). Hematoxylin and eosin staining was used for determination of histopathology. Immunohistochemistry method was used for detection of TLR expression.

Immunohistochemistry method

In the immunohistochemistry method, sections were extracted from paraffin, rehydrated with decreasing intensity of alcohol and transferred to citrate buffer for antigen retrieval. After blocking endogenous peroxidase with 3% hydrogen peroxide, slides were incubated overnight with primary antibodies including TLR3, TLR4, TLR7, TLR8 and TLR9 at a dilution of 1/2000, 1/4000, 1/300, 1/1000 and 1/1000, respectively. Then the sections were washed with TBS, and exposed to appropriate secondary antibody HRP anti Rabbit/mouse IgG (Dako, Denmark). The action was developed using the 3, 3 diaminobenzidine tetrahydrochloride chromogen (DAB, Dako). In the next step, the slides were counterstained with Hematoxylin and exposed in water, followed by immersing in graded alcohol and xylene. Evaluation of negative control was done by replacing the primary antibody with fetal bovine serum in each series.

Scoring

The staining of tumor cells was scored +3 defined



A: Negative staining B: Weak staining

C: Strong staining D: Moderate staining

Figure 1. Staining of tumor cells in ovarian cancer: A; negative staining, B: weak staining, C: strong staining, D: moderate staining.

strong staining, +2 moderate staining, +1 weak staining and 0 negative staining. Figure 1 shows staining of tumor cells in ovarian cancer.

Immunoreactivity score (IS)

Determination of IS is performed according to following formula.

$IS = \sqrt{P * I}$ (P: Percent of TLR stained cells, I: Color Intensity)

Statistical analysis

Statistical analysis was done using SPSS version 19 (IBM Corp, Armonk, NY, USA). The comparison of quantitative variables was performed by Mann Whitney test. Correlation between TLRs was performed through Spearman's rank correlation coefficient.

Results

In the current study, demographic and clinical characteristics of case and control groups are shown in table 1. As shown in table 1, 86.3 and 89.3% of the patients in the case and control groups were greater than 40 years old. Mean age of individuals in case

Table 1. Demographic and clinical characteristics of case and control group

Variables	Case	Control
	Number (percent)	
Age (yr)		
<40	9 (13.63%)	7 (10.60)
>40	57 (86.3%)	59 (89.3%)
Grade		
I	34 (51.5%)	---------
II	18 (27.2%)	
III	14 (21.2%)	
Type of cancer		
Serous	61 (92.42%)	-----
Mucinous	5 (7.58%)	
Menopause		
Yes	24 (36.36%)	43 (65.15%)
No	42 (63.63%)	23 (34.8%)

and control was 53 and 56 years old, respectively. Moreover, 92.4% of the patients had serous ovarian cancer. Furthermore, 36.3 and 65.1% of the patients in the case and control group were menopause, respectively. In addition, 51.5% of the patients in case group had grade II.

Quantitative and qualitative analysis of TRL3

Figure 2 shows the mean immunoreactivity score of TLR3. The mean IS of TLR3 in the case and control groups was 0.6±0.99 and 0.16±0.1, respectively. Moreover, a significant difference was seen between the case and control in terms of IS of TLR3 (quantitative variables) (p=0.001).

Quantitative and qualitative analysis of TRL4

Figure 3 shows the mean immunoreactivity score of TLR4. The mean IS of TLR4 was 0.63±0.95 and 0.77±1.27, respectively. Furthermore, no significant difference was seen between the case and control groups in terms of IS of TLR4 (quantitative variable) (p=0.905).

Quantitative and qualitative analysis of TRL7

Figure 4 shows the mean immunoreactivity score of TLR7. The mean IS of TLR7 in case and control was 1.03±1.05 and 0.66±1.07, respectively. Moreover, a significant difference was seen between the case and control in terms of IS of TLR7 (p=0.013).

Quantitative and qualitative analysis of TRL8

Figure 5 shows the mean immunoreactivity score of TLR8. The mean IS of TLR8 in case and control was 0.14±0.42 and 0.30±0.78. Moreover, no significant difference was seen between the case and control in terms IS of TLR8 (p=0.36).

