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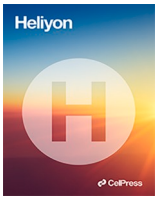

Research article

Prognostic significance of serum Chemerin and neutrophils levels in patients with oral squamous cell carcinoma

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ABSTRACT

Objectives: Chemerin, as a novel multifunctional adipokine, is proposed to be involved in high cancer risk and mortality. The present study was aimed to evaluate the prognostic value of serum Chemerin and neutrophils in patients with oral squamous cell carcinoma (OSCC).

Materials and methods: 120 patients with OSCC were included in this prospective cohort study. The levels of serum Chemerin were measured by enzyme-linked immunosorbent assay (ELISA). We also explored the possible effects of Chemerin on neutrophils' chemokines in OSCC using a real-time PCR, western blotting.

Results: Levels of serum Chemerin, neutrophils and NLR were significantly higher among non-survivors compared to survivors of OSCC (both $P < 0.05$). Higher serum Chemerin levels were associated with advanced TNM stage, lymph node metastasis, differentiation and tumor recurrence (both $P < 0.05$). Serum Chemerin levels correlated with neutrophils and NLR levels ($r = 0.708$, $r = 0.578$, both $P < 0.05$). Based on ROC analysis, Chemerin + NLR predicted OSCC patient mortality with 81.54 % sensitivity and 87.27 % specificity, with an AUC of 0.8898. In a Kaplan-Meier analysis, high serum Chemerin levels, high neutrophil levels and high NLR levels were associated with shorter overall and disease-free survival (both $P < 0.05$). A univariate and multivariate Cox regression analysis showed that serum Chemerin and neutrophils were independent risk factors for OSCC. (both $P < 0.05$). QRT-PCR and western blotting results showed that Chemerin upregulated the expression of chemokines IL-17 and CXCL-5 in neutrophils (both $P < 0.05$).

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Conclusions: Our study suggests that measurement of serum Chemerin and neutrophils might be a useful diagnostic and prognostic biomarker for OSCC patients. Chemerin may promote neutrophils infiltration in OSCC through upregulation of chemokines IL17 and CXCL-5.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common tumor in oral cavity and has become a significant public health problem, with a 5-year survival rate of 50–60 % only. According to past studies, it is a highly heterogeneous and fatal disease. OSCC may be promoted by immune microenvironment via contributing to inflammation and immune regulation [1,2]. The peripheral immune system of patients with OSCC exhibits altered levels of immune cells and serological characteristics [3,4]. Thus, a variety of biomarkers for diagnosis and prognosis were proposed, and targeted immunotherapy was applied in clinical therapy. Several peripheral blood parameters associated with inflammation, including neutrophil-to-lymphocyte ratio (NLR) [5,6], Platelet–lymphocyte ratio (PLR) [7], C- reactive protein (CRP) [8], have been shown to be useful in assessing the prognostic value of disease. Recent studies have revealed that neutrophils play a critical role in tumor initiation, growth, proliferation, and metastatic spread in the tumor microenvironment [9,10]. Neutrophils was suggested as a prognostic marker in several types of cancers, including gastric cancer [11,12], ovarian cancer [13], colorectal [14] and pancreatic cancer [15]. Our previous study found that elevated neutrophils infiltrating in the tumor site adversely affected patients' survival with OSCC [16,17]. However, to date, the roles of blood neutrophils and NLR in OSCC prognosis are less well known.

First discovered in psoriatic skin lesions, chemerin is an effective chemoattractant protein encoded by retinoic acid receptor 2 [18]. Recent studies have found that serum Chemerin achieves relatively good diagnostic and prognostic value in the breast cancer [19], Pancreatic ductal adenocarcinoma (PDAC) [20]and colorectal cancer [21]. Wang et al. [22]revealed that Chemerin levels were significantly higher in gastric cancer patients than in healthy individuals and Gastric cancer with advanced clinical stages and non-intestinal type was associated with elevated serum Chemerin levels. Weigert et al. [23] revealed Chemerin levels are higher in the serum of Inflammatory Bowel Disease (IBD) patients than in healthy controls, suggesting that Chemerin may regulate intestinal inflammation. A serum Chemerin biomarker might be helpful for diagnosing and prognosticating non-small cell lung cancer (NSCLC) [24] and colorectal adenoma (CRA) [25], Involved in NSCLC tumor-promoting networks, inflammatory pathways, and cancer-related metabolic pathways [26].

Previous studies have shown that Chemerin binds to ChemR23 and chemotaxis dendritic cells and macrophages to trigger rapid immune responses and inflammatory immune responses [27,28]. The research suggested that Immune surveillance may be enhanced by chemerin expression in tumor cells [29,30]. Our previous studies demonstrated that When chemerin is overexpressed in OSCC, neutrophils are attracted to tumor sites, promoting tumor progression through neutrophil-mediated mechanisms, and strong Chemerin expression + high TANs density in OSCC tissues predict poor clinical outcomes [16].

However, there are few studies on the relationship between Chemerin, neutrophil/NLR and the clinical prognosis of OSCC patients. The purpose of this study was to investigate the effects of Chemerin, neutrophils and NLR on the prognosis of patients with oral squamous cell carcinoma (OSCC), and the potential mechanism of serum Chemerin affecting neutrophils chemotaxis.

2. Material and methods

2.1. Study population and serum chemerin measurement

One hundred-twenty patients (64 males and 56 females) with primary tongue squamous cell carcinoma participated in this study at the Affiliated Hospital of Qingdao University between 2018 and 2019. Inclusion criteria: first confirmed in the Affiliated Hospital of

Table 1
Primer sequences and size of PCR products.

Gene	Sequence
GAPDH	F:5′-CGGAGTCAACGGATTTGGTCGTAT-3′ R:5′-AGCCTTCTCCATGGTGGTGAAGAC-3′
IL-6	F:5′-CAAAGATGGCTGAAAAAGATGGA-3′ R:5′-CTGTTCTGGAGGTACTCTAGGT-3′
IL-8	F:5′-CTTGGCAGCCTTCCTGATTTCT-3′ R:5′-GTTTTCCTTGGGGTCCAGACAG-3′
IL-17	F:5′-TCCCACGAAATCCAGGATGC-3′ R:5′-GGATGTTCAGGTTGACCATCAC-3′
CXCL-5	F:5′-GAGAGCTGCGTTGCGTTTGTTTAC-3′ R:5′-CCGTTCTTCAGGGAGGCTACCACT-3′
CXCL-6	F:5′-CCCAAAGCTTGAGTTTCCTGC-3′ R:5′-AGTGGTCAAGAGAGGGTTCG-3′
MCP-1	F:5′-CTCAGCCAGATGCAATCAATGC-3′ R:5′-CCTCAAGTCTTCGGAGTTTGGG-3′

Qingdao University without surgery, chemotherapy or radiotherapy, endocrine therapy or targeted therapy, and so forth; The clinical characteristic information is presented in Table 1 and Table 2. The study protocol was approved by the ethics committee of the Affiliated Hospital of Qingdao University. Written informed consent was obtained from all patients. These studies adhered to the guidelines of the Declaration of Helsinki.

