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The prognosis of head and neck squamous cell carcinoma related to immunosuppressive tumor microenvironment regulated by IL-6 signaling

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ABSTRACT

Evasion of immune surveillance is a significant factor in head and neck squamous cell carcinoma (HNSCC) carcinogenesis. IL-6 signaling is a critical mechanism for the induction of dysfunctional immune responses. In the present study, we examined the role of IL-6 in the prognosis of HNSCC regarding the immunosuppressive tumor microenvironment. We retrospectively analyzed the clinical outcomes of HNSCC patients and examined its correlation with the levels of IL-6 in tumors and circulating myeloid-derived suppressor cells (MDSCs) in peripheral blood. Furthermore, the relationships between IL and 6, programmed death ligand (PD-L1) expression, and immune response were examined *in vitro* and *in vivo*. Our data revealed that IL-6 overexpression was associated with the increased risk of developing disease failure and poor prognosis for HNSCC. The immunoreactivity of IL-6 in HNSCC specimens was positively linked to the staining of PD-L1 and the level of circulating MDSCs. By cellular and animal experiments, there were augmented radiation-induced increases in the expression of PD-L1 and the activation of MDSCs noted in IL-6-positive tumors. When IL-6 signaling was inhibited, the levels of PD-L1 and MDSC recruitment were significantly down-regulated. Furthermore, the neutrophil-to-lymphocyte ratio (NLR) was positively correlated with the levels of IL-6 and PD-L1 in tumor, and circulating MDSCs. In conclusion, IL-6 is a significant predictor of treatment outcome in HNSCC patients, and plays an important role in the induction of immunosuppressive tumor microenvironment mediated by increased MDSCs and PD-L1 expression. Furthermore, IL-6 combined with NLR can assist the clinician to make an informed decision regarding treatment options.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous disease occurring in various sites, including the oral cavity, oropharynx, and hypopharynx [1,2]. Treatment failure and locoregional recurrence are common and account for the majority of deaths [3,4]. Identification of the potential molecular markers for predicting treatment response and prognosis is important for the effective management of HNSCC.

Abundant epidemiological data revealed a strong correlation between inflammation and the incidence of cancers [5,6]. Host inflammatory responses were reported to play an important role in tumor development and progression [7]. Myeloid-derived suppressor cells (MDSCs), a heterogeneous mixture of immature myeloid cells, were reported to markedly expand in tumor-bearing mice and patients with cancer, and contribute to an immunosuppressive tumor microenvironment (TME) [8,9]. MDSCs suppress immunity by perturbing both innate and adaptive immune responses, and have a remarkable ability to inhibit T-cell responses. MDSCs recruitment is associated with an increased rate of metastasis and recurrence in HNSCC. Significant impairment in immune recognition of tumor cells and evasion from immune response is a significant factor in HNSCC carcinogenesis [10,11]. There is emerging evidence that signaling through PD-L1 plays an essential role for the immune escape of cancers functionally linked to MDSCs [12,13]. Upregulation of PD-L1 allows cancer cells to escape from the host immune systems by functionally inactivating T-cell immune surveillance [14]. The expression of PDL1 is reported to be high in HNSCC tumors often, and checkpoint inhibitors have demonstrated consistent improvements in patient outcomes in metastatic HNSCC. Various immunosuppressive cytokines have been reported to contribute to the dysfunction of antitumor immunity and are investigated as prognostic indicators for cancer patients [15]. IL-6 has

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been reported to be over-expressed in several human malignancies and linked to poor prognosis and the resistance to anticancer therapies [16]. We previously observed that overexpressed IL-6 may be a significant predictor for treatment resistance in HNSCC [17]. IL-6 is a multifunctional cytokine which plays an important role in a wide range of biological activities in different types of cells, including inflammatory cells and tumor cells [18]. Furthermore, IL-6-mediated STAT3 activation was reported to regulate the expansion of MDSC and the expression of PD-L1 in tumor and immune cells [18–21]. Accordingly, IL-6 signaling should be a critical mechanism for the induction of dysfunctional immune responses in the TME to initiate a positive condition for cancer. Therefore, in the present study, we focused our work to assess the predictive value of IL-6 for the prognosis of HNSCC and its effects on the tumor immune response to provide new insights into the development of immune-based therapy.

Materials and methods

Tissue specimens and characteristics of patients

The Institutional Review Board of our hospital approved this study. The written consents were signed by the patients for their specimen and information to be stored in the hospital and used for research. We excluded patients who had M1 disease or nonprimary HNSCC at diagnosis. A total of 248 patients with a histologically confirmed diagnosis of HNSCC and who received curative treatment were enrolled in our study. The planned treatments included definitive radiotherapy and chemotherapy (CCRT) or surgery combined with adjuvant treatment, according to the guidelines proposed by the oncology team at our hospital. Of these patients, 173 patients were diagnosed with locally advanced (stage III to IV) oral squamous cell carcinoma (OSCC) and received curative treatment, including surgery and adjuvant radiotherapy, and 75 patients were diagnosed with pharyngeal cancer and received definite CCRT. The planning dose was 60–66 Gy for adjuvant radiotherapy and greater than 66 Gy for definite RT. The specimens collected from the 248 patients at diagnosis were retrospectively collected for immunochemical analysis. When blocks were available, hematoxylin and eosin-stained slides were re-evaluated by a pathologist to assess the quality of slides. Data concerning the clinicopathological characteristics and treatment outcomes were collected (Tables 1a–1c). In addition, the NLR was calculated by dividing the absolute neutrophil count by the absolute lymphocyte count. To assess the predictive value of the NLR, we set the cut-off value for the NLR at 3.0 based on our previous published reports [22]. Accordingly, all HNSCC patients were divided into two groups according to the pretreatment NLR: high (NLR ≥ 3) and low (NLR < 3) groups.

Immunohistochemical staining (IHC)

Tissue sections from formalin-fixed, paraffin-embedded tissues were cut into 4-μm sections for IHC. The specimens were assessed using the semi-quantitative immunoreactive score (IRS). The criterion for positive staining is a specimen with an IRS scoring grade greater or equal to 2. The details were described in Supplementary methods.

