Expression of Toll-like Receptor 4, Survivin and Caspase 3 in Ovarian Cancer

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ABSTRACT

Ovarian cancer is the most lethal gynecologic malignancy. Recent studies have shown that expression levels of toll like receptor (TLR) 4, survivin and caspase-3 in cancerous tissue may have a prognostic value in many cancer types including ovarian cancer. In the present study, we aimed to investigate the relationship between immunohistochemical expression of TLR4, survivin and caspase-3 and also to assess the effects on clinicopathological variables and survival parameters. Eighty four patients with ovarian cancer who treated between 2011 and 2019 were reviewed retrospectively. TLR4, survivin and caspase-3 levels that were obtained with immunohistochemistry from the tumor embedded in paraffin blocks. TLR4 expression was found higher in advanced stage compared to the early stage disease (p= 0.034). TLR4 positivity was inversely associated with lower caspase-3 expresion (p= 0.03) and survivin expression was closely related to capsule rupture (p= 0.037). Kaplan-Meier analysis demonstrated that stage, grade, lymphovascular invasion, and platinum sensitivity were significantly associated with progression free survival. Stage and adjuvant chemotherapy were also found to be significantly associated with overall survival. In Cox multivariate analysis, comparison according to lower versus higher TLR4 expression demonstrated that higher TLR4 expression was significantly associated with decreased overall survival (HR: 0.303; 95% CI: 0.109 to 0.847; p= 0.023). But, there was no association between survival parameters and either survivin or caspase-3 levels. As a result, TLR4 expression may have a prognostic role in ovarian cancer.

Keywords: Toll-like receptors, Survivin, Caspase

INTRODUCTION

The incidence of ovarian cancer increases with age, being most prevalent in the sixth and seventh decades of life.[1] At the time of diagnosis, more than 70% of patients present with advanced disease. Debulking surgery followed by adjuvant platinum-based chemotherapy is the standard of care in advanced epithelial ovarian cancer.[2] Recurrence is one of the major causes of high mortality in ovarian cancer. Chemotherapy plays a critical role for adjuvant treatment and in the palliative care of these patients with recurrent advanced disease.[3] Toll-like receptors are molecules that are located on cell surface and intracellular area. They belong to pattern recognition receptors (PRRs) class, which detect and respond to microbial antigens. TLRs are expressed on leukocytes and various solid tissue cells. The cellular localization of TLRs varies in many tumors. TLR2/1, TLR2/6, TLR4, TLR5, TLR10, and TLR11 are localized to cell surfaces, whereas TLR3, TLR7, TLR8, and TLR9 are localized within endosomes.[4,5,6] TLR4 expression in ovarian tumors gradually increases from benign and borderline to malignant, pathology, which indicated that TLR4 might be involved in the progression from normal ovarian tissue to cancer.

It has been reported that TLR4 is associated with poor overall survival, probably due to its association with advanced stage and higher grade. This can be explained by the activation of the TLR4/MyD88/NF-κB pathway that can contribute to an inflammatory microenvironment.[8]

TLR-based therapies include the TLR7 agonist and oligodeoxynucleotides binding to TLR9 have been applied to vaccination, cancer therapy, and immunotherapy for allergic disease. TLR can be an interesting target for treatment of a variety of pathologies including cancers, viral infection, immune diseases, and inflammation.[9,10]

Survivin blocks caspase activity, thereby inhibiting apoptosis. High expression levels of this antiapoptotic protein have been shown to predict poor prognosis and reduced survival rate in many cancer types including ovarian cancer.[11,12]

There has been no data in the literature evaluating the correlation between TLR and apoptotic markers in apoptosis such as survivin and caspase-3. In our study, we investigated immunohistochemical (IHC) expression of toll like receptor (TLR) 4, survivin, caspase-3 and their association with prognostic clinicopathological variables and survival data in ovarian cancer.

MATERIALS AND METHODS

Patients

Patients (n= 84) with FIGO stage I-IV epithelial ovarian cancer who undergone surgery or biopsy and treated at Pamukkale University Faculty of Medicine, Department of Medical Oncology between 2011 and 2019 were included to the analysis. The clinicopathologic variables, demographic data and chemotherapy regimens were retrospectively reviewed from the patient files.