All serum samples were obtained between August 2018 and December 2019 at the Affiliated Hospital of Qingdao University in Qingdao. Serum Chemerin levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

2.2. Cell culture

As previously described [14], neutrophils were isolated from the peripheral blood of healthy blood donors in the Affiliated Hospital of Qingdao University after obtaining written informed consent. Neutrophils were cultured at 37 °C in 5 % CO2 humidified atmosphere of Roswell Park Memorial Institute (RPMI-1640), containing 10 % FBS and 1 % penicillin streptomycin. OSCC cell lines (Cal27, SCC9 and SCC15) were purchased from the Shanghai Institute of Chinese Academy of Sciences and cultured in DMEM medium (DMEM) containing 10 % FBS and 1 % penicillin-streptomycin at 37 °C and 5 % CO_2. Cells were maintained in a standard humidified incubator with 5 % CO2 at 37 °C. 1×10^6 OSCC cells were seeded with neutrophils (1:3 ratio) in six-well plates. Neutrophils were collected after 48 h of co-culture system. As previously described [14], Recombinant human Chemerin (R-Chemerin, abcam, USA) had the greatest effect on chemotactic neutrophils at a concentration of 100 ng/mL. R-Chemerin (100 ng/mL) was used to determine the Chemokine expression levels on neutrophils.

2.3. Lentiviral transduction

The lentivirus transfection vectors were constructed and transfected into carcinoma cells as our previous study. Chemerin was overexpressed by lentivirus (Chemerin). Empty lentivirus was used as negative control (NC). SCC 9 and Cal 27 cells were divided into three groups for transfection: Chemerin, NC and Parental. Western blot was used to detect the expression of Chemerin in each group.

2.4. Quantitative real-time (qRT)-PCR

The total RNA extraction and qRT-PCR processes were performed as described previously [15]. The sequences of the primers are listed in Table 1. Primers were synthesized by the Shanghai Sangon Biological Engineering Technology & Services Co.

2.5. Western blot analysis

Western blot assays were performed as described previously [15]. Anti-IL-6 protein (dilution 1:750, abcam, USA), anti-IL-8 protein (dilution 1:1000, abcam, USA), anti-IL-17 protein (dilution 1:1000, abcam, USA), anti-CXCL-5 protein (dilution 1:750, abcam, USA), anti-CXCL-6 protein (dilution 1:1000, abcam, USA), and anti-MCP-1 protein (dilution 1:2000, abcam, USA) were used.

2.6. Statistical analysis

Statistical analysis Data are represented as mean ± standard deviation. Non-normally distributed data were analyzed using the Wilcoxon's test. The data of normal distribution were analyzed using student's *t*-test. Chi square test was used for categorical variables, and unpaired *t*-test was used for continuous variables. The relationship between neutrophil/NLR and Chemerin was determined by Pearson's correlation test. The sensitivity and specificity of different potential predictors of mortality were evaluated by using the

Table 2
Relationship between mortality and blood indicators in OSCC patients.

Variables	Survivor (n = 65)	Non-survivor (n = 55)	*t*	*p*
Chemerin(ng/mL)	46.90 ± 9.05	67.88 ± 16.92	8.646	<0.001***
neutrophils(× 10*9/L)	3.06 ± 0.95	4.14 ± 1.42	5.009	<0.001***
lymphocytes(× 10*9/L)	2.13 ± 0.52	2.04 ± 0.51	- 1.023	0.308
NLR	1.48 ± 0.47	2.18 ± 1.03	4.833	<0.001***
albumin(g/L)	40.59 ± 4.81	41.72 ± 3.94	1.391	0.167
fibrin(g/L)	3.34 ± 0.69	3.47 ± 0.66	1.081	0.282
Platelets(× 10*9/L)	230.23 ± 67.89	210.18 ± 70.11	0.068	0.795
PLR	113.84 ± 39.45	108.98 ± 46.49	0.451	0.503
leukocyte(× 10*9/L)	6.18 ± 1.73	6.47 ± 1.73	0.921	0.359
CRP (mg/L)	3.77 ± 1.36	4.09 ± 1.33	1.280	0.203
monocytes(× 10*9/L)	0.65 ± 0.36	0.60 ± 0.25	- 0.931	0.354
eosinophils(× 10*9/L)	0.19 ± 0.16	0.20 ± 0.18	0.553	0.582
basophils(× 10*9/L)	0.024 ± 0.015	0.023 ± 0.016	- 0.446	0.657

***$p < 0.001$.
Abbreviations: NLR, neutrophil–lymphocyte ratio; PLR, Platelet–lymphocyte ratio; CRP, C-reactive protein.

Receiver Operating Characteristic (ROC) curve analysis. The area under the curve (AUC) is used to evaluate the accuracy of prognosis and ranged from 0.5 to 1.0 - higher values indicated higher discrimination. Kaplan-Meier method and Log Rank test were used to statistically analyze the survival time and survival rate of patients. Cox regression model was used to analyze the risk factors affecting survival. SPSS 23.0 for Windows (IBM, Chicago, IL, USA) is used for data analysis. The significance level of all statistical tests was set as a two-sided *P*-value of 0.05.

3. Results

3.1. Clinical characteristic and serum chemerin levels in OSCC patients

The clinical characteristics and main laboratory examination results of survivors and non-survivors are listed in Table 2. As shown in Table 2, the levels of serum chemokine, neutrophils and NLR in dead patients were significantly higher than those in surviving patients (both, $P < 0.001$). Additionally, among the patients with OSCC (Table 3), Chemerin levels were significantly higher in patients with T3+4 disease than in patients with T1+2 disease ($P = 0.006$), in patients with poor differentiation than in patients with moderate-well differentiation ($P < 0.001$), in patients with lymph node metastasis (LN+) than in patients without lymph node metastasis (LN−) ($P = 0.016$) and in patients with recurrence than in patients without recurrence ($P < 0.001$). However, no significant correlation was found between serum Chemerin levels and age, sex or tumor size (Table 3).