MDSC isolation and flow cytometry analysis

Peripheral blood samples were obtained from 130 patients with pathologically and clinically confirmed HNSCC. To assess the proportion of MDSCs among peripheral blood mononuclear cells (PBMCs), multicolor fluorescence-activated cell sorting (FACS) was performed using the FACS Caliber flow cytometer (BD Biosciences, San Jose, CA, USA). Human low-density neutrophils and granulocytic MDSCs are closely related and, presently, there is no generally accepted consensus on mutually exclusive definitions for these cell types [23]. In majority of oncological studies, human granulocytic MDSCs are characterized as

Table 1a
Clinico-pathological characteristics of 248 HNSCC patients.

	No. of patients		
	IHC-IL-6(−)	IHC-IL-6 (+)	*p* value
Patients	126	122	
Age			0.207
< 56y/0	69	57	
≥56 y/o	57	65	
Differentiation			0.007*
WD	62	37	
MD	40	49	
PD	20	32	
Unknown	4	4	
Clinical T stage			0.256
T1-T2	68	57	
T3-T4	58	65	
LN metastasis			0.140
Clinical negative	56	43	
Clinical positive	70	79	
Diagnosis			0.582
Pharynx	36	39	
Oral cavity	87	83	
NLR			< 0.001*
< 3	79	46	
≥3	47	76	
Disease failure			< 0.001*
Control	103	59	
Failure (LR + DM)	23	63	
Overall survival			< 0.000*
Alive	105	68	
Dead	21	54	

Events [a] = dead or alive with disease failure.
LR = loco-regional failure; DM = Distant metastasis.

$CD14^-$ $CD15^+CD11b^+HLA\text{-}DR^-$ cells [24]. Accordingly, the human MDSC subset characterized as $CD11b^+$ $HLA\text{-}DR^-$ $CD14^-$ cells was sorted from the peripheral blood. The percentage of MDSCs was measured using multicolor flow cytometry, and isotype-specific antibodies were used as negative controls. We then sorted $CD11b^+$ $HLA\text{-}DR^-$ myeloid cells to analyze the expression level of PD-L1 from 96 patients before and two weeks after radiotherapy (RT dose ≥60 Gy). The details were described in Supplementary methods.

Cell culture and reagents

The human hypopharyngeal cancer cell line, FaDu, was obtained from the American Type Culture Collection. The human oral cancer cell line SCC4 was obtained from the Bioresource Collection and Research Center (BCRC). The origin of SCC4 is from squamous cell carcinoma of the mouth floor. The IL-6 silencing vector and control vector were purchased from Santa Cruz (Santa Cruz, CA). Stable cancer cells were generated by transfecting cells with either the IL-6 silencing vectors or control vectors, followed by selection with puromycin for four weeks. Human recombinant IL-6, IL-6-neutralized antibody and the JAK inhibitor AG490 were purchased from R&D Systems (Minneapolis, MN) and Sigma (St. Louis, MO), respectively. In addition, the PD-L1-neutralizing antibody were obtained from Biolegend (San Diego, CA).

T-cell suppression assay

The suppressive function of MDSC was measured by their ability to inhibit the proliferation of autologous T cells in the following Suppression Assay. The details were described in Supplementary methods.

Table 1b
Clinico-pathological characteristics of 173 patients with pathologic stage 3–4.

	No. of patients		
	IHC-IL-6(−)	IHC-IL-6 (+)	*p* value
Patients	90	83	
Age			0.111
< 56y/0	50	36	
≥56 y/o	40	47	
Differentiation			0.222
WD	47	34	
MD	29	37	
PD	10	11	
Unknown	4	1	
Pathologic T stage			0.195
T1-T2	39	28	
T3-T4	51	55	
Pathologic LN metastasis			0.039
Negative	51	34	
Positive	39	49	
PD-L1 staining			< 0.001
Negative	51	10	
Positive	39	73	
NLR			0.012
< 3	52	32	
≥3	38	51	
Disease failure			< 0.001*
Control	74	37	
Failure (LR + DM)	16	46	
Overall survival			< 0.001*
Alive	72	38	
Dead	18	45	

Table 1c
Clinico-pathological characteristics of 75 patients with pharyngeal cancer with definite CCRT.

	No. of patients		
	IHC-IL-6(−)	IHC-IL-6 (+)	*p* value
Patients	36	39	
Age			0.927
< 56y/0	19	21	
≥56 y/o	17	18	
Differentiation			0.002*
WD	15	3	
MD	11	12	
PD	10	21	
Unknown	0	3	
Clinical T stage			0.138
T1-T2	21	16	
T3-T4	15	23	
LN metastasis			0.463
Clinical negative	9	7	
Clinical positive	27	32	
PD-L1 staining			< 0.001
Negative	27	12	
Positive	9	27	
IL-6 level			< 0.001*
Mean ± STD	6.37 + /-0.52	13.01 + /-0.48	
NLR			< 0.001*
< 3	27	14	
≥3	9	25	
Disease failure			0.024*
Control	29	22	
Failure (LR + DM)	7	17	
Overall survival			0.001*
Alive	3	16	
Dead	33	23	

Tumor xenograft

Eight-week-old male athymic nude mice were used for the experiments, with the approval of our Hospital Experimental Animal Committee. Cells (5 × 106) were subcutaneously implanted in the dorsal gluteal region. The coexpression of myeloid-cell lineage differentiation antigen Gr1 and CD11b were characterized MDSCs in mice [25].To determine the effect of irradiation *in vivo*; the tumor-bearing mice were irradiated for 12 Gy [26], and the targeted protein or MDSCs were measured at the indicated time. For local irradiation, mice were anesthetized and restrained to irradiate a field including the thigh bearing ectopic tumor. Control mice were subjected to sham irradiation. The effects of IL-6 on MDSC recruitment were also investigated by an i.p injection of anti-IL-6 or isotype antibody (0.5 mg/mouse, once a week) or IL-6 (100 ng per mice, thrice a week). In addition, the intraperitoneal injection of a PD-L1 neutralization antibody (250 μg per mouse, two times per week) was given to evaluate the effect of PD-L1 blockade *in vivo*.

