Clinical roles of interleukin-6 and STAT3 in oral squamous cell carcinoma

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Abstract

The effect inflammation has on cancer prognosis is marked by the presence of cytokines and chemokines. Interleukin-6 (IL-6) is one a multifunctional cytokine that regulates inflammatory responses. We investigated the roles of IL-6 and STAT3 and examined the relationship between IL-6 signaling and clinicopathological factors in patients with oral squamous cell carcinoma (OSCC). We retrospectively examined 116 patients who underwent radical surgery for OSCC. IL-6 and STAT3 expression were detected by immunohistochemistry. IL-6 and STAT3 positivity were detected by IHC, at 78.4 and 80.2%, respectively. IL-6 expression was significantly associated with pattern of invasion (P = 0.004), vascular invasion (P = 0.003), and pathological nodal status (P = 0.019). Multivariate logistic regression analysis revealed that IL-6 expression was significantly associated with vascular invasion (P = 0.044). Meanwhile, there was no significant association between STAT3 expression and clinicopathological factors and no significant relationship between IL-6 and STAT3 expression. IL-6 expression was significantly associated with 5-year disease-free survival. These results suggest that IL-6 is involved in lymphangiogenesis and recurrence in OSCC.

Keywords IL-6・STAT3・Oral squamous cell carcinoma・Lymphangiogenesis

Introduction

Oral squamous cell carcinoma (OSCC) accounts for 2−3% of all cancers and is the 11th most common cancer worldwide [1]. OSCC remains a major cause of morbidity and mortality in patients with head and neck cancer. Despite excellent functional and survival outcomes in patients with early-stage oral cancer, patients with advanced-stage disease continue to have poor survival, with only a 5% improvement in overall survival in the last 20 years [2]. Therefore, more effective treatment strategies are necessary to improve the survival of patients with OSCC. With advances in tumor immunology, immunotherapy has become an emerging option for cancer therapy [3,4]. Recently, several studies have reported that biomarkers involved in inflammation and the immune system are useful for understanding the biological behavior of OSCC [5–10].

Previous studies have reported that inflammation affects cancer prognosis [11–19]. IL-6 is a multifunctional cytokine that regulates inflammatory responses [20]. IL-6 plays an important role in many tumor functions, including development, migration, invasion, growth, proliferation, apoptosis, progression, angiogenesis, and differentiation of tumor cells [21]. IL-6 activates the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, phosphatidylinositol-3 kinase (PI3K) pathway, and the mitogen-activated protein kinase (MAPK) pathway [22]. IL-6 binds to IL-6R, which forms a complex with gp130, to activate STAT3 via the JAK/STAT pathway [12, 21, 23]. Activated STAT3 controls proliferation, survival, inflammation, invasion, metastasis, and angiogenesis in normal cells [21,23,24]. However, activated STAT3 induces pro-survival and pro-proliferative signaling and contributes to tumor growth of cancer cells [24]. However, few studies have examined the relationship between IL-6 signaling and OSCC biological characteristics. Therefore, we investigated the roles of IL-6 and STAT3 in OSCC and examined the relationship between IL-6 signaling and clinicopathological factors of patients with OSCC.

Materials and Methods

Patient characteristics

We analyzed data from 116 patients with OSCC who had their first visit to our department between April 2008 and March 2013 and were treated, usually with surgery. Ethical approval was obtained from the institutional review board of Nagasaki University Hospital (IRB no.: 15061128). Patients who underwent preoperative chemotherapy or radiation therapy or who had insufficient preoperative records were excluded. All patients underwent extensive pretreatment evaluations, including blood chemistry analysis, complete blood count, chest X-ray, computed tomography (CT), and/or magnetic resonance imaging (MRI) of the head and neck area, and thoracoabdominal CT, and staging using the TNM classification of malignant tumors, UICC 7th edition [25].

The pattern of invasion (POI), which was evaluated as types 1–4 as defined previously by Bryne et al. [26], was examined at the host/tumor interface. Perineural invasion was defined as the presence of tumor cells within any of the three layers of the nerve sheath (the epineurium, perineurium, and endoneurium). Vascular invasion was defined as the clear presence of tumor cells within a vascular space (lymphatic space or blood vessel), and the tumor had to be adhered to the vessel endothelium or attached to a thrombus in the vessel.