3.2. Predictive ability of serum chemerin, neutrophils, and NLR

As shown in Fig. 1, serum Chemerin levels positively correlated with serum neutrophils and NLR levels ($r = 0.708$, $r = 0.578$, both, $P < 0.001$). The ROC curve analysis of using serum chemokines alone to predict mortality showed that the optimal cut-off chemokine was 55.39 (ng/mL), AUC was 0.8877, sensitivity was 84.62 %, and specificity was 83.64 % (Fig. 2). The predictive ability of serum chemotaxis was better than that of neutrophils or NLR. Next, we analyzed the prediction accuracy of different biomarker combinations, and found that the combination of Chemerin and other markers can improve the prediction sensitivity. The best AUC obtained by using the combination of Chemerin and NLR is 0.8898 (Fig. 3). Table 4 shows the prediction accuracy of a single marker or a combination of markers.

3.3. Analysis of serum chemerin level on the survival of patients with OSCC

To evaluate the effect of neutrophil infiltration and Chemerin expression on OSCC patient survival, we followed up all 120 patients. The median follow-up time was 36 months. During the observation period, 54 patients died of OSCC.

Kaplan and Meier analysis shows that low serum Chemerin level group's overall and disease-free survival time were longer than high low serum Chemerin level group ($P < 0.001$, Figs. 4A and 5A), the survival rates in the two groups were 86.4 % and 23.00 %; two groups of average overall and disease-free survival time were 46.95 & 35.71 months and 45.15 & 27.88 months respectively. Low neutrophils level group's overall and disease-free survival time were longer than high neutrophils level group ($P < 0.001$, Figs. 4B and 5B), the survival rates in the two groups were 73.3 % and 35.0 %; two groups of average overall and disease-free survival time were

Table 3
Clinicopathological characteristic and serum Chemerin levels in OSCC patients.

Variables		Number	Low Chemerin level	High Chemerin level	χ^2	*P*
Gender						
	Male	64	29(45.3 %)	35(54.7 %)	0.815	0.367
	Female	56	30(53.6 %)	26(46.4 %)		
Age (years)						
	<61	72	33(45.8 %)	39(54.2 %)	0.800	0.371
	≥61	48	26(54.2 %)	22(45.8 %)		
TNM stage						
	I,II	58	36(62.1 %)	22(37.9 %)	7.477	0.006**
	III,IV	62	23(37.1 %)	39(62.9 %)		
Differentiation						
	Poor	25	4(16.0 %)	21(84.0 %)	13.899	<0.001***
	Moderate-Well	95	55(57.9 %)	40(42.1 %)		
Lymph node metastasis						
	Yes	52	40(58.8 %)	28(41.2 %)	5.855	0.016*
	No	68	19(36.5 %)	33(63.5 %)		
Tumor size (d/cm)						
	<5	70	36(51.4 %)	34(48.6 %)	0.344	0.588
	≥5	50	23(46.0 %)	27(54.0 %)		
Tumor recurrence						
	Yes	38	8(21.1 %)	30(78.9 %)	17.586	<0.001***
	No	82	51(62.2 %)	31(37.8 %)		

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

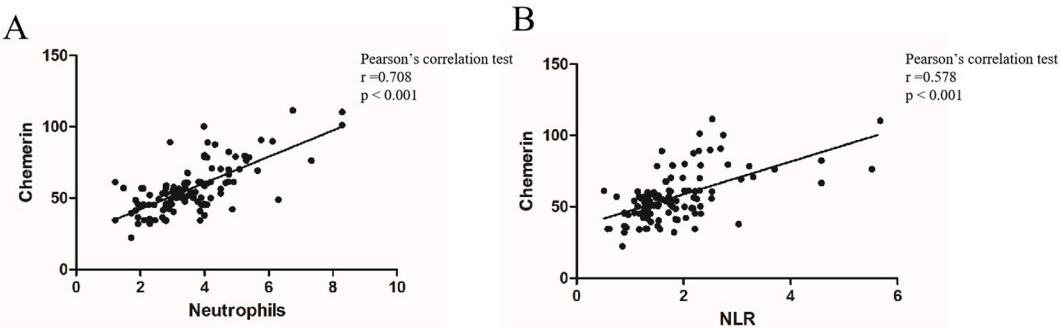

Fig. 1. Correlations of the Chemerin with Neutrophils. Notes: Correlations between neutrophil and neutrophil–lymphocyte ratio (NLR) levels in patients with OSCC and serum levels of Chemerin were assessed by Pearson's correlation test, (A) neutrophils, r = 0.708, $P < 0.001$; and (B) NLR, r = 0.578, $P < 0.001$.

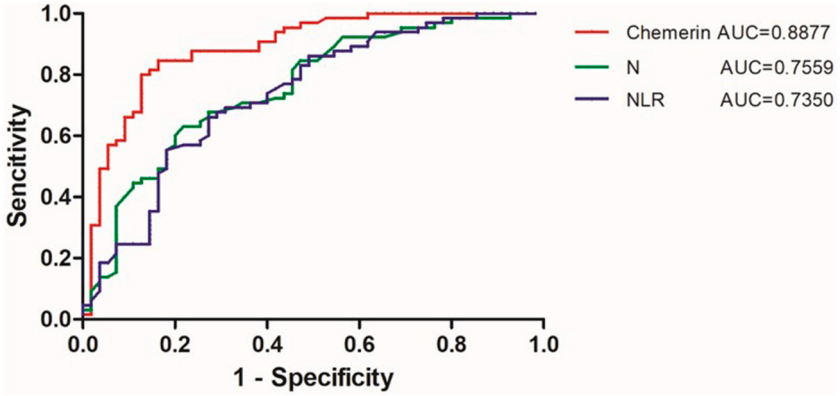

Fig. 2. ROC curves of the Chemerin , N and NLR for predicting OSCC patients. The receiver operating characteristic (ROC) curves for single predictors had the following areas: Chemerin, 0.8877; Neutrophil, 0.7559; NLR, 0.7350. All AUCs are shown for each ROC curve. AUC, area under ROC curve.

