Chemerin promotes invasion of oral squamous cell carcinoma cancer by stimulating IL-6/TNF-α production via STAT3 signaling pathway

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Abstract

Aims:
The primary hallmark of oral cancer is cervical lymph node metastases. Previously, we discovered that elevated serum chemerin levels were related to oral squamous cell carcinoma (OSCC) with lymph node metastases. However, the mechanisms by which chemerin promotes OSCC metastasis are unknown. In this work, we focused on inflammation to investigate the mechanisms of chemerin-mediated OSCC metastasis.

Methods:
Serum from 10 pairs of OSCC patients with and without cervical lymph node metastases was collected before surgery. A Luminex liquid suspension assay was used to quantify the concentration of 27 different types of cytokines. Chemerin and inflammatory factors were validated by ELISA in both blood serum and cell culture supernatant. The relationship between chemerin and inflammatory factors was analysed. Western blot was used to measure the amount of phosphorylated STAT3 protein expression. Migration and invasion were investigated using the transwell assay.

Results:
Compared with the group without metastases, the levels of IL-6 (P = 0.006), IL-15 (P = 0.020), GM-CSF (P = 0.036), RANTES (P = 0.032), TNF-α (P = 0.005) and VEGF (P = 0.006) were significantly higher in OSCC patients with metastases. Serum chemerin level was found to correlate significantly with IL-6, GM-CSF, TNF-α and VEGF. Furthermore, treatment with recombinant chemerin significantly induced the secretion of IL-6 and TNF-α via activation of the STAT3 signalling pathway in OSCC cells. An IL-6/TNF-α neutralising antibody also reduced chemerin-induced migration and invasion of OSCC cells.

Conclusion:
These finding suggested that chemerin contributed to OSCC development might be associated with increasing IL-6 and TNF-α through activation of the STAT3 pathway.

1. Introduction
Invasion and metastasis are crucial biological features of malignant tumor progression that have a significant impact on the survival and prognosis of cancer patients. Oral squamous cell carcinoma (OSCC) is the most common malignant tumor in the oral and maxillofacial region, and its development is a complicated multistep process[1]. Although comprehensive therapies such as surgical treatment, radiation, chemotherapy, and biological immunotherapy have made significant progress over the years,
they can only restrain tumor cell development to a certain extent. In clinical practice, however, there is currently a lack of efficient control measures for the occurrence of invasion and metastasis of malignant tumors.

For many years, the 5-year survival rate of patients with OSCC has been approximately 50%[2, 3]. The main problem is that local recurrence after therapy, early metastasis to the cervical lymph nodes and distant metastasis are still the main reasons affecting the curative effect of OSCC. Therefore, if we can strengthen the research of the molecular mechanism of OSCC pathogenesis and identify some biomarkers that can predict the occurrence, invasion and metastasis of OSCC, we will be able to provide accurate diagnosis and treatment to improve the survival rate and quality of life.

Numerous studies have demonstrated that adipocytokines are associated with a poor prognosis in cancer patients, including those with OSCC[4, 5]. Chemerin, encoded by RARRES2, is a recently identified chemokine that modulates chemotaxis and macrophage and dendritic cell activation, thereby affecting inflammation and adipocyte metabolism[6]. Chemerin has been documented to play an important role in cancer progression[7]. We have previously observed that elevated serum chemerin level was positively correlated with cervical lymph node metastasis and a poor outcome in OSCC patients[8]. However, the potential mechanisms regarding the chemerin induced OSCC progression remain unknown. Chronic inflammation has also been linked to tumor recurrence, angiogenesis, and metastasis in OSCC[9]. It has shown that tumor cells produce cytokines and chemokines that promote tumor progression and growth[10]. However, the mechanisms by which inflammation promotes cancer progression are poorly understood.

In the present study, we investigated whether chemerin triggers OSCC inflammation and dramatically accelerates OSCC progression. Understanding the regulation of chemerin in OSCC progression may identify new targets for OSCC therapy.

2. Materials and methods

2.1 Blood samples and Luminex liquid suspension assay

Serum samples from 10 pairs of OSCC patients with or without lymphatic metastasis prior to anticancer therapy were obtained from the First Affiliated Hospital of Sun Yat-sen University between March 2019 and December 2020. Blood was collected and centrifuged at 3,000g for 15 minutes before being kept at 80°C until further use. The patients’ blood samples were delivered to Wayen Biotechnologies (Wayen Biotechnologies, China) for the Luminex liquid suspension test to measure inflammatory markers.

2.2 Cell culture and treatment

Human OSCC cell lines SCC25 and SCC9 were obtained from ATCC (ATCC, Manassas, VA, USA) and cultured in DMEM (Gibco, BRL, San Francisco, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% antibiotic-antimycotic and maintained at 37°C in a humidified
incubator containing 5% CO₂. The SCC25 and SCC9 cells were treated with recombinant human chemerin (R-chemerin; 2324-CM-025, R&D Systems, USA) at various concentrations (10, 50, 100 ng/ml) for 24 h. For the inhibitor treatment, we treated SCC25 and SCC9 with STAT3 inhibitor, cryptotanshinone (10 µM, C5624, Sigma, USA) for 24 h.

2.3 Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-6, TNF-α, VEGF, IL15, GM-CSF, RANTES, and chemerin in the blood samples and culture medium were detected using an ELISA kit (R&D Systems) according to the manufacturer's instructions. All samples were assayed in duplicate under the same conditions, and absorbance was read at 450 nm using a microplate reader (Bio-Rad Laboratories). The sensitivity of the assay was in the range of 0.1–5000 pg/mL.

2.4 Transwell migration and invasion assay

Transwell assays using BD BioCoat Cell Culture Inserts and BD BioCoat BD Matrigel™ Invasion Chambers (354656 and 354480, BD Biosciences, USA) were used to assess OSCC cell migration and invasion, respectively. The SCC25 and SCC9 cells were suspended with DMEM and seeded at a density of 1 × 10⁵ cells per insert (8-µm, 24-well insert, Millipore, USA) in a total volume of 100 µl of a serum-free medium. The chemerin, IL-6 and TNF-α neutralizing antibody (10µg/ml, R&D Systems, USA) supplemented with 10% FBS was added into the lower chamber. After 24 h of incubation at 37°C, migrating or invading cells were fixed with 4% paraformaldehyde for 10 min and then stained with DAPI for 30 min. Cells were counted under high magnification (×200).

2.5 Western Blot Analysis

After