Immunohistochemistry (IHC)

Biopsy or surgical specimens were taken from the patient with OSCC, formalin-fixed, and paraffin-embedded after surgery. Specimens were cut into 4-μm serial sections. Serial sections were deparaffinized in xylene, soaked in 10 mM citrate buffer (pH 6), and placed in the autoclave at 121°C for 5 min for antigenicity activation. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol at room temperature for 30 min. The sections were incubated with primary antibodies (IL6, Abcam, Cambridge, UK; 1:600 dilution and STAT3, 124H6, Cell Signaling Technology, MA, USA;

1:600 dilution) overnight at 4°C. Immunohistochemical staining was performed with the EnVision system (EnVision+, DAKO, Glostrup, Denmark). Reaction products were visualized using diaminobenzidine (DAB) solution, counterstained with Mayer's hematoxylin, dehydrated, cleared with xylene, and mounted. Immunohistochemistry expression analyses were performed by calculating the sum of distribution scores and intensity scores. The distribution score was defined as the estimated fraction of positively stained tumor area (0: none, 1: <20%, 2: 20–50%, 3: 50–80%, and 4: >80%). The intensity score was defined as the estimated staining intensity (0: no staining, 1: weak, 2: moderate, and 3: strong). Total scores ranged from 0 to 7. Score of 5 or more were determined positive expression by Receiver Operating Characteristic (ROC) analysis.

Measurement of C-reactive protein (CRP) level and neutrophil-to-lymphocyte ratio (NLR)

All serum CRP levels and complete blood counts were measured during preoperative examination. NLR values were calculated using neutrophil and lymphocyte counts. The optimal cut-off value for CRP was 3 mg/L, and the NLR cutoff was 2.4, as previously reported [7,14].

Statistical Analysis

All analyses were performed using SPSS 22.0 (Japan IBM, Tokyo, Japan). Categorical data were assessed by Chi-squared test. Multivariate logistic regression analyses were used to determine the associations between various factors and IL-6 expression. Survival times were calculated from the date of surgery. Disease-free survival (DFS) was defined as the period from the date of surgery to cancer recurrence. Disease-specific survival (DSS) was defined as the period from the date of surgery to death or the end of observation. DFS and DSS of the entire cohort were estimated using the Kaplan-Meier method and compared by log-rank test. Multivariate analyses were assessed using the Cox proportional hazards model. Significant factors from univariate analyses were included in multivariate analysis. P values less than 0.05 were considered significant.

Results

Patient characteristics

Patient demographic characteristics are summarized in Table 1. The mean age at diagnosis was 67.4 years (range, 30–95 years). The male-to-female ratio was 1.27, with 65 male subjects. Diffuse invasion was found in 42 of 116 patients (36.2%), vascular invasion was found in 54 patients (46.6%), and perineural invasion was found in 27 patients (23.3%). Pathological lymph node metastasis was noted in 42 patients (36.2%). Local recurrence developed in 11 patients (9.5%) during the follow-up period, which was a mean 48.9 months for all participants (range, 2–88 months).

IL-6 and STAT3 expression by IHC

IL-6 was strongly expressed in stroma and cancer cell membranes, but it was not expressed in cancer cell cytoplasm (Fig. 1a). STAT3 was strongly expressed in cancer cell membranes and nuclei (Fig. 1b). IL-6 and STAT3 positivity rates by IHC were 78.4 and 80.2%, respectively.

Associations between IL-6 and STAT3 expression, clinicopathological factors, and survival

IL-6 and STAT3 expression levels are summarized in Table 1. IL-6 expression was significantly associated with POI, vascular invasion, and pathological nodal status. Multivariate logistic regression analysis revealed that IL-6 expression was significantly associated with vascular invasion ($P = 0.044$) (Table 2). Meanwhile, there was no significant association between STAT3 expression and clinicopathological factors. There was no significant relationship between IL-6 and STAT3 expression (Table 3). Univariate analyses by log-rank test revealed that POI ($P < 0.001$), vascular invasion ($P < 0.001$), perineural invasion ($P < 0.001$), pathological nodal status ($P < 0.001$), and IL-6 (Fig. 2, $P = 0.010$) were significantly associated with 5-year DFS. Predictors associated with 5-year DFS in univariate analyses were included in the Cox proportional hazards model, and this multivariate

analysis showed that POI and pathological nodal status were independent predictors of 5-year DFS. Univariate analyses by log-rank test revealed that clinical stage ($P = 0.032$), POI ($P < 0.001$), vascular invasion ($P = 0.026$), perineural invasion ($P = 0.004$), and pathological nodal status ($P < 0.001$) were significantly associated with 5-year DSS. According to the Cox proportional hazards multivariate analysis, pathological nodal status was the only independent predictor of 5-year DSS.