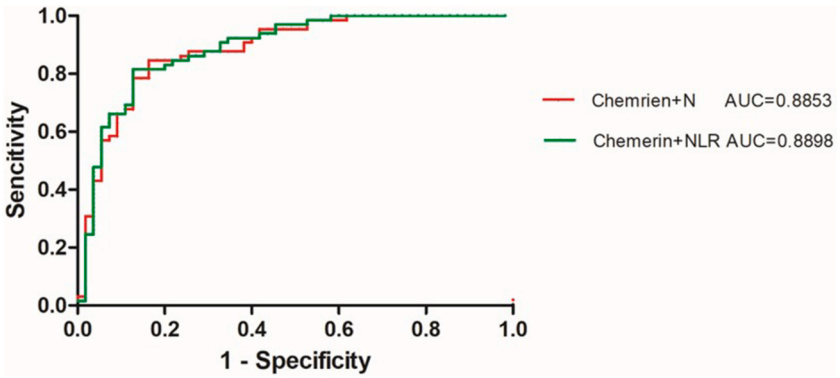

Fig. 3. ROC curves of the Chemerin, N(Neutrophil) and NLR (Neutrophil–lymphocyte ratio) combinations for predicting mortality of OSCC patients. The receiver operating characteristic (ROC) curves for combined predictors had the following areas: Chemerin + N, 0.8853; Chemerin + NLR, 0.8898. All AUCs are shown for each ROC curve. AUC, area under ROC curve.

45.43 & 40.00 months and 42.69 & 29.89 months respectively. Low NLR level group's overall and disease-free survival time were longer than high low NLR level group ($P < 0.001$, Figs. 4C and 5C), the survival rates in the two groups were 73.3 % and 35.0 %; two groups of average overall survival disease-free time were 44.85 & 37.62 months and 42.39 & 30.29 months respectively.

Further analysis showed that in OSCC patients with high clinical stage, the overall survival and disease-free survival of the low serum Chemerin level group were longer than the high serum Chemerin level group ($P < 0.001$, Figs. 4D and 5D); the survival rates of

Table 4
Prognostic accuracy of the Chemerin, N, and NLR.

Prognostic marker	Cut-off	Sensitivity	Specificity	PPV	NPV	AUC
Chemerin	55.39(ng/mL)	84.62 %	83.64 %	81.39 %	86.56 %	0.8877
N	3.165(× 10*9/L)	63.08 %	78.18 %	70.95 %	71.48 %	0.7559
NLR	1.537	66.15 %	72.73 %	67.22 %	71.78 %	0.7350
Chemerin + N		84.62 %	83.64 %	81.39 %	86.56 %	0.8853
Chemerin + NLR		81.54 %	87.27 %	84.42 %	84.83 %	0.8898

Abbreviations: N, neutrophil; PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve.

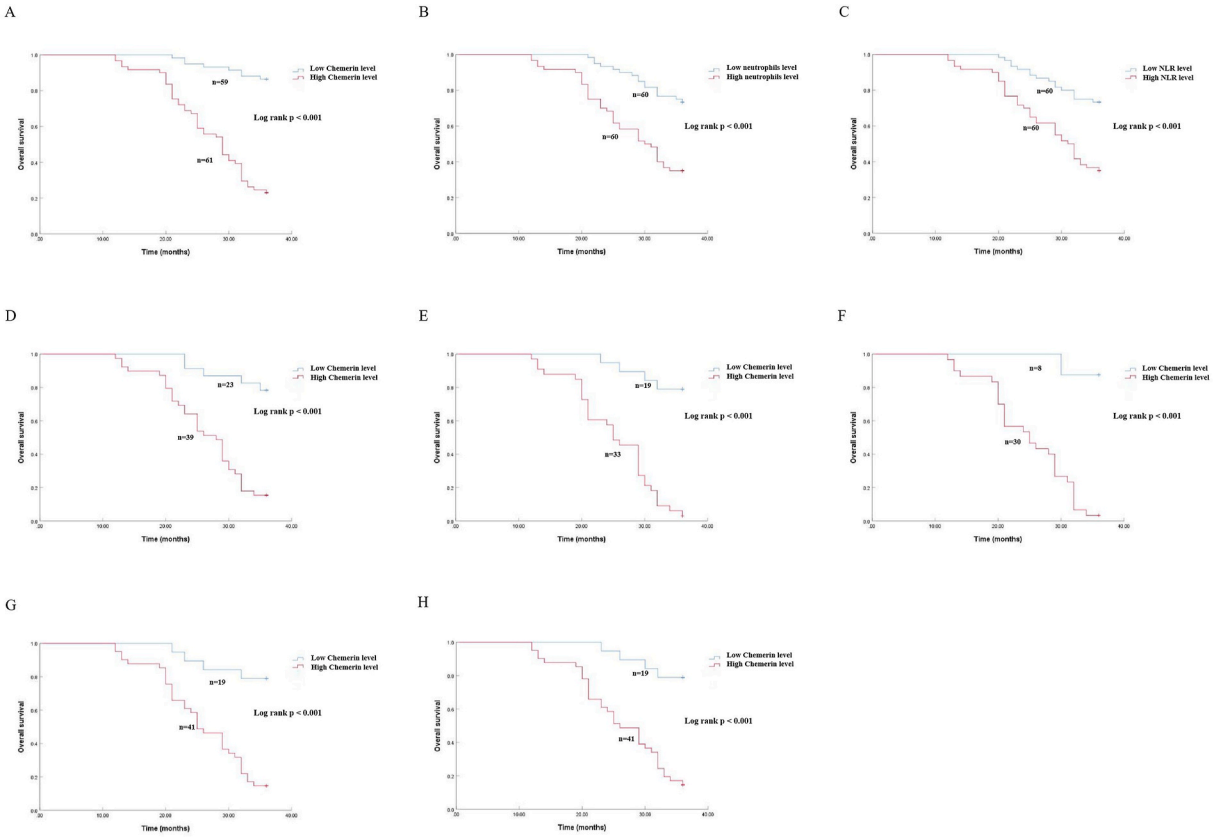

Fig. 4. The overall Survival time analysis of the effect of Chemerin and Neutrophils level on OSCC patients. (A–C) The overall cancer-related survival of OSCC patients in the High Chemerin level group, High Neutrophils level group, High NLR level group was significantly reduced, compared with the low Chemerin level group, low Neutrophils level group and low NLR level group. (D–F) In TNM stage III and IV OSCC, lymph node metastasis and tumor recurrence OSCC patients, the overall cancer-related survival of OSCC patients in the High Chemerin level group was significantly reduced. (G–H) In high neutrophils level, high NLR level OSCC patients and the overall cancer-related survival of OSCC patients in the High Chemerin level group was significantly reduced. ($P < 0.001$, log-rank test).