Statistical analysis

The significance of differences between samples was determined using Student's t-tests. Data are presented as the means ± standard error of the mean (SD). Survival probability was analyzed statistically using the Kaplan–Meier method. The significance of between-group differences was assessed using the log-rank test. Multivariate analyses were performed using a Cox regression model for survival.

Results

The expression of PD-L1 in HNSCC

Recently, the role of PD-L1 in HNSCC has been brought into the spotlight. However, the expression profile and clinical significance of PD-L1 in HNSCC remain to be elucidated [27]. Therefore, we evaluated the expression of PD-L1 immunohistochemically in 248 HNSCC patients and the association of this expression with clinicopathologic characteristics. Fig. 1a showed the representative positively and negatively stained slides for PD-L1 in human cancer specimens at diagnosis compared with adjacent non-malignant epithelial tissues. Of the 248 HNSCC patients, there were 148 (59%) patients who showed an overexpression of PD-L1. Compared to the positivity of PD-L1 in pharyngeal cancer, there was a higher percentage of PD-L1 overexpression noted in OSCC (65% in OSCC versus 48% in pharyngeal cancer, $p = 0.013$). The positive staining of PD-L1 was significantly associated with a higher incidence of loco-regional failure in HNSCC, especially in pharyngeal cancer (Fig. 1b). Other clinical factors, including histologic grade, clinical T classification and lymph node metastasis did not differ significantly between PD-L1-positive and PD-L1-negative groups. Radiotherapy has a crucial role in the treatment of HNSCC. Accordingly, we proposed that PD-L1 might be a predictor of the treatment response in HNSCC. We further examined the effect of irradiation on the expression of PD-L1 for HNSCC *in vitro* and *in vivo*. For human cancer cell lines FaDu and SCC, RT increased the expression of PD-L1, and the increases were positively associated with the irradiation dose (Fig. 1c–e).

Role of IL-6 in the expression of PD-L1

PD-L1 regulation is an area of active investigation aiming to exploit the current immunotherapy successes even further. The molecular mechanism for regulation of PD-L1in HNSCC still requires further investigation. Activated IL-6/STAT3 was reported to induce immunosuppression by upregulating PD-1/PD-L1 in cancer. Fig. 1d and e showed that irradiation increased the PD-L1 expressions associated

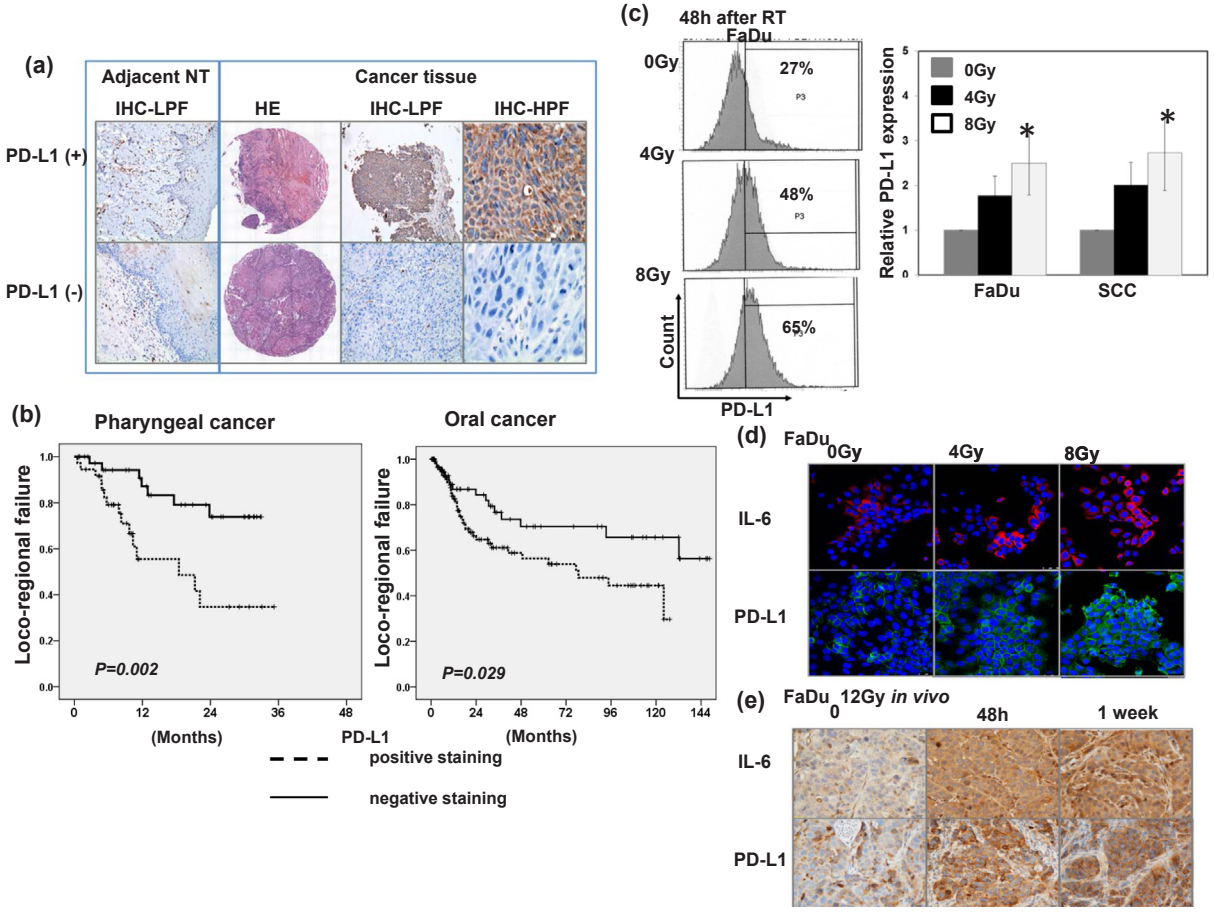


Fig. 1. The expression of PD-L1 in HNSCC. (a) Representative images of IHC staining with an anti-PD-L1 antibody of HNSCC and adjacent non-malignant epithelium from TMA blocks (LPF, low power field (100×); HPF, high power field (400×)). (b) The differences of locoregional recurrence after curative-intent treatment according to the staining of PD-L1 in patients with pharyngeal cancer or oral cancer. The curative-intent treatment included surgery or definitive RT. (c) The levels of PD-L1 were evaluated by FACS for human FaDu and SCC cancer cells at 48 h after RT with 0, 4, and 8 Gy *in vitro*. Representative slides are shown, *, $P < 0.05$. The Y-axis shows the ratio of PD-L1-positive cells at each condition divided by that under control condition, respectively. In addition, the levels of PD-L1 and IL-6 were evaluated by (d) IF at 48 h after RT with 0, 4, and 8 Gy *in vitro*, and (e) IHC staining of xenograft tumors at the indicated times after 12 Gy irradiation. Representative slides are shown.