Discussion

IL-6 is a multifunctional cytokine that regulates immune responses [20]. IL-6 production is increased in response to various stimuli, such as infection and inflammation. Cancer stimulates inflammation that can lead to increased expression of IL-6-modulating factors, such as IL-1β, COX-2, PGE2, and TGF-β [22]. The IL-6 receptor is a type I cytokine receptor complex that consists of the ligand-binding IL-6Ra chain (also called CD126) and the gp130 (also called CD130), which is responsible for downstream signaling [21]. IL-6 activates the JAK/STAT, PI3K, and the MAPK pathways [8,22]. This signaling influences cell migration, malignant tumor growth and invasion, anti-apoptotic signaling, angiogenesis, and bone remodeling [8–10,22,27,28].

Shinriki et al. demonstrated that IL-6 signaling via the PI3K-Akt pathway stimulates VEGF-C synthesis and lymphangiogenesis in OSCC [27]. VEGF-C is an essential chemotactic and survival factor during embryonic and inflammatory lymphangiogenesis [29]. Furthermore, several studies have reported that the VEGF-C is associated with lymph node metastasis, and its expression is a prognostic factor for various cancers including oral cancer [30–32]. In this study, we examined the association between IL-6 expression and clinicopathological factors in patients with OSCC by IHC. Our data indicate that IL-6 expression is associated with POI, vascular invasion, and pathological nodal status in OSCC. In particular, vascular invasion strongly correlated with IL-6 expression, suggesting that IL-6 is involved in lymphangiogenesis in OSCC.

On the other hand, several studies have reported that IL-6 activates the JAK/STAT3 pathway in various cancers [12,15,24,28]. Yadav et al. demonstrated that IL-6 promotes epithelial-mesenchymal transition (EMT) changes via the JAK/STAT3 pathway [28]. STAT3 is downstream mediator of IL-6 and represses E-cadherin transcription via the snail-related zinc-finger transcription factor [28]. E-cadherin loss in tumor cells is a widely known hallmark of EMT. Additionally, some studies have reported that IL-6 overexpression plays transcriptional and regulatory roles in invasion and metastasis, leading to poor prognoses for patients with head and neck cancers [9,17,22,24,28]. In this study, however, the relationship between IL-6 and STAT3 expression was not significant. In addition, STAT3 was not significantly associated with clinicopathological factors. Therefore, our results suggested that IL-6 promotes lymphangiogenesis via the PI3K-Akt pathway rather than the JAK/STAT3 pathway.

Some studies reported that post-operative serum or salivary IL-6 levels could predict recurrence and survival in patients with head and neck cancer [9,10,33]. Sato et al. indicated that post-operative salivary IL-6 level is a useful marker for OSCC locoregional recurrence [10]. In this study, IL-6 expression was significantly associated with 5-year DFS, suggesting that IL-6 evaluation by IHC could predict time to recurrence, including locoregional recurrence and distant metastasis. However, any correlation between IL-6 expression and local recurrence was not clear, leading us to conclude that IL-6 was primarily involved in lymphangiogenesis and metastasis of OSCC.