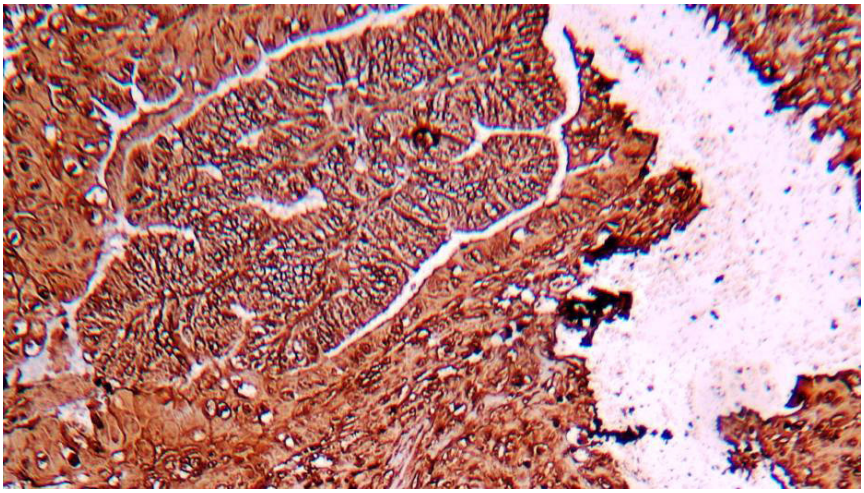
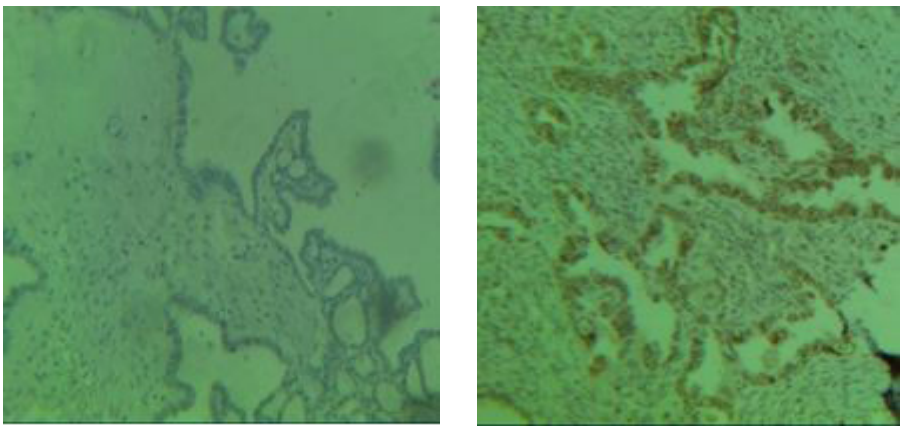


Figure 2. TLR3 expression (score 3).



A: Negative TLR4 (40×) B: Strong TLR4 (40x)

Figure 3. TLR 4 immunostaining; A: Negative TLR4 (40×), B: Strong TLR4 (40×).

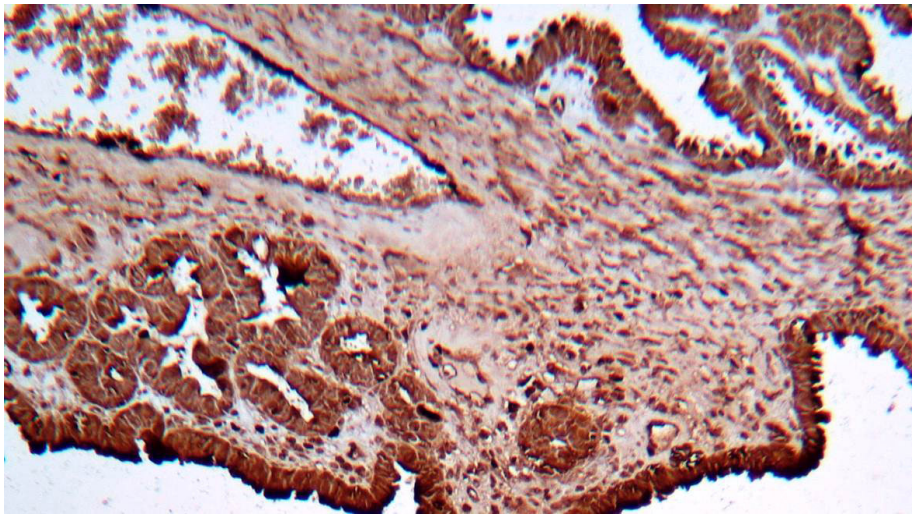


Figure 4. TLR 7 expression (score 3).