the two groups were 78.3 % and 15.4 %, the average overall and disease-free survival time of the two groups were 46.59 & 34.33 months and 43.03 & 25.55 months. In OSCC patients with lymph node metastasis, the overall survival and disease-free survival of the low serum Chemerin level group were longer than the high serum Chemerin level group ($P < 0.001$, Figs. 4E and 5E), the survival rates of the two groups were 78.9 % and 3.0 %, the average overall and disease-free survival time of the two groups were 46.39 & 32.24 months and 43.05 & 22.00 months. In OSCC patients with recurrent, the overall survival and disease-free survival of the low serum Chemerin level group were longer than the high serum Chemerin level group ($P < 0.001$, Figs. 4F and 5F); the survival rates of the two groups were 87.5 % and 3.3 %, the average overall and disease-free survival time of the two groups were 47.63 & 31.10 months and 44.55 & 21.15 months. In OSCC patients with high neutrophil level, the overall and disease-free survival of the low serum Chemerin level group were longer than the high serum Chemerin level group ($P < 0.001$, Figs. 4G and 5G); the survival rates of the two groups were 78.9 % and 14.6 %, the average overall and disease-free survival time of the two groups were 45.76 & 32.98 months and 42.81 & 23.95 months. In OSCC patients with high NLR level, the overall and disease-free survival of the low serum Chemerin level group were longer than the high serum Chemerin level group ($P < 0.001$, Figs. 4H and 5H); the survival rates of the two groups were 78.9 % and

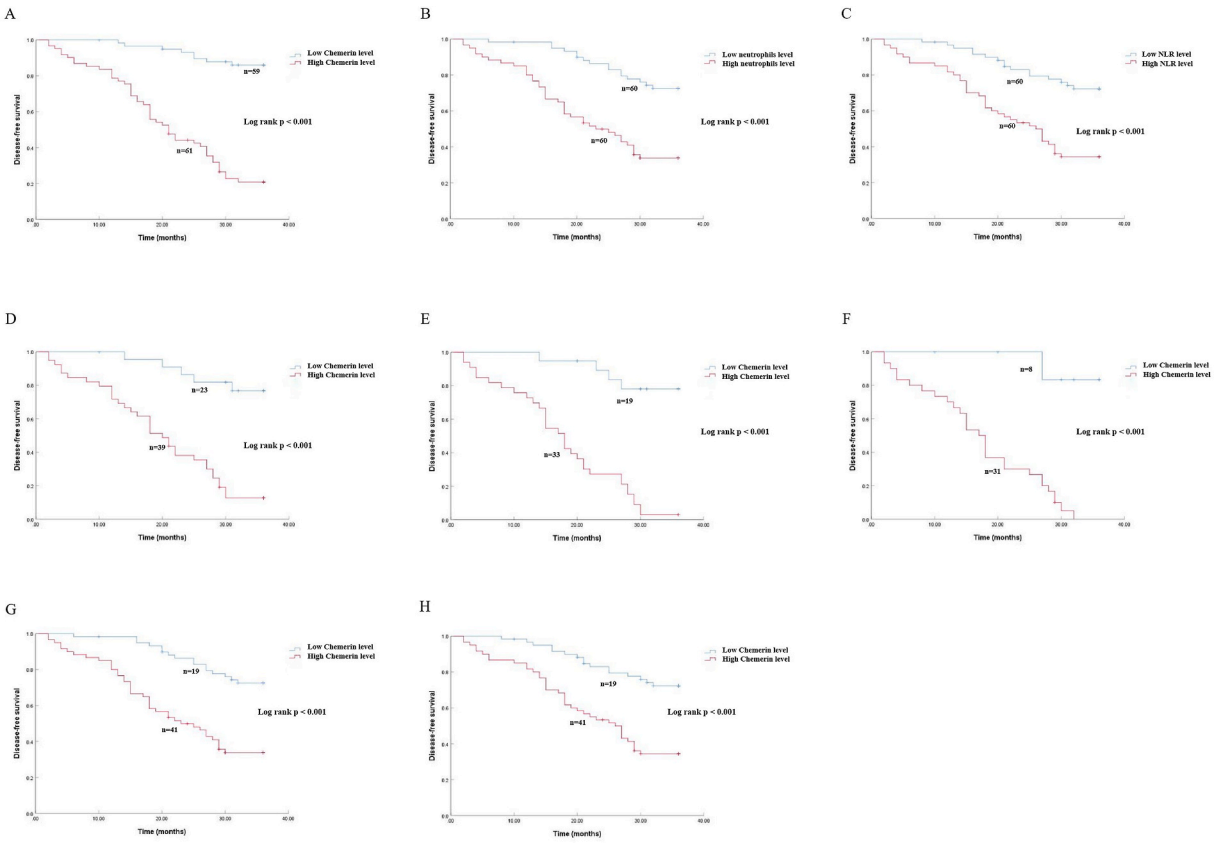

Fig. 5. The disease-free Survival time analysis of the effect of Chemerin and Neutrophils level on OSCC patients. (A–C) The disease-free cancer-related survival of OSCC patients in the High Chemerin level group, High Neutrophils level group and High NLR level group was significantly reduced, compared with the low Chemerin level group, low Neutrophils level group and low NLR level group. (D–F) In TNM stage III and IV OSCC, lymph node metastasis and tumor recurrence OSCC patients, the disease-free cancer-related survival of OSCC patients in the High Chemerin level group was significantly reduced. (G–H) In high neutrophils level, high NLR level OSCC patients and the disease-free cancer-related survival of OSCC patients in the High Chemerin level group was significantly reduced. ($P < 0.001$, log-rank test).

Table 5
Univariate and multivariate overall survival analysis in OSCC patients.