Immunohistochemistry (IHC)

Immunohistochemistry was performed on 3-micron sections cut from routinely processed formalin-fixed, paraffin-embedded tissue blocks. The tissue sections were deparaffinized and rehydrated, pretreated with 0.01 M citrate buffer (pH 6), and then stained for caspase 9 (Bioss, 1/100 dilution, Rabbit Polyclonal Primary Antibody), survivin (Bioss, 1/100 dilution, Rabbit Polyclonal Primary Antibody), TLR4 (Toll - like receptor 4) (Bioss, 1/150 dilution, Rabbit Polyclonal Primary Antibody) antibodies by Ventana Benchmark ULTRA ™ automated immunostainer. In addition, the ultraView Universal DAB detection kit was used for all staining. Positive and negative controls were used for each antibody, based on the manufacturer's prerequisites. The slides were evaluated by two double-blinded pathologists (FB, YAK) with a Nikon eclipse e200 microscope. Tumor histotype was verified by light microscopic examination of H&E stained slides. Staining patterns were analyzed for each antibody; the percentage of positive staining and intensity (grade 0-3+) were determined at 40x magnification. c3 and survivin, were considered "positive" nuclear staining and TLR-4 was considered "positive" for cytoplasmic staining.

A final expression score for TLR4, survivin, and caspase-3 (range 0-300) was the product of staining intensity (range 0-3, corresponding to absent, weak, moderate or strong, respectively) multiplied by the proportion of tumor staining for that intensity (range 0-100). Median score was accepted as the cut off level. Median calculated scores (40.8, 244, and 192.6 for for TLR4, caspase-3, and surviving, respectively) were used to categorize patients into two groups as "low expression" and "high expression" groups.

This study was approved by the Pamukkale University Ethics Committee and all patients signed written informed consent.

Statistical Analysis

The SPSS software (SPSS version 23; IBM, Chicago, USA) package was used for the statistical analysis. Descriptive statistics were presented as median and percentage. Parameters that parametrical comparisons were performed on were presented in mean and standard deviation. Significance of differences between two groups was evaluated with a Mann-Whitney U test. Associations between quantitative data were determined with a Spearman's correlation test. Progression-free and overall survival were calculated using the Kaplan-Meier method. Cox proportional hazards regression was used to estimate hazard ratios (HRs) and

Table 1. Patient and tumor characteristics

Characteristics	n	%
Histology		
Serous	61	72.6
Borderline	7	8.3
Granulosa	7	8.3
Müsinous	2	2.4
Endometrioid	3	3.6
Clearcell	4	4.8
FIGO stage		
1	22	26.2
2	9	10.7
3	37	44.1
4	16	19
Primary surgery		
Complete debulking	77	91.7
Biopsy	7	8.3
Histological grade		
Low grade (G1-G2)	28	33.3
High grade (G3)	56	66.7
Capsule rupture		
Absent	27	32.1
Present	57	67.9
Lymphovascular invasion		
Absent	31	36.9
Present	53	63.1
Hormone receptor		
Negative	22	26.2
Positive	62	73.8
Adjuvant chemotherapy		
Present	69	82.1
Absent	15	17.9
Platinum sensitivity		
Sensitive	70	83.3
Resistant	14	16.7
TLR4		
Low < 40.8	53	63.1
High ≥ 40.8	31	36.9
Caspase-3		
Low < 244	27	32.1
High ≥ 244	57	67.9
Survivin		
Low < 192.6	42	50.0
High ≥ 192.6	42	50.0
Relapse	51	60.7

95% confidence intervals (CIs) for association of expression values with disease outcome. Log-rank test was used for statistical significance and p< 0.05 was considered statistically significant.