CRP and NLR are indicators of inflammatory response and often checked at during preoperative examination. Tumor growth can cause tissue inflammation and an immune response to tumor antigens [11,12]. Furthermore, IL-6 secreted by cancer cells induces CRP production, which is a tumor antigen that links chronic inflammation and tumor growth [14–17]. Leukocyte count is usually increased in response to infection, inflammation, allergic reaction, and malignancy. Although neutrophils have anti-tumor effects, lymphocytes are most responsible for controlling cancer progression, and increasing NLR is less efficacious [18]. Therefore, NLR elevation has been suggested to be associated with poor prognosis. Some studies report a relationship between prognosis and both high preoperative

CRP levels and NLR [5,7,19]. Tumor cells produce various cytokines and chemokines that induce inflammatory responses, including a diverse leukocyte population, cytokines, and cytotoxic mediators [11]. This study found no relationship between IL-6 or STAT3 expression and increasing preoperative CRP levels or NLR in OSCC. In our study, the proportion of patients with high CRP levels was small compared with that of other reports [5,7]. In cases of oral cancer, when the differential diagnosis includes acute dental infection, antibiotics are administered early. Inflammation-related oral cancer becomes suspected when chronic inflammation remains, with no CRP level elevation. Some studies reported that elevated NLR is associated with worse prognosis [7,19]. Neutrophils induced by GCSF from the tumor aid in tumor progression by producing chemical mediators, such as VEGF, and causing a relative reduction in lymphocyte number and a decrease in lymphocyte-dependent cellular immune reactions [18,34,35]. Because lymphocyte-mediated cellular immune responses are reduced when NLR is high, high NLR is often seen in tumor progression and is a predictor of poor prognosis [18]. In this study, the relationship between IL-6 and STAT3 expression and increased preoperative NLR was not significant. Recent studies have reported that elevated NLR could be used to predict response to neoadjuvant chemoradiation and prognosis in rectal cancer [36,37]. Therefore, if patients with lower baseline NLR values underwent neoadjuvant chemoradiation, they would be more likely to have better responses. We could not evaluate the relationship between NLR value and neoadjuvant therapy efficacy because patients who underwent neoadjuvant chemoradiation or chemotherapy were not included in our cohort.

In conclusion, our data indicate that IL-6 expression is associated with POI, vascular invasion, and pathological nodal status in OSCC. In particular, vascular invasion strongly correlated with IL-6 expression but not STAT3. Therefore, the present study suggests that IL-6 involved in lymphangiogenesis and recurrence in OSCC. Further studies to elucidate the mechanism of IL-6-mediated VEGF-C synthesis may provide information leading to the development of treatment targeting the PI3K-Akt pathway and improved outcomes in patients with OSCC.

Conflict of interest

The authors have no conflicts of interests to declare in this study.

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Figure Legends

Fig. 1 Representative immunohistochemical staining for IL-6 and STAT3 in OSCC. **a**: IL-6 was strongly positive in cell stroma and on cancer cell membranes(intensity score 3), but there was no IL-6 staining in the cancer cell cytoplasm. **b**: STAT3 was strongly expressed in cancer cell membranes and nuclei (intensity score 3).

Fig. 2 Kaplan-Meier curves for 5-year disease-free survival according to IL-6 expression in patients with OSCC. The IL-6 overexpression group had a significantly lower disease-free survival rate than that of the low expression group ($P = 0.010$).