with up-regulated IL-6. Accordingly, we further assess the regulation of PD-L1 expression by IL-6 signaling in HNSCC. As shown in Fig. 2a–c and Supplementary Fig. 1a–c, the inhibition of IL-6 by silencing vector significantly decreased the expression of PD-L1 in SCC4 and FaDu cancer cells with or without irradiation. Furthermore, the addition of recombinant IL-6 augmented the expression of PD-L1 in stable transfectants of human cancer cells. To investigate the pathway that mediated the effect of IL-6 signaling on PD-L1, we blocked STAT3 activation with JAK2 inhibitor (50 μM AG490) for 24 h *in vitro*. As shown in Fig. 2d, when the STAT3 pathway was inhibited, the decreases in PDL1 protein levels were comparable to those induced by the IL-6-silencing vector. We further correlated the expressions of PD-L1 and IL-6 in the HNSCC. As shown in Fig. 2e, there is a positive link between IL-6 and PD-L1 expression analyzed by IHC in a series of 248 patients with HNSCC. In addition, the staining of PD-L1 in tumor positively correlated with the level of IL-6 in serum from 130 HNSCC patients (Fig. 2f). Based on the data, we suggested that the activated IL-6/STAT3 pathway might be responsible for the up-regulation of PD-L1 in HNSCC.

Role of IL-6 in the recruitment of MDSCs and its PD-L1 expression

Tumor cell-extrinsic effects of IL-6 have been demonstrated in anti-tumor immune responses through myeloid-lineage cells and T cells [28]. The accumulated evidence indicates that MDSCs contribute to the negative regulation of immune responses [8,29], and associated with the prognosis of HNSCC [30]. We used tumor-bearing mice to examine the role of IL-6 in the recruitment of MDSCs and its PD-L1 expression with or without irradiation. As shown in Fig. 3a–c and Supplementary Fig. 2a, the inhibition of IL-6 attenuated the MDSC recruitment and the level of PD-L1 of MDSC with or without irradiation *in vivo*. We further evaluated the percentage of $CD11b^+$ $HLA\text{-}DR^-$ $CD14^-$ cells, a subset of MDSCs, in the peripheral circulation of 130 patients with HNSCC using flow cytometry. Supplementary Fig. 2b shows the representative flow cytometry data for MDSC analysis obtained from one HNSCC patient with an IL-6-negative tumor and one with an IL-6-positive tumor. As shown in Fig. 3d and e, the percentage of circulating MDSCs correlated with the staining of IL-6 in the tumor and the level of IL-6 in the serum of HNSCC patients. We then sorted $CD11b^+$ $HLA\text{-}DR^-$ myeloid cells to analyze the expression level of PD-L1. As shown in Fig. 3f, RT increased the percentage of PD-L1-positive cells relative to the total number of $CD11b^+$ $HLA\text{-}DR^-$ myeloid cells in HNSCC patients. Furthermore, the increase of the PD-L1-positive myeloid cells after RT was significantly higher in patients with elevated circulating IL-6 (i.e., greater than the median value) compared with patients with lower IL-6 levels in serum. MDSCs are characterized by the ability to suppress T cell functions including proliferation [31]. Therefore, we further examined the suppressive ability of T cell proliferation in MDSCs from HNSCC patients with high or low circulating IL-6. As shown in Supplementary 3, increased IL-6 was associated with higher suppressive function of MDSCs in T cell proliferation, which could be abrogated by anti-PD-L1.