RESULTS

A total of 84 patients were included in this study. The majority of the patients (n= 77, 91.7%) were treated with debulking surgery. The median age was 55 years (range: 24-81 years) and 63.1% of patients were presented at advanced-stage. The most common (66.7%) histological subtype was serous carcinoma. Relapsed/recurrent disease was observed in 51 (60.7%) patients and 83.3% of patients had platinum sensitive disease. Of the cases, 62 (73.8%) had positive hormone expression. Median follow up time was 35 months (range: 1-159 months). The median disease-free survival of the cases was 18 months (range: 1-159 months). Of the total, 18 (21.4%) patients died during follow-up. The median overall survival of the cases was 27 months. Demographic characteristics of patients were presented in Table 1. Increased TLR4, caspase-3 and survivin staining was observed in 36.9%, 67.9% and 50% of the cases, respectively. TLR4 expression was significantly more frequent in advanced stage than early stage (p= 0.034). Increased survivin expression was also more common in patients with capsule rupture (p= 0.03).

Higher survivin expression was associated with capsule rupture (p= 0.03). There was no significant difference in TLR4, caspase-3, survivin between high and low grade ovarian cancer (Table 2). In higher expressed TLR4 group, mean caspase-3 level was 229 (range: 131-327) and in lower expressed TLR4 group mean caspase-3 level was 269 (range: 204-334). TLR4 positivity was inversely associated with lower caspase-3 expresion (p= 0.03). When we analyzed the patients as serous carcinoma and non-serous carcinoma, no statistical significance was found between TLR 4, survivin and caspase 3 levels (p> 0.005).

Kaplan-Meier survival analysis was conducted to compare the effects of stage, grade, LVSI, platinum sensitivity and expression of TLR4, caspase-3, and surviving on overall and progression-free survival. Univariate analysis of these parameters with Log Rank test revealed significant survival distribution differences for stage, grade, LVSI, and platinum sensitivity regarding disease-free survival (p< 0.001, p= 0.006, p= 0.01, and p< 0.001, respectively) (Table 3). In group with low TLR4 expres-

Table 2. Associations between histopathological characteristics of patients and TLR4, Survivin, Caspase-3

			TLR4		Survivin		Caspase-3	
		n	Mean±SD	p	Mean±SD	p	Mean±SD	p
Stage	1-2	31	56.32±58.48	0.034	197.90±107.27	0.26	243.87±94.43	0.64
	3-4	53	31.75 ±50.72		189.52±98.84		244.15±87.86	
Grade	Low	28	42.14±53.46	0.75	186.07±114.82	0.79	243.57±92.10	0.96
	High	40	40.16±55.76		195.89±91.98		244.28±89.48	
Capsule rupture	Absent	27	30.48±44.49	0.34	160.18±107.63	0.037	205.92±108.28	0.09
	Present	57	45.71±58.63		207.98±92.59		262.10±73.96	
Lymphovascular	Absent	31	45.32±51.60	0.23	189.19±105.61	0.50	247.74±83.25	0.98
invasion	Present	53	38.18±56.73		194.62±96.87		241.88±94.09	
Hormone receptor	Negative	22	31.81±45.37	0.56	167.72±110.59	0.62	227.72±100.47	0.30
	Positive	62	44.01±57.63		201.45±94.77		249.83±85.80	

* Univariate analysis is obtained and analysed by using Mann Whitney U and Kruskal-Walles tests.

sion median follow-up time was 40 months (range: 2-78 months) and it was 26 months (range: 6-46 months) in group with high TLR4 expression. Low and high expression of TLR4, caspase-3 and survivin had comparable median progression-free and overall survival times (Figure1, 2).

Cox regression analysis was further performed to investigated the independent effect of these parameters on disease-free and overall survival. In this analysis increased TLR4 expression was found to be independently associated with poorer overall survival (HR: 3.236, 95% CI: 1.081-9.690, $p< 0.05$) (Table 4).

DISCUSSION

Ovarian cancer is the most lethal cancer of the female reproductive system and requires new targeted therapies. Biomarkers have been monitored to evaluate the therapeutic effects, including cell proliferation, apoptosis, signal recognition, and transduction. Unfortunately, so far, a limited number of studies were interested in the expression and the function of TLRs, survivin and caspase in the ovarian cancer. TLR4 plays an essential role in immunity. The role of inflammation and related pathways had been consistently documented in carcinogenesis and progression of several cancers. Based on their important role in inflammation and tissue regeneration, we hypothesized that TLR4 might play a role in defining the prognosis of ovarian tumors. This study demonstrated that high TLR4 expression was significantly more frequent in advanced stage when compared to early stage disease indicating that TLR4 may play a role in progression of ovarian cancer.