Variables	Univariate analysis		Multivariate analysis	
	Hazard ratio (95 % CI)	*P* value	Hazard ratio (95 % CI)	*P* value
Sex	1.364 (0.795–2.340)	0.259		
Age (years)	0.679 (0.386–1.192)	0.177		
TNM stage	2.709 (1.526–4.810)	0.001**	2.025 (1.105–3.709)	0.022*
Differentiation	0.193 (0.112–0.336)	<0.001***	0.608 (0.298–1.241)	0.172
Lymph node metastasis	3.489 (1.994–6.106)	<0.001***	1.492 (0.747–2.977)	0.257
Tumor size (d/cm)	1.232 (0.725–2.095)	0.441		
Tumor recurrence	4.107 (2.400–7.027)	<0.001***	1.179 (0.610–2.281)	0.624
Serum Chemerin(ng/mL)	1.053 (1.039–1.066)	<0.001***	2.025 (1.105–3.709)	0.001**
neutrophils(× 10*9/L)	1.680 (1.407–2.007)	<0.001***	0.802 (0.567–1.133)	0.211
lymphocytes(× 10*9/L)	0.766 (0.450–1.304)	0.325		
NLR	2.180 (1.700–2.795)	<0.001***	1.766 (1.183–2.635)	0.005**
albumin(g/L)	1.045 (0.985–1.109)	0.144		
fibrin(g/L)	1.201 (0.827–1.757)	0.344		
Platelets(× 10*9/L)	0.996 (0.992–1.000)	0.078		
PLR	0.998 (0.991–1.004)	0.517		
leukocyte(× 10*9/L)	1.106 (0.950–1.289)	0.195		
CRP (mg/L)	1.144 (0.941–1.392)	0.177		
monocytes(× 10*9/L)	0.614(0.249–1.513)	0.289		
eosinophils(× 10*9/L)	1.338 (0.312–5.745)	0.695		
basophils(× 10*9/L)	0.022 (0.000–818776.466)	0.669		

HR, hazard ratio; , *p< 0.05, **p < 0.01, ***p < 0.001.

14.6 %, the average overall and disease-free survival time of the two groups were 46.47 & 33.51 months and 43.17 & 24.18 months.

3.4. Cox model analysis of prognostic factors in patients with OSCC

Cox proportional hazard model was used to analyze the independent prognostic factors affecting the survival of patients with OSCC. The results showed that TNM stage ($P = 0.022$), serum Chemerin ($P = 0.001$) and NLR ($P = 0.005$) were independent factors affecting the overall survival time of patients with OSCC (Table 5); serum Chemerin ($P = 0.003$) and NLR ($P = 0.011$) were independent factors affecting the disease-free survival time in OSCC patients (Table 6).

3.5. Lentiviral transfection

The expression of Chemerin in SCC9, SCC15 and Cal27 cell lines was detected by Western blot. As shown in the figure (Fig. 6A), Low levels of Chemerin expression in SCC9 and Cal27 cells, so SCC9 and Cal27 cell lines were transfected with lentiviruses that overexpressed Chemerin (Fig. 6B and C).

3.6. Chemerin from tumor cells can induce upregulation of neutrophil chemokines in neutrophils

To explore if there are other chemotaxis mechanisms underlying the effect of Chemerin on neutrophils, a series of neutrophil chemokines were detected using western blotting and qRT-PCR. We found that R-chemerin up-regulated the expression of CXCL-5 and IL-17 on neutrophils in a co-culture system but had no effect on IL-6, IL-8, CXCL-6 and MCP-1 expression. (Fig. 7A). Overexpression of Chemerin on OSCC cells produced the same results for neutrophils (Fig. 7B and C).

3.7. Discussion

Neutrophils are the first line of defense against invading pathogens, and also the most abundant leukocytes subsets [31]. Neutrophils play key roles in the tumor microenvironment (TME) through various mechanisms, such as promoting extracellular matrix remodeling, thrombosis, T cell-dependent anti-tumor immunity, and angiogenesis [32]. To date, a sizeable minority of studies on neutrophils in peripheral blood of cancers have found that the ratio of high neutrophils to lymphocytes (NLR) is related to the low survival rate of various cancers in advanced stage [33,34]. Chemerin is not only a new multifunctional adipokine, but also a chemoattractant [18]. Studies have found that Chemerin is involved in fat metabolism, regulation of inflammation, angiogenesis, cell chemotaxis and proliferation and migration [35–38]. However, the relationship between serum Chemerin and neutrophil level in OSCC patients has not been extensively studied. In the current study, we explored the relationship between Chemerin and neutrophils in peripheral blood of OSCC patients and their effects on the diagnosis and prognosis of OSCC patients.

In this study, we first evaluated the diagnostic and prognostic value of serum Chemerin levels in OSCC patients. Serum Chemerin, neutrophils and NLR levels were higher in non-survivors than in survivor patients with OSCC. Adverse clinicopathological variables such as higher TNM stage, poor differentiation, lymph node metastasis, and tumor recurrence were associated with high serum Chemerin levels. We observed that levels of serum Chemerin were positively correlated with neutrophils and NLR levels. ROC analysis showed that the sensitivity and specificity of Chemerin + NLR in predicting mortality of OSCC patients were 81.54 % and 87.27 %, respectively, and AUC was 0.8898. The results showed that the combined detection of Chemerin and NLR had better diagnostic value than single index of Chemerin, neutrophils and NLR. Kaplan-Meier analysis revealed that high serum Chemerin level, high neutrophils level and higher NLR levels were associated with shorter overall survival and disease-free survival. Further analysis showed that in OSCC patients with high clinical stage, lymph node metastasis, recurrent, high neutrophil level and high NLR level, the overall survival and disease-free survival of the low serum Chemerin level group were longer than high serum Chemerin level group. Cox regression analysis showed that TNM stage, serum Chemerin and NLR were independent factors affecting the overall survival time of patients with OSCC; serum Chemerin and NLR were independent factors affecting the disease-free survival time of patients with OSCC. Our results showed that serum Chemerin and neutrophils may serve as serum biomarkers for diagnosis and prognosis in OSCC patients.