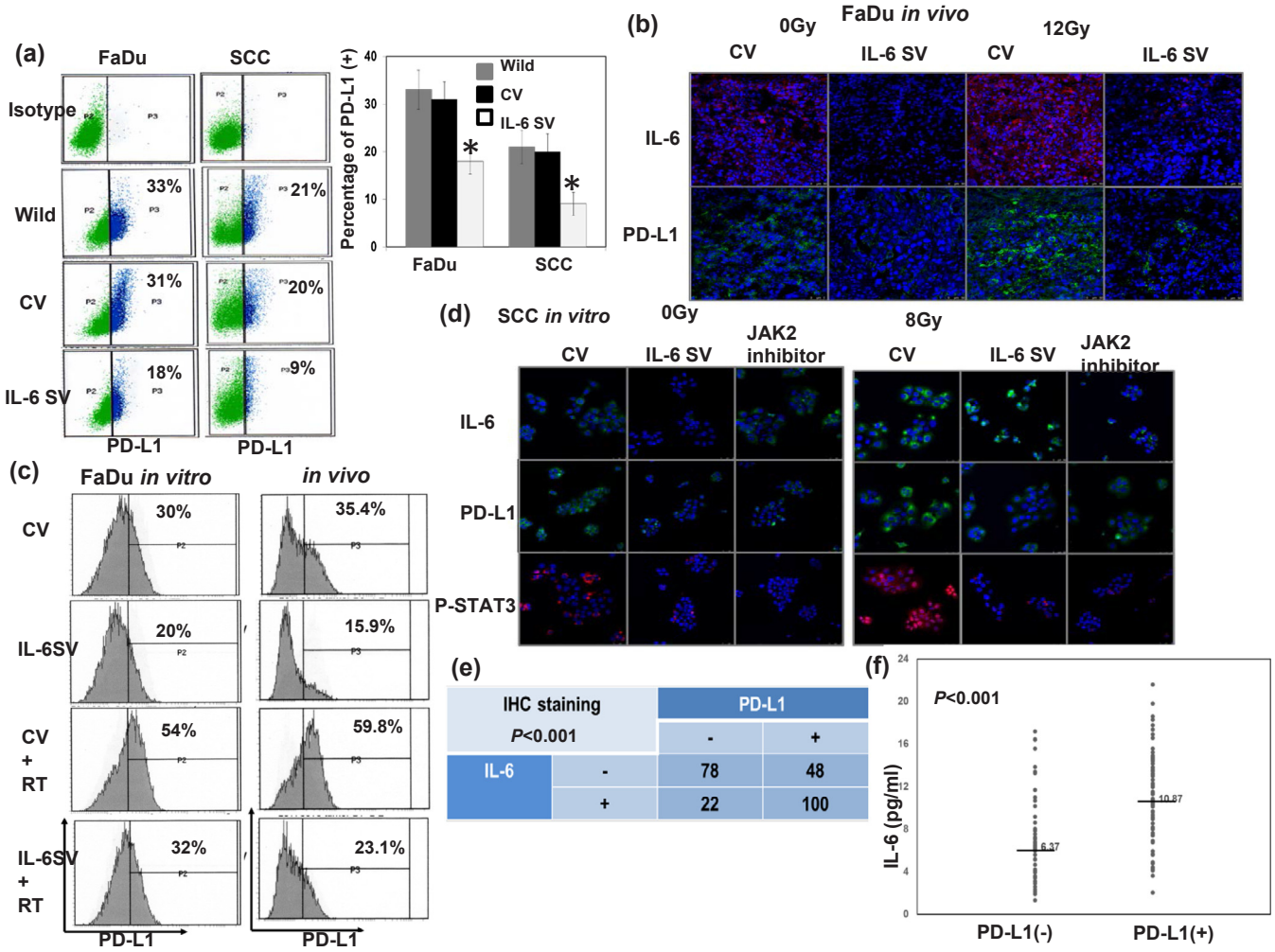


Fig. 2. Role of IL-6 in the expression of PD-L1. The levels of PD-L1 were evaluated by (a) FACS for human FaDu and SCC transfectants *in vitro* (CV, cells transfected with control vector; IL-6 SV, cells transfected with IL-6 silencing vector). Each column is shown as the means of 3 separate experiments; bars, SD. *, $P < 0.05$. (b) Effect of IL-6 inhibition on PD-L1 expression of tumor xenograft with or without irradiation was evaluated by IF (Blue, DAPI; Green, PD-L1; Red, IL-6). (c) Effect of IL-6 and irradiation on PD-L1 expression was evaluated by FACS *in vitro* and *in vivo*. (d) Effect of IL-6 inhibition and JAK2 inhibitor on PD-L1 expression of cancer cells with RT 0 or 8 Gy was evaluated by IF (Blue, DAPI; Green, PD-L1; Red, IL-6). (e) A significant positive correlation was observed in the cancer specimen that stained positive for IL-6 and PD-L1. (f) IL-6 levels measured using ELISA in serum from cancer patients with PD-L1 negative and positive staining. The lines indicate the median values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Expression of IL-6 correlates with the prognosis of HNSCC

As observed, we found that IL-6 was associated with the immunosuppressive TME in HNSCC. We further examined whether the elevated IL-6 levels were associated with the treatment response and prognosis in HNSCC patients. Using immunohistochemical analysis, Fig. 4a shows the representative positively and negatively stained slides for IL-6 in human cancer specimens at diagnosis compared with adjacent non-malignant epithelial tissues. Of the 248 HNSCC patients, there were 122 (49%) patients who showed an overexpression of IL-6. Table 1a demonstrated that there was a positive correlation between IL and 6-positive staining and the higher incidence of poor differentiation and disease failure. Among these patients, 83 (48%) had positive IL-6 immunoreactivity out of 173 OSCC cases and 39 (52%) had positive IL-6 immunoreactivity out of 75 pharyngeal cancer cases. The positive staining for IL-6 was significantly linked to the incidence of pathologic lymph node metastasis and disease failure for OSCC (Table 1b) and correlated with poor differentiation and a higher disease failure rate in pharyngeal cancer (Table 1c). As shown in Fig. 4b and Table 1d, positive staining of IL-6 was significantly associated with a reduced survival rate by univariate and multivariable Cox regression analysis. Furthermore, in multivariate analysis, IL-6, but not PD-L1, possessed the predictive power in loco-regional control in both pharyngeal cancer and OSCC patients (Supplementary Tables 1). These findings suggest that IL-6 could be a predictor for prognosis in HNSCC patients with curative intent treatment.

Predictive role of NLR on levels of IL-6, PD-L1 and circulating MDSCs

The NLR may reflect host inflammatory responses and changes in the tumor microenvironment [32]. We previously reported that the values of NLR significantly correlated the levels of circulating MDSCs [22]. Fig. 5a–b demonstrated that the levels of MDSCs and IL-6 in peripheral circulation were significantly higher in the NLR ≥ 3 than the NLR < 3 group. We further assessed the usefulness of the NLR for predicting the expression levels of IL-6 and PD-L1 in tumors. As shown in Fig. 5c, there was a significantly higher NLR in patients with IL-6-positive tumors compared with patients with IL-6-negative tumors. Furthermore, Fig. 5d demonstrated that the expression rate of IL-6 and PD-L1 in tumor specimens was significantly higher in the NLR ≥ 3 group than in the NLR < 3 group.