Li et al. reported that TLR4 is associated with advanced and high-grade disease in serous ovarian cancer.[8] Our results are concordant with these findings. Li et al. also found a gradual rising trend in TLR4 expression from benign to malignant ovarian tumors, and therefore they argued that TLR4 might be involved in the development and progression from ovarian normal tissue to cancer. We documented no significant association between TLR4 expression and platinum sensitivity, which contradicts previous findings.[13] We hypothesized that different selection criteria and surgical practices in different studies may cause this discrepancy. Consistent with our findings, Vlad et al reported that median TLR4 expression was significantly higher in cases with peritoneal carcinomatosis than in cases without peritoneal carcinomatosis.[14] Similar association between increased TLR4 expression and advanced disease was also reported in other cancers; high expression of TLR4 relates to increased risk of tumor progression in colorectal cancer patients and cutaneous malignant melanoma patients.[15,16] Another study in ovarian cancer patients also documented that TLR4 expression

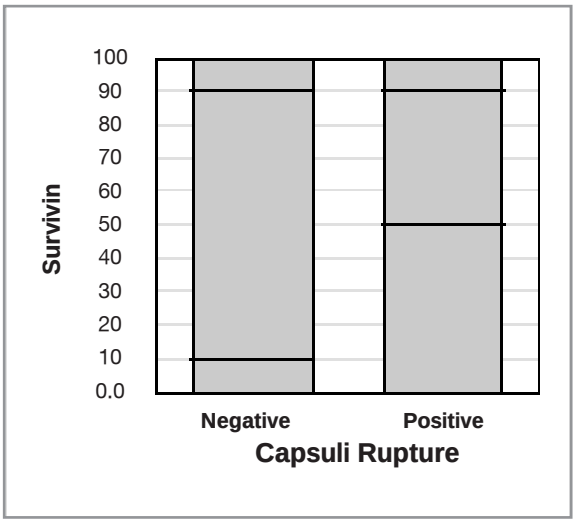

Figure 1. Survivin score was also associated with capsule rupture

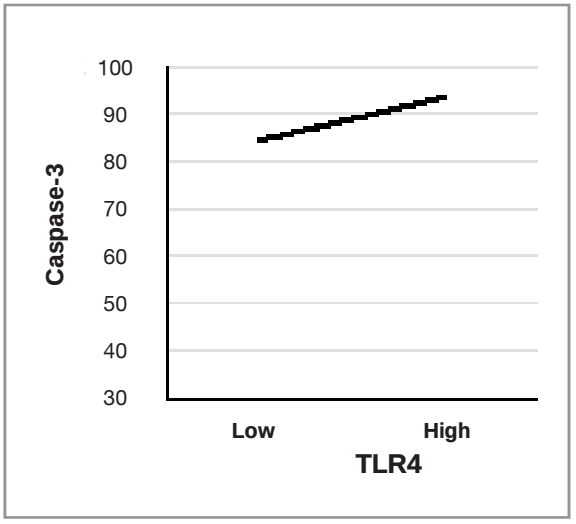

Figure 2. TLR4 was associated with Caspase-3

significantly increased when compared to precancerous tissues.[17,18] All these findings indicate that TLR4 related pathways play an important not only in differentiation of the ovarian neoplasms, but also may contribute to the development of ovarian cancer from benign or premalignant tissues.

Association of TLR expression pattern with the prognosis of the ovarian cancer patients was also investigated at several centers. All these reports consistently documented poor survival parameters with increased TLR4 expression.[8,13,14] Also a recent meta-analysis showed that higher expression levels

Table 3. Prognostic factors related to progression free survival and overall survival

Characteristics		n	p	
			Progression free survival	Overall survival
Stage	Early	31	< 0.001	< 0.001
	Late	53		
Grade	Low	28	0.006	0.33
	High	56		
Lymphovascular invasion	Negative	31	0.01	0.98
	Positive	53		
Adjuvant chemotherapy	Present	69	0.56	0.002
	Absent	15		
Platinum sensitivity	Sensitive	70	< 0.001	0.36
	Resistant	14		
TLR4	Low < 40.8	53	0.92	0.18
	High ≥ 40.8	31		
Caspase-3	Low < 244	27	0.84	0.46
	High ≥ 244	57		
Survivin	Low < 192.6	42	0.76	0.29
	High ≥ 192.6	42		