Previous studies have shown that neutrophils can secrete immunoreactive molecules (such as b2-integrin [39], oncostatin M [40] and neutrophil elastase [41]) to potentiate the migration, invasion and spread of cancer cells. Polarized neutrophils also produced higher levels of the pro-angiogenic molecule FGF2 to support angiogenesis and tumor growth in gastrointestinal cancer liver metastasis mouse models [42]. Chemokines are cancer-related inflammatory factors [43]that promote tumor progression through various pathways such as leukocyte recruitment, tumor cell invasion, proliferation and metastasis [44]. It has been reported that Chemerin can induce immune cells such as macrophages, cytotoxic natural killer cells and immature dendritic cells to chemotaxis to inflammatory sites [45]. Our previous study found that Chemerin promotes OSCC proliferation and invasion by activating EMT and JAK2/STAT3 signaling pathways by neutrophils [16]. However, the detailed mechanisms of Chemerin underlying neutrophil infiltration were not clear. We hypothesized that chemokines may play an important role in this process. Therefore, We examined the effect of Chemerin on the expression of chemokines in neutrophils [46](Fig. 6). The results found that Chemerin significantly upregulated the expression of IL-17 and CXCL-5 in neutrophils but had no effect on IL-6, IL-8, CXCL-6 and MCP-1 expression [47–51]. This indicates that Chemerin can also up-regulate the expression of other neutrophil chemokines and so attracts neutrophils to the tumor site. Previous research has shown that combined expression for TAN and IL-17 appears to be associated with a metastasis-prone phenotype in OSCC. In addition, IL-17 can serve as a valuable predictor of prognosis for patients with OSCC [52]. Another important and widely recognized CXC chemokine CXCL-5 (epithelial neutrophil-activating peptide-78) is associated with neutrophil infiltration and poor prognosis in

Table 6
Univariate and multivariate disease-free survival analysis in OSCC patients.

Variables	Univariate analysis		Multivariate analysis	
	Hazard ratio (95 % CI)	*P* value	Hazard ratio (95 % CI)	*P* value
Sex	1.343 (0.783–1.066)	0.285		
Age (years)	0.734 (0.418–1.290)	0.282		
TNM stage	2.743 (1.545–4.810)	0.001***	1.813 (0.987–3.330)	0.055
Differentiation	0.177 (0.101–0.310)	<0.001***	0.585 (0.281–1.219)	0.153
Lymph node metastasis	3.497 (1.998–6.120)	<0.001***	1.418 (0.698–2.881)	0.334
Tumor size (d/cm)	1.267 (0.945–2.154)	0.384		
Tumor recurrence	4.421 (2.577–7.584)	<0.001***	1.832 (0.754–2.718)	0.272
Serum Chemerin(ng/mL)	1.053 (1.039–1.066)	<0.001***	1.035 (1.012–1.060)	0.003**
neutrophils(× 10*9/L)	1.681 (1.410–2.003)	<0.001***	0.859 (0.612–1.205)	0.379
lymphocytes(× 10*9/L)	0.763 (0.447–1.301)	0.320		
NLR	2.071 (1.633–2.625)	<0.001***	1.616 (1.117–2.339)	0.011*
albumin(g/L)	1.040 (0.980–1.106)	0.170		
fibrin(g/L)	1.203 (0.819–1.176)	0.346		
Platelets(× 10*9/L)	0.998 (0.992–1.005)	0.627		
PLR	0.997 (0.993–1.001)	0.116		
leukocyte(× 10*9/L)	1.109 (0.953–1.291)	0.180		
CRP (mg/L)	1.148 (0.946–1.394)	0.163		
monocytes(× 10*9/L)	0.667 (0.273–1.629)	0.374		
eosinophils(× 10*9/L)	1.478 (0.356–6.132)	0.591		
basophils(× 10*9/L)	0.020 (0.000–811446.243)	0.661		

HR, hazard ratio; , *p< 0.05, **p< 0.01, ***p < 0.001.

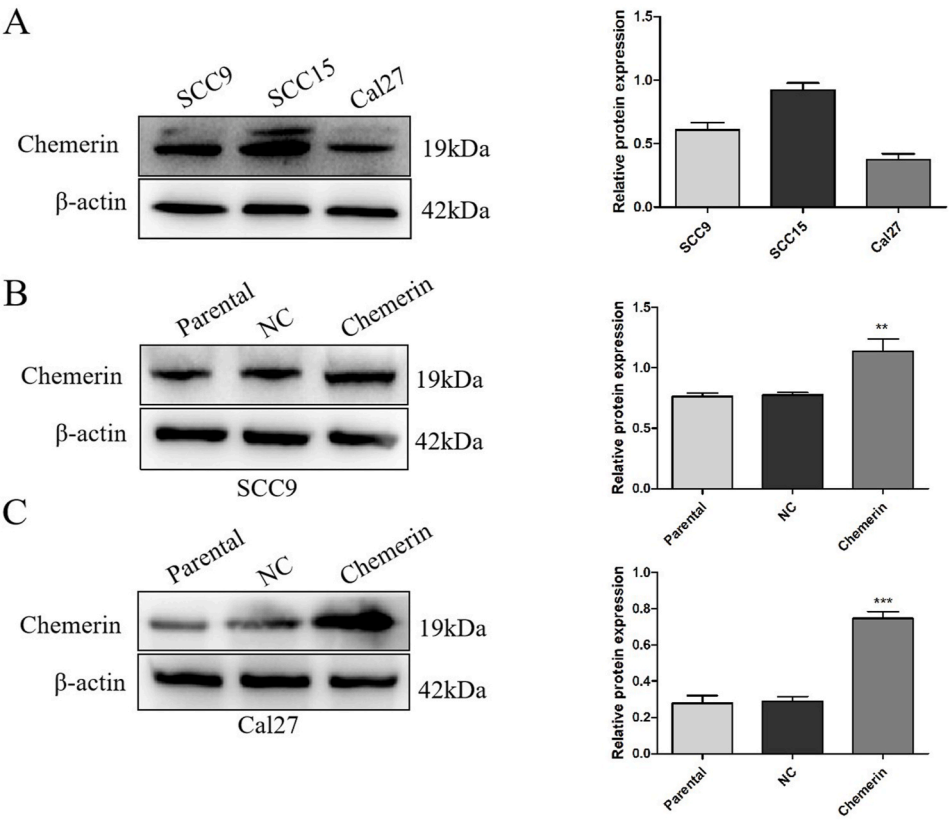

Fig. 6. Over-expression efficiency of Chemerin in OSCC cell lines. (A)Western blot analysis of Chemerin protein expression in SCC9, SCC15 and Cal27 cells. β-actin was used as the control. (B, C) SCC9 and Cal27 cells were used to over-express Chemerin by lentivirus, and Western blot analysis showed the expression of Chemerin increased after Chemerin transfection. *P < 0.05; **P < 0.01; ***P < 0.001. Compared with the parental group. NC, negative control. Parental, the blank control group was cells without gene transfection.