Fig. 1a

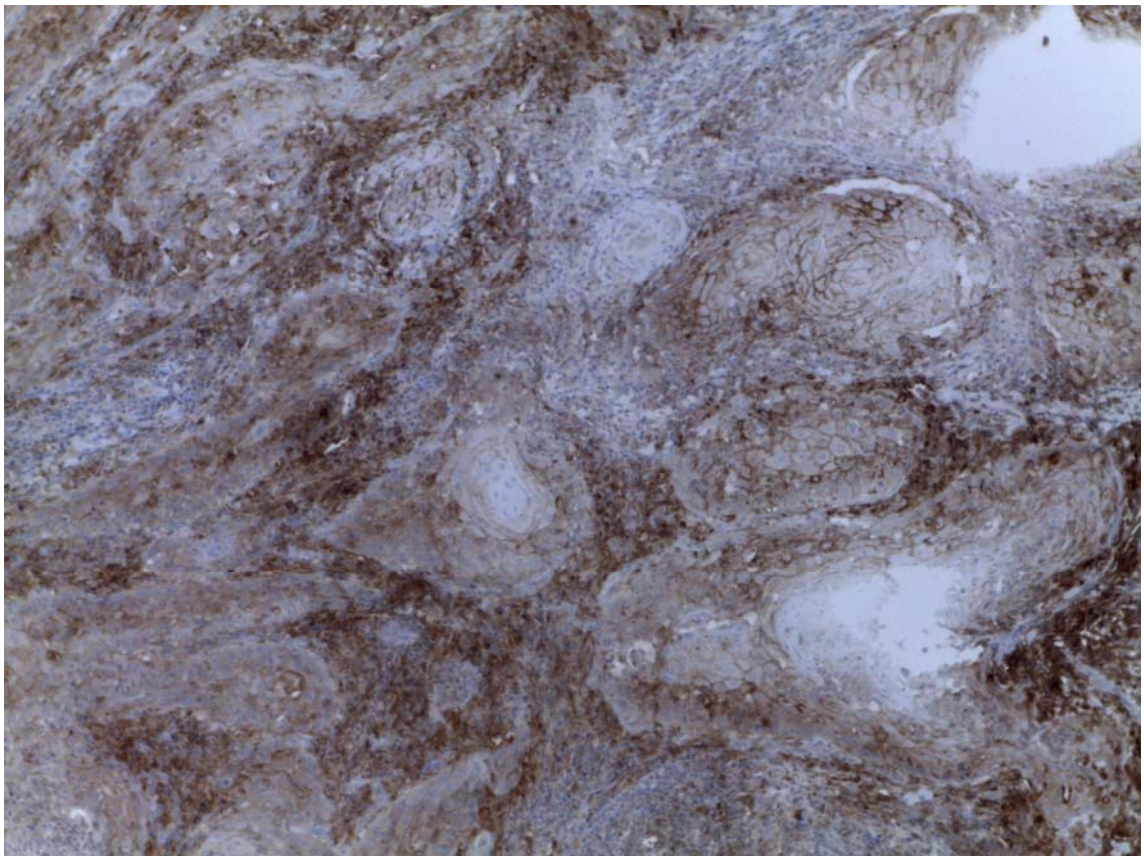

Fig. 1b

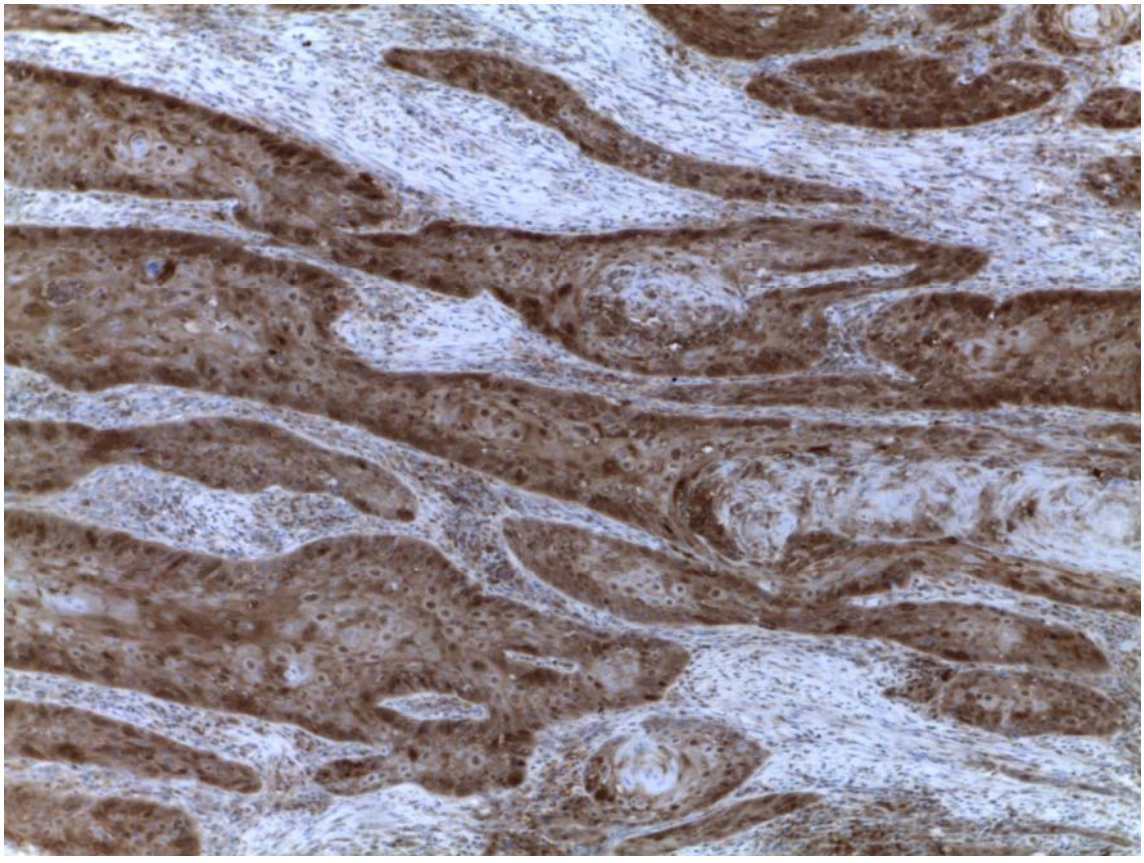

Fig. 2

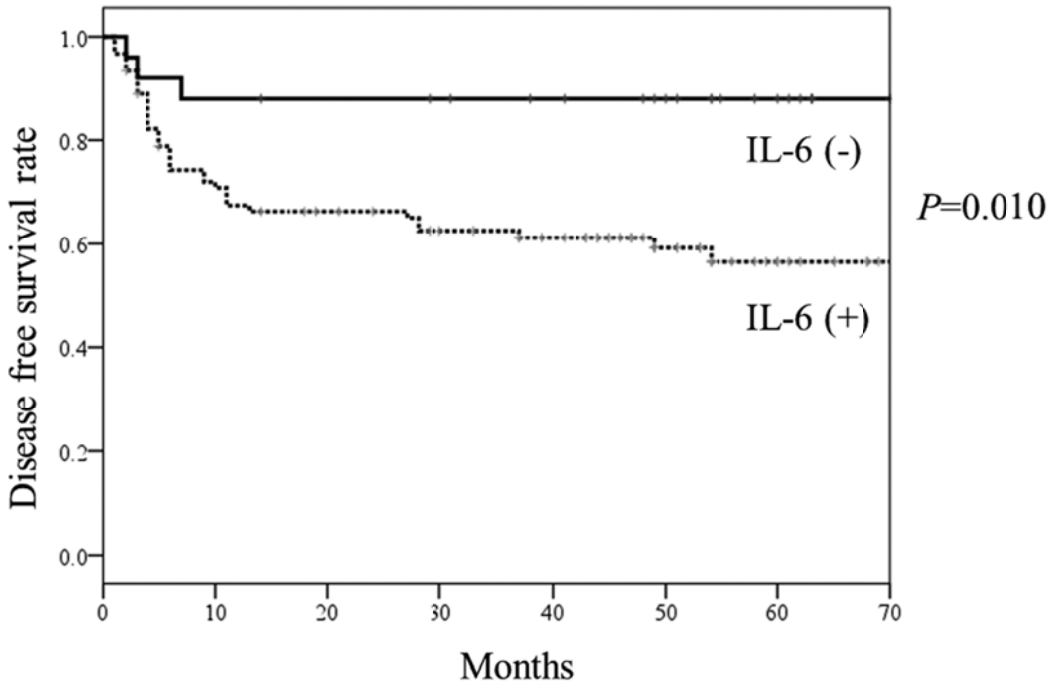
Disease free survival rate
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
Months
IL-6 (-)
IL-6 (+)
P=0.010

Table 1 Association of IL-6 and STAT3 with clinicopathological factors

Characteristics	No. of cases (%)	IL-6			STAT3		
		Negative	Positive	*P*	Negative	Positive	*P*
Age (years)				0.250			0.239
<65	47(40.5)	13	34		12	35	
≥65	69(59.5)	12	57		11	58	
Gender				1.000			0.646
Male	65(56.0)	14	51		14	51	
Female	51(44.0)	11	40		9	42	
Clinical T stage				0.277			0.399
T1–2	92(79.3)	22	70		20	72	
T3–4	24(20.7)	3	21		3	21	
Clinical stage				0.088			0.625
Stage I-II	80(69.0)	21	59		17	63	
Stage III-IV	36(31.0)	4	32		6	30	
POI				**0.004**			0.631
1-3	74(63.8)	22	52		16	58	
4	42(36.2)	3	39		7	35	
Vascular invasion				**0.003**			0.818
No	62(53.4)	20	42		13	49	
Yes	54(46.6)	5	49		10	44	
Perineural invasion				0.183			0.273
No	89(76.7)	22	67		20	69	
Yes	27(23.3)	3	24		3	24	
Pathological nodal status				**0.019**			0.147
No	74(63.8)	21	53		18	56	
Yes	42(36.2)	4	38		5	37	
Local recurrence				0.452			0.225
No	105(90.5)	24	81		19	86	
Yes	11(9.5)	1	10		4	7	
CRP				0.587			0.779
<3	91(78.4)	21	70		19	72	
≥3	25(21.6)	4	21		4	21	
NLR				0.369			1.000
<2.4	64(55.2)	16	48		13	51	
≥2.4	52(44.8)	9	43		10	42	