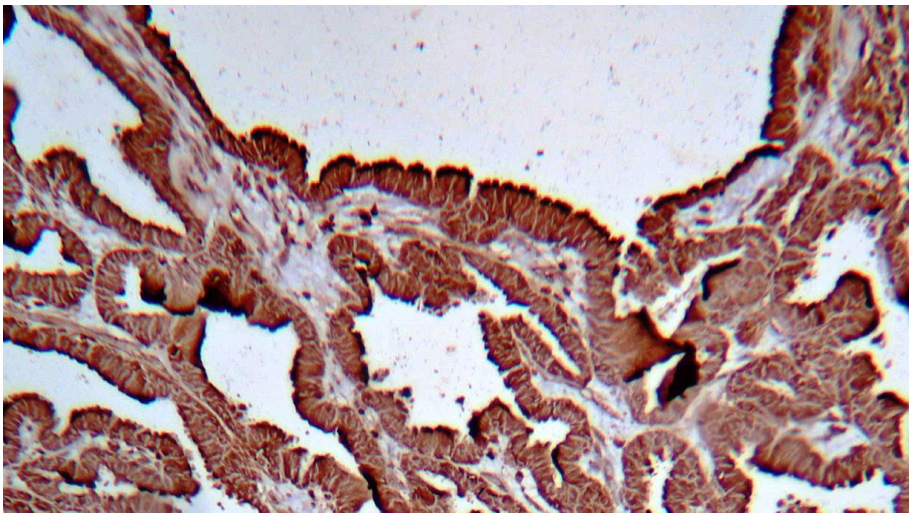


Figure 5. TLR 8 expression (score 3).

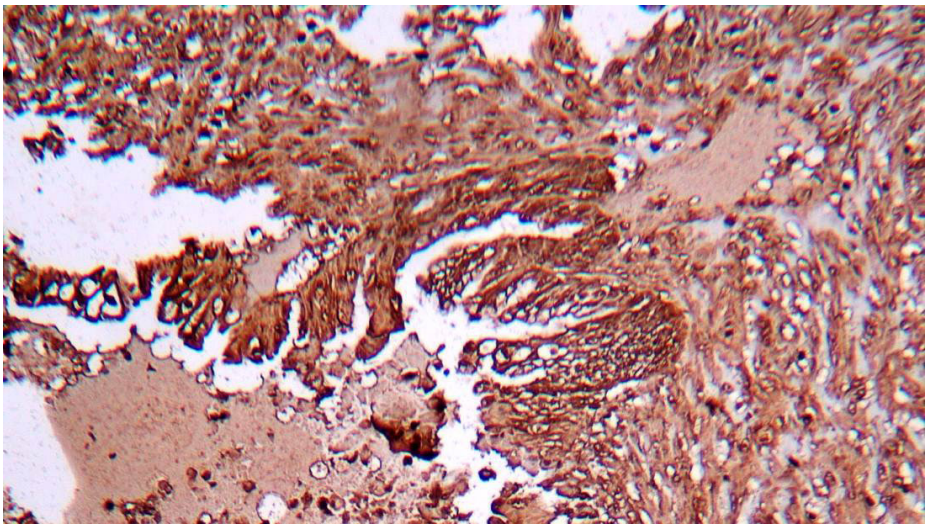


Figure 6. TLR 9 expression (Score 3).

Quantitative and qualitative analysis of TRL9

Figure 6 shows the mean immunoreactivity score of TLR9. The mean IS of TLR9 in case and control was 0.19±0.48 and 0.23±0.67, respectively. No significant difference was seen between the case and control in terms IS of TLR9 (p=0.07).

Moreover, the correlation between TLR7 with TLR4 and TLR9 with TLR8 showed that a positive correlation was observed between TLR4 and TLR7 in the case group (r=0.398, p=0.001). Moreover, a positive correlation was observed between TLR8 and TLR9 in the case group (p=0.001, r=0.414). In addition, a significant correlation was seen between TLR8 and TLR9 in the control group (p<0.001, r=0.784).

Discussion

In the current study, we evaluated TLRs, including TLR-3 in mucinous and serous ovarian cancer and observed a significant difference between the case and control groups in terms of TLR3. In this regard, a high IS of TLR3 was observed in tumor cells compared to normal cells. Very few studies have been conducted regarding these TLRs in ovarian cancer.