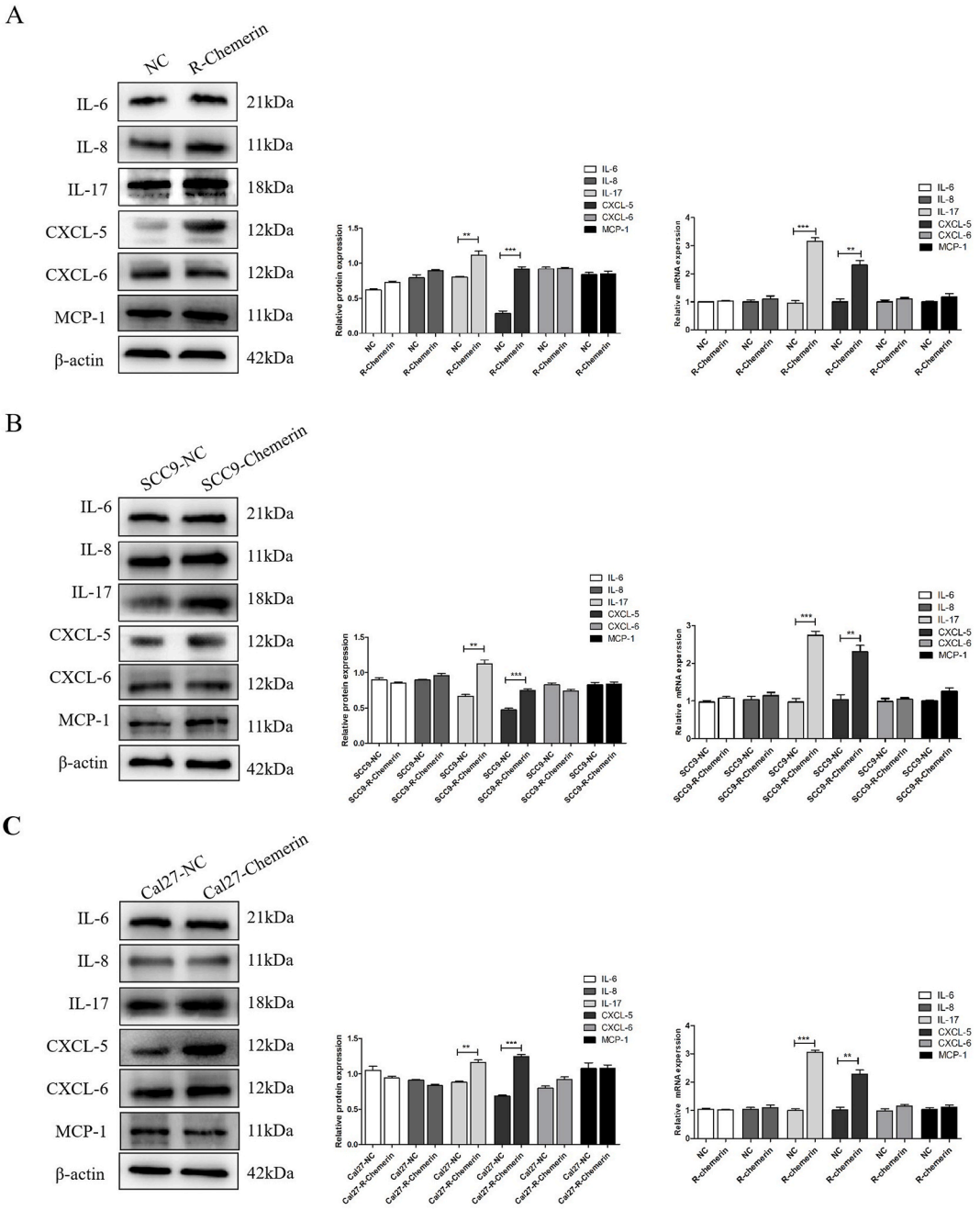

Fig. 7. Chemerin promotes chemokine expression on neutrophils. (A)The mRNA and protein expression of IL-17 and CXCL-5 was significantly higher in R-chemerin group than in NC group, while the expression of IL-6, IL-8, CXCL-6 and MCP-1 had no obvious change. (B, C) The mRNA and protein expressions of IL-17 and CXCL-5 in Chemerin overexpression group were significantly higher than those in NC group in SCC9 and Cal27 cells, while the expressions of IL-6, IL-8, CXCL-6 and MCP-1 were not significantly changed. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Compared with the NC group. NC, negative control.

hepatocellular carcinoma [53]. These studies and our research suggest that Chemerin may up-regulate the expression of IL-17 and CXCL-5 to attract neutrophils to the tumor site, which may represent a complicated chemotaxis pattern and explain the potent chemotactic effect of Chemerin on neutrophils. In conclusion, our results found serum Chemerin, neutrophils, and NLR levels were significantly higher in non survivors than in OSCC survivors, and high serum Chemerin level was strongly correlated with TNM stage, lymph node metastasis, differentiation and tumor recurrence. Furthermore, the prognosis of OSCC patients with high levels of serum Chemerin and neutrophils was poor. This may be caused by the high expression of Chemerin in the tumor site through a complex chemotactic mechanism, which attracts more neutrophils to the tumor site and promotes the development of tumor. Our results

suggested that serum Chemerin may be a valuable clinical biomarker for the diagnosis, progression, and prognosis assessment of OSCC.

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Data availability statement

The original contributions presented in this study can be obtained upon request by contacting the corresponding author via email.

Ethics approval and consent to participate

This study was performed in line with the principles of the Declaration of Helsinki. Informed consent was obtained from all individual participants included in the study. Written informed consent for publication was obtained from the patients enrolled in the study. Approval was granted by the Ethics Committee of The Affiliated Hospital of Qingdao University (10.14.2020/QYFYWZLL25964).

CRediT authorship contribution statement

Xiaoyuan Hu: Writing – original draft. **Ning Wang:** Writing – review & editing, Conceptualization. **Fei Gao:** Methodology. **Shengyou Ge:** Data curation. **Mei Lin:** Methodology. **Xuan Zhang:** Data curation. **Tongtong Li:** Software. **Tao Li:** Software. **Changting Xu:** Resources. **Caixiu Huang:** Resources. **Guicai Liang:** Methodology. **Wei Shang:** Supervision. **Fenggang Xiang:** Methodology. **Yuanyong Feng:** Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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