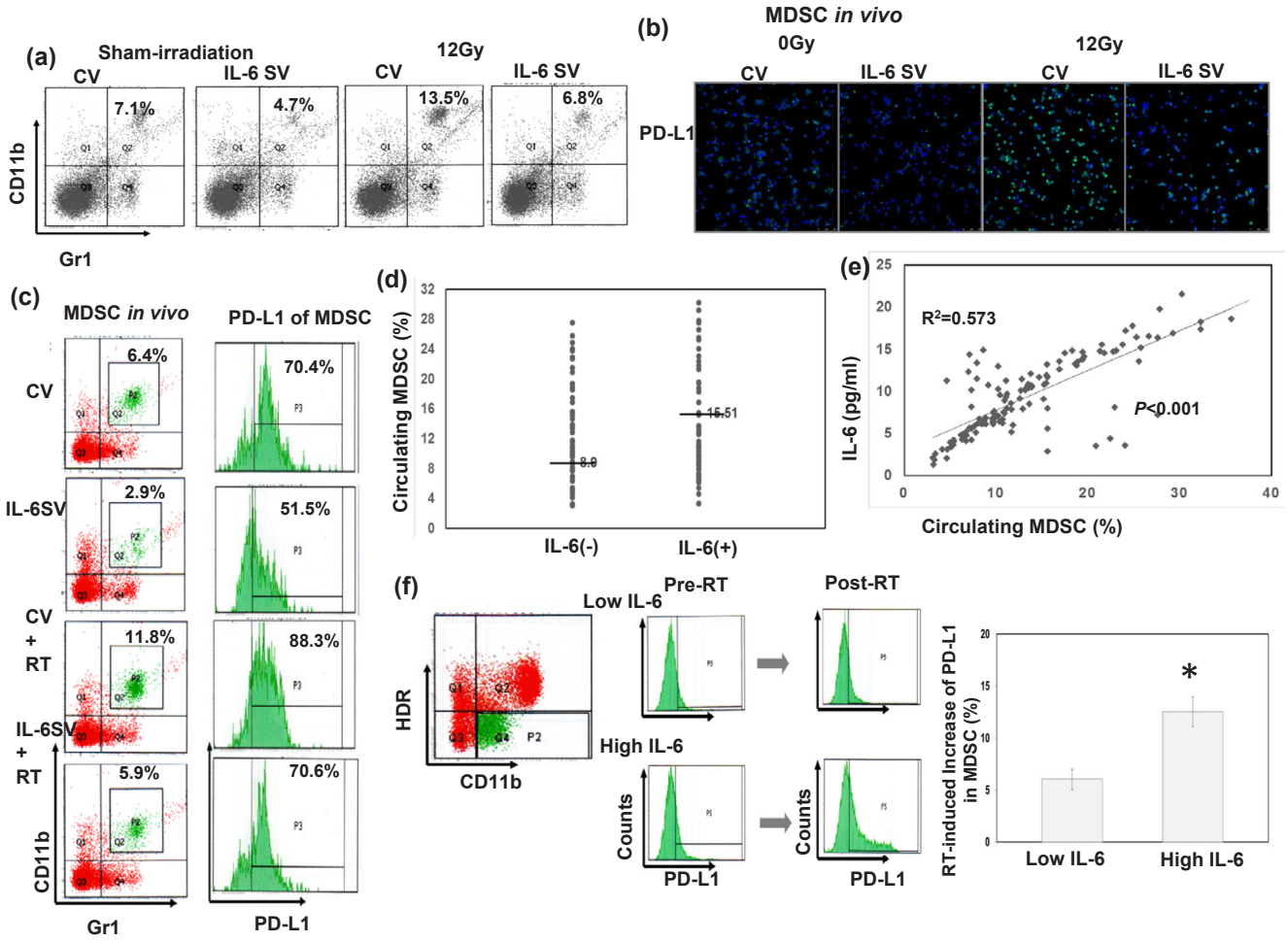


Fig. 3. IL-6 linked with the MDSCs recruitment and PD-L1 expression. (a) Effect of IL-6 on the recruitment of MDSC in tumor evaluated by FACS using GrI-CD11b staining in mice bearing tumors with or without IL-6 silencing vectors. The results are shown by representative slides. (b) Effect of IL-6 on PD-L1 expression of CD11b + cells sorted from mice bearing tumor with or without irradiation (Blue, DAPI; Green: PD-L1). (c) The level of CD11b + Gr1 + cells (MDSCs) from tumor-bearing mice with or without irradiation was evaluated by FACS. Furthermore, CD11b + Gr1 + cells were gated (P2 area), then the PD-L1 expression was analyzed. (d) Circulating MDSC levels in the group of patients with IL-6-positive tumor compared to those in IL-6-negative group. The lines indicate the median values ($p < 0.001$). (e) There was a positive correlation between the levels of MDSC and IL-6 in the peripheral circulation. (f) The PD-L1 expression of CD11b + HDR − cells gated from PBMC of HNSCC patients before and after RT was evaluated by FACS. *, $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Discussion

The tumor microenvironment plays an important role in cancer development and progression and may be associated with systemic inflammation [33]. Some cancers evade immune surveillance by developing ways of excluding the T-cells from the tumor microenvironment [14]. However, HNSCC has been found to be one of the most immune-infiltrated cancer types [34], suggesting there are other mechanisms underlying the immunosuppressive microenvironment generated by the tumor. The upregulation of PD-L1 can occur in tumor cells and allows cancer cells to escape from host immune systems by functionally inactivating T-cell immune surveillance [35,36]. The inhibition of this interaction can enhance T-cell response and mediate clinical anti-tumor activity [37,38]. The expression of PDL1 is often high in HNSCC tumors, with a wide range [39]. Immune checkpoint inhibitors have demonstrated durable improvements in patient outcomes in HNSCC, with being granted FDA approval. In addition, PD-L1 was reported to be associated with resistance to anticancer therapies [40,41]. RT increases PD-L1 expression in tumor cells and suppressor cells to consequently suppress radiation-induced immune responses [42,43]. However, the prognostic role of PD-L1 expression in HNSCC still remains unclear. In the present study, we demonstrated that RT augmented the expression of PD-L1 using cellular and animal experiments. Regarding our clinical data, 60% of the samples had positive PD-L1 staining, and the positive staining of PD-L1 was significantly associated with a higher rate of loco-regional failure. Accordingly, we suggested that PD-L1 plays a role in the interaction of radiotherapy and the immune system in HNSCC.

PD-L1 regulation is an area of active investigation. IL-6 is reported to plays a role in immunity regulation including resting T cells, and IL-6/STAT3 signaling is involved in the regulation of PD-L1 expression in some cancers [18–21,44]. There was a positive correlation between the expression levels of PD-L1 and IL-6 based on our clinical specimens analyzed by IHC and ELISA. We further examined the role of IL-6 in PD-L1 regulation in HNSCC. By cellular and animal experiments, the expression of PD-L1was obviously decreased when suppressing IL-6 expression. Furthermore, inhibition of STAT3 activation could attenuate the expression of PD-L1 comparable to that induced by IL-6 inhibition. Therefore, we suggest that IL-6-STAT3 signaling is critical in the upregulation of PD-L1 expression in HNSCC.