Bolded values indicate $p < 0.05$.

POI, Pattern of invasion; *CRP*, C-reactive protein; *NLR*, Neutrophil-to-lymphocyte ratio.

Table 2 Association of IL-6 expression with clinicopathological factors by multivariate analysis

Characteristics	IL-6		
	HR	CI	*P*
POI			0.114
1-3	Ref.		
4	3.84	0.73-20.3	
Vascular invasion			**0.044**
No	Ref.		
Yes	3.30	1.03-10.6	
Pathological nodal status			0.945
No	Ref.		
Yes	0.95	0.19-4.70	

Bolded values indicate $p < 0.05$.

POI, Pattern of invasion; *HR*, hazard ratio; *CI*, confidence interval.

Table 3 Relationship between IL-6 expression and STAT3 expression

	STAT3 expression		
	Negative (n=23)	Positive (n=93)	
IL-6 expression			*P*=1.000
Negative (n=25)	5	20	
Positive (n=91)	18	73	

Table 4 Univariate and multivariate analyses of risk factors influencing survival

Characteristics	Disease free survival				Disease specific survival			
	Univariate		Multivariate		Univariate		Multivariate	
	5-year DFS	p^a	HR (95% CI)	p	5-year DSS	p	HR (95% CI)	p
Age (years)		0.424	-	-		0.332	-	-
<65	65.7%				75.4%			
≥65	62.2%				87.0%			
Gender		0.203	-	-		0.329	-	-
Male	70.6%				83.5%			
Female	55.5%				81.2%			
Clinical T stage		0.065	-	-		0.588	-	-
T1–2	67.7%				83.5%			
T3–4	50.0%				76.6%			
Clinical stage		0.050	-	-		**0.032**		0.753
Stage I-II	71.6%				88.6%		Ref.	
Stage III-IV	46.6%				61.7%		1.20 (0.38−3.80)	
POI		**<0.001**		**0.002**		**<0.001**		0.227
1-3	90.1%		Ref.		94.6%		Ref.	
4	17.2%		5.90 (1.97−17.7)		60.7%		3.03 (0.50−18.3)	
Vascular invasion		**<0.001**		0.642		**0.026**		0.746
No	78.0%		Ref.		89.7%		Ref.	
Yes	47.7%		1.19 (0.58−2.43)		73.7%		1.22 (0.36−4.14)	
Perineural invasion		**<0.001**		0.612		**0.004**		0.572
No	75.2%		Ref.		87.3%		Ref.	
Yes	26.6%		*0.84 (0.42−1.67)*		65.9%		0.71 (0.22−2.33)	
Pathological nodal status		**<0.001**		**0.004**		**<0.001**		**0.031**
No	91.5%		Ref.		96.8%		Ref.	
Yes	17.2%		5.11 (1.70−15.4)		56.0%		14.0(1.27−154)	
CRP		0.522	-	-		0.066	-	-
<3	62.0%				87.5%			
≥3	70.7%				65.4%			
NLR		0.482	-	-		0.351	-	-
<2.4	65.8%				78.1%			
≥2.4	62.0%				86.6%			
IL-6		**0.010**		0.268		0.126	-	-
Negative	88.0%		Ref.		95.8%			
Positive	56.5%		2.29 (0.53−9.95)		77.9%			

STAT3		0.338	-	-		0.184	-	-
Negative	72.8%				93.8%			
Positive	61.7%				78.7%			

[a]Bolded values indicate $p < 0.05$.

POI, Pattern of invasion; *CRP*, C-reactive protein; *NLR*, Neutrophil-to-lymphocyte ratio.