Sheyhidin *et al* suggested that high expression of TLR3 in tumor cells leads to higher lymph node metastasis and increased invasion (8). However, Hasimu *et al*, in 2011 evaluated TLR3 in cervical lesion and reported that the expression of TLR3 was not significantly different in the case (squamous cell carcinoma, and cervical intraepithelial neoplasia) and the control groups (12), which was inconsistent with our study. It seems that type of disease and different sample size were the reasons of this difference. Husseinzadeh *et al* reported that TLR3 has a dual role; contributing tumor elimination *via* the up-regulation of interferons α, β and Natural Killer cell (NK) activation and tumor progression (13).

Our study also demonstrated no difference between the 2 groups (the case and control group) concerning TLR4, but another study revealed that TLR-4 is expressed in a group of patients with cervical intraepithelial neoplasia and cervical squamous cell carcinoma (12). Other findings proposed that TLR4 holds a promise as a therapeutic target for serous ovarian cancer (14). Muccioli *et al* found that TLR4 is up-regulated in many ovarian epithelial tumors and numerous ovarian cancer cell lines which were not consistent with our study (15). Yang *et al* obtained a similar result and reported that over-expression of TLR4 in tumor cells plays a main role in progression and metastasis (16). Perhaps the difference between the studies was due to different grade and stage, and the type of malignancy. Husseinzadeh *et al*, revealed that TLR3, and TLR4 are highly expressed in both normal and neoplastic ovarian epithelium (13).

In the current study, the mean IS of TLR7 in tumor cells was greater than normal cells. Zhou *et al*, in 2009 reported that expression of TLR7 in benign and malignant epithelium of patients is weak (10). Sheyhidin *et al* stated that a significant increase in TLR7 mRNA level was observed in Esophageal Squamous Cell Carcinoma (ESCC) samples (8). Lee *et al*, in another study reported that TLR7 is expressed in ovarian cancer cell line (SNU251 cell line) (17). The findings of this study were consistent with our study. While TLR7 expression was increased in tumor cells, there was no significant difference between the case and control group in terms of TLR8 and TLR9 expression in our study. Zhou *et al*, declared that expression of TLR8 was observed in benign and malignant epithelium of some patients with ovarian cancer (10). Hasimu *et al* revealed that the expression levels of TLR7 were significantly higher in CIN and CSCC than in normal controls (12). The findings of the mentioned study were consistent with our study.

Moreover, Fehri *et al* evaluated the role of TLR9 in gynecologic cancer and revealed that TRL9 promotes tumor progression and invasiveness of cervical tissue (18). Berger *et al* reported that there is a significant association between TLR9 expression with poor differentiation (19) and disease aggressiveness in ovarian cancer. Chang *et al* reported that toll-like receptor 9 agonist increases anti-tumor immunity and prevents tumor-associated immunosuppressive cell numbers (20). Another study found variable expression of TLR8 in benign and neoplastic tissues (10); however, the level of TLR 9 was weak (10). Lee *et al*, reported that expression of TLR9 was weak in SNU251 cell line (17). Therefore, the findings of different studies were various and several factors such as the type of cancer, race, the sample size, grade, and stage are involved in this difference.

Coefficient correlation between TLR4 and TLR7,

TLR9, and TLR8 indicates that expression of these biomarkers increases together in the presence of infection. It seems that the TLR receptors mentioned above recognize different groups of viruses. Therefore, this finding can be justified in this way that along with the entry of the viral agent, there may be another co-infection which is a prerequisite and also a requirement for causing cancer along with the viral agent. In other words, this cancer is a multi-factor cancer, which means that along with the virus, there is another microorganism with the virus to stimulate other TLRs. In addition, it is possible that a series of unknown viruses entering the ovary have synergistic effects and involve various types of TLRs at the same time.

Conclusion

According to the results of this study, high IS of TLR7 and TLR3 in ovarian cancer patients may confirm the likelihood of association of viral infection with ovarian cancer. Also, considering that TLR3 is one of the receptors that have viral and bacterial ligands, there is a possibility that a bacterial infection is also involved in the development of ovarian cancer.

Furthermore, the expression of TLR4, TLR8 and TLR9 was not different in ovarian tissue of the case and control group. Of course, it is also worth noting that the most common viral ligands were evaluated in the study, but there is the probability that other infectious agents also trigger these receptors. However, the proof of this issue requires further research.

It is proposed to conduct this study with RT-PCR technique on the paraffin block sample and compare the results with the above results in future studies. In addition, the most important limitation of the study was the use of a sample fixed with formalin and kept in a paraffin block (FF-PE), which sometimes the lifespan of some samples reaches 5 years. This time may be accompanied by degradation of Ag TLR structure.

Conflict of Interest

None.

Funding

None.

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