The identification and inhibition of key drivers of immunosuppression have the potential to improve patient outcome when combined with radiotherapy. Evidence indicates that IL-6 signaling induces dysfunctional immune system responses in the tumor

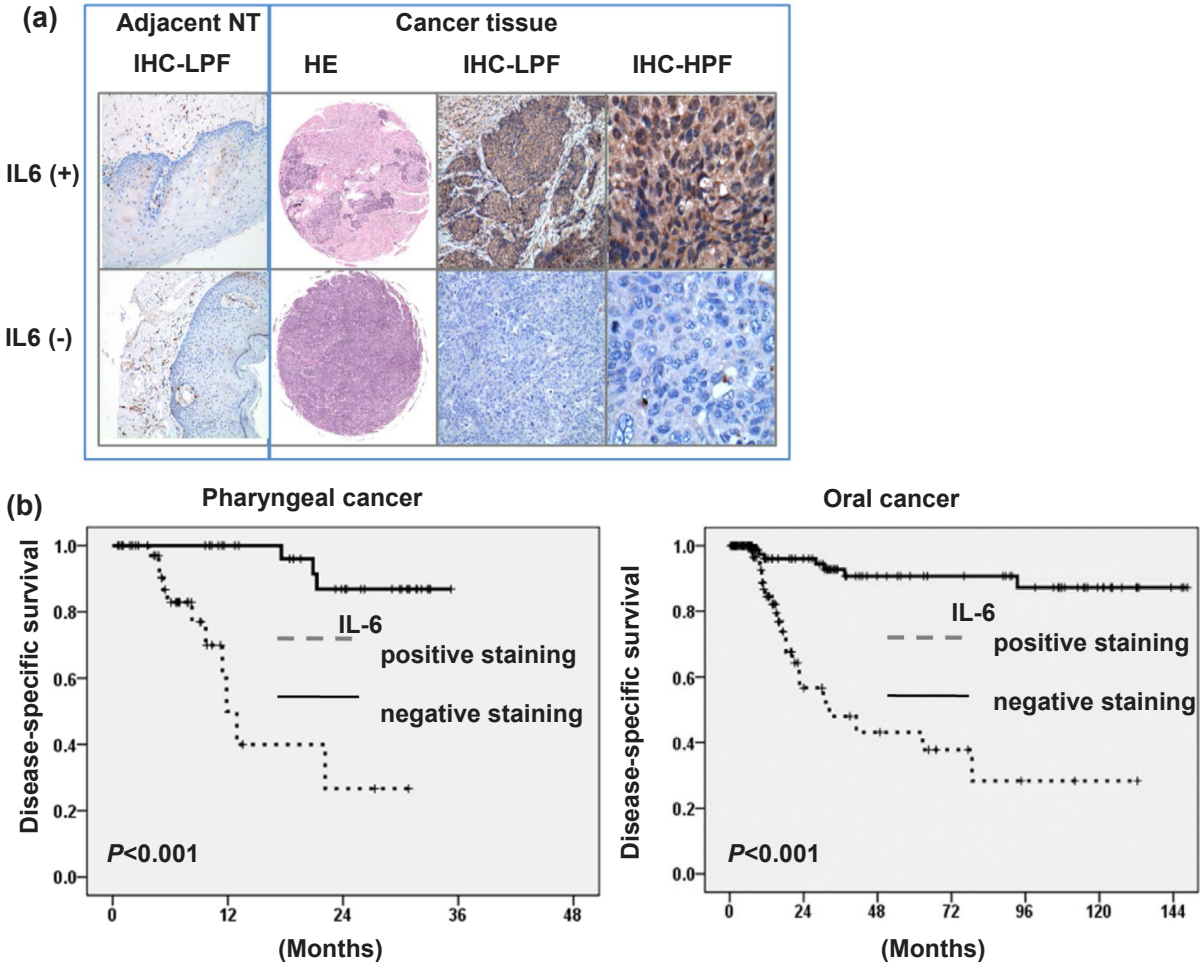


Fig. 4. Role of IL-6 in the prognosis of HNSCC. (a) Representative images of IHC staining with an anti-IL-6 antibody of HNSCC and adjacent non-malignant epithelium from TMA blocks (LPF, low power field (100×); HPF, high power field (400×)). (b) The differences of disease-specific survival according to the staining of IL-6 in patients with pharyngeal cancer or OSCC.

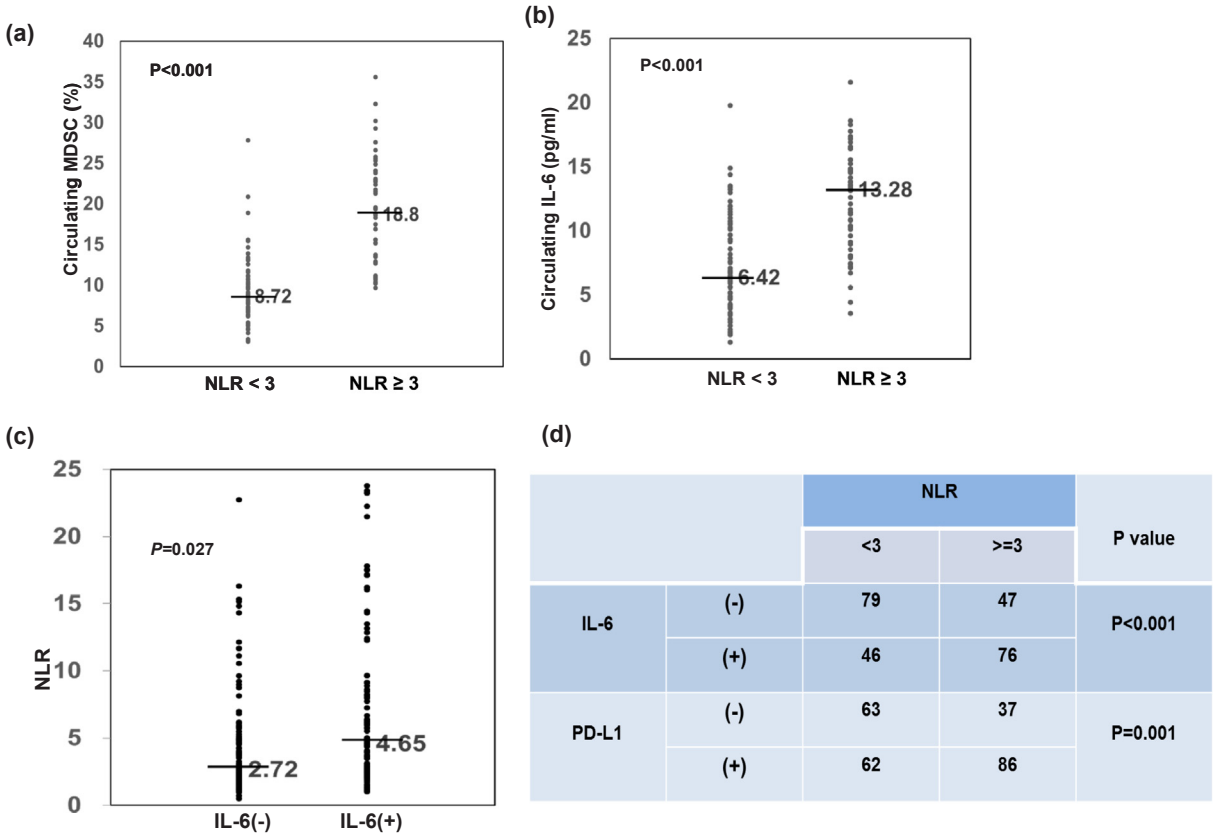
Table 1d
Multivariate analysis to determine molecular markers associated with Overall survival of HNSCC patients.