* p values are obtained by Kaplan Meier analysis. p< 0.05 is accepted to be statistically significant

Table 4. Prognostic factors for progression free survival and overall survival of ovarian cancer in multivariate analysis

	Prognostic factors	Hazard Ratio	95% CI	p
Progression free survival	Stage	4.161	1.826-9.480	0.001*
	Grade	3.866	1.144-13.062	0.030*
	Platinum sensitivity	0.246	0.103-0.587	0.002*
	TLR4 expression	1.001	0.516-1.945	0.997
	Caspase-3 expression	0.809	0.444-1.475	0.489
	Survivin expression	0.841	0.452-1.564	0.584
Overall survival	Stage	6.011	1.294-27.920	0.022*
	Grade	9.799	1.282-74.902	0.028*
	Platinum sensitivity	1.097	0.185-6.514	0.919
	TLR4 expression	3.236	1.081-9.690	0.036*
	Caspase-3 expression	0.583	0.201-1.686	0.319
	Survivin expression	2.884	0.836-9.941	0.094

p values are obtained by Cox multivariate analysis. * p< 0.05 is accepted to be statistically significant

of TLR4 or TLR7 in tumor tissues could predict poorer survival.[19] In our study, Cox multivariate analysis but not univariate comparison demonstrated that high TLR4 expression was associated with decreased overall survival. Since several tumor parameters including TLR4 expression may interact with each other, we believe that multivariate analysis is more accurate for defining the individual and independent effects of these parameters. Therefore, our result showing an independent adverse effect of increased TLR4 expression on overall survival is supported by previous reports. The originality of our finding is the fact that this adverse effect is independent of stage, grade or platinum sensitivity status.

Caspase-3 functions at the end stage of the apoptotic process and survivin enhances cell survival via targeting the terminal effect of caspase-3.[11] Therefore, caspase-3 and survivin together counterbalances each other in the apoptotic process though sensitive feedback mechanisms. The effect of survivin on ovarian cancer biology was assessed in a meta-analysis total of 1097 patients that documented higher survivin expression in ovarian cancer than in the normal tissues. Survivin was associated with stage and tumor grade of ovarian carcinoma.[20] However we were unable show a significant difference between survivin and stage or grade. One explanation for this discrepancy may lie in heterogeneity of the study populations between studies. Therefore, further studies are needed to define the role of survivin in ovarian cancer biology in more specific patient groups. Cellular studies suggest that there may be a link between survivin expression and sensitivity to taxotere treatment.[21] Accordingly, Li et al showed that survivin expression indicates poor prognosis.[22] In our study there was no association between survivin and survival. Ferrandina et al however, showed that survivin status does not seem to be helpful in the prognostic characterisation of ovarian cancer.[23] Interestingly in our study we demonstrated for the first time survivin was associated with capsule rupture, which was not associated with worse prognosis. There may be several explanations for this disparate result, one of which is the discussion that capsule rupture may not be associated with survival. Another overlooked factor may be the surgical technical differences; whether optimal debulking was achieved or not. In example, a more extensive surgery may overcome the poor prognostic effect of capsule rupture that was found to be associated with higher survivin expression in our study. Therefore, further prospective studies should be conducted including detailed information regarding the surgical details of the initial operation.

Caspase-3 is also important in apoptosis and has the potential to predict the clinical response to

chemotherapy.[24] Hu et al reported that high expression of caspase-3 had a significant shorter overall survival compared with low caspase-3 expression in ovarian cancer, gastric cancer, cervical cancer and colorectal cancer.[25] In our study we did not find any correlation between caspase-3 and survival. However, we showed that caspase-3 was correlated with higher TLR4 expression (p= 0.03). To our knowledge, this is the first study to document the relationship between caspase-3 and TLR4 expression. One important finding in this study was the documentation of the independent effect of high TLR4 expression on worse survival, when other factors including survivin and caspase-3 are controlled. Further research on this subject is required to dissect the molecular mechanisms that explain the effect of TLR4 in ovarian cancer biology independent of survivin and caspase-3.

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