Variable	HR*	95% CI	P value
IL-6			
Negative staining	Ref		
Positive staining	5.71	3.22–10.10	< 0.001*
LN metastasis			
No	Ref		
Yes	1.79	1.06–3.03	0.031*
Surgery			
No	Ref		
Yes	0.44	0.24–0.80	0.007*
RT			
No	Ref		
Yes	1.45	0.63–3.33	0.386
Chemotherapy			
No	Ref		
Yes	0.63	0.36–1.12	0.108
Loco-regional failure			
No	Ref		
Yes	2.21	1.33–3.66	0.002*

microenvironment [16,18]. Activation of IL-6/STAT3 has been shown to be a key factor in maintaining immune tolerance in myeloid cells, and it is the main transcription factor that regulates the expansion of MDSC [18,45]. MDSCs are thought to promote cancer progression and present an important barrier that limits the full potential of immune-based cancer therapies [46]. MDSC recruitment was reported to be a negative prognostic factor in HNSCC [11,30]. In the present study, we demonstrated that higher circulating MDSCs were noted in patients with IL-6-positive tumors. In vivo, the expression level of IL-6 positively linked to the level of MDSC recruitment and the increase induced by RT. IL-6 inhibition attenuated the RT-induced MDSCs recruitment and PD-L1 expression in tumors and MDSCs. Moreover, FACS revealed that the use of IL-6 blockade significantly decreased in the activation of MDSCs and the levels of PD-L1 in CD11b+ cells. Therefore, we suggest that IL-6 modulated the local tumor microenvironment mediated by increased MDSCs recruitment and PD-L1 expression, thereby allowing cancer cells to acquire an advanced malignant phenotype and resistance to treatment.

To further investigate the clinical significance of IL-6 in HNSCC, we explore the role of IL-6 in treatment response and its predictive value for prognosis. Based on the clinical outcome analysis of 173 patients with locally advanced OSCC and 75 patients with pharyngeal cancer, the enhanced expression of IL-6 was correlated with a higher incidence of pathologic LN involvement, a higher disease failure rate, and a lower survival rate. Based on multivariate analysis, the levels of IL-6, lymph node involvement, and RT dose possessed the ability to predict the risk of loco-regional recurrence. The data demonstrated the role of IL-6 in predicting prognosis and the treatment efficiency of HNSCC.

Neutrophils and lymphocytes, which constitute the predominant



		NLR		P value
		<3	>=3	
IL-6	(-)	79	47	P<0.001
	(+)	46	76	
PD-L1	(-)	63	37	P=0.001
	(+)	62	86	

Fig. 5. Predictive role of NLR on levels of IL-6 and circulating CD11b + CD14 − HLA-DR − Cells. (a) There was a higher circulating MDSC levels, and (b) circulating IL-6 levels in the group of patients with NLR ≥ 3 compared to the group of patients with NLR < 3. Lines indicate the median values. (c) The NLR levels in the groups of HNSCC patients without and with IL-6 positive staining in tumor specimen. (d) The positive staining of IL-6 and PD-L1 in tumor specimens was significantly higher in the NLR ≥ 3 group than in the NLR < 3 group.

proportion of total circulating leukocytes, play a key role in host systemic immune responses and have effects on tumor progression in the tumor microenvironment [47]. An elevated NLR in many solid tumors, including HNSCC, has been associated with reduced survival [32,48]. The NLR is a cheaper and faster laboratory measurement than any other biomarker, and it does not require any additional cost. Accordingly, we further examined the correlation between IL-6 levels in tumors and serum and the NLR. The data showed that the NLR was significantly correlated with IL-6 levels in the serum and tumors and the expression of PD-L1. Furthermore, we found a positive correlation between the NLR and circulating MDSC levels in HNSCC patients. The findings indicated that the NLR could predict the levels of IL-6, circulating MDSCs, and PD-L1 in HNSCC patients. The suppression of IL-6 levels may enhance the immune response, thereby resulting in improved clinical outcomes in patients with refractory HNSCC. Immune checkpoint inhibitors have demonstrated consistent improvements in patient outcomes in advanced HNSCC. With the increasing use of immunotherapy, patient selection has become an important issue in assessing efficacy. Less optimal patient selection might limit clinical efficacies when applying agents targeting PD-1/PD-L1. Thus, it is imperative to identify clinically feasible parameters highly relevant to the level of PD-L1. Based on NLR relevant to IL-6 and PD-L1 levels, we suggest the NLR to be an important biomarker for patients that can assist the clinician and patient to make informed decisions regarding treatment options for HNSCC patients.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2019.02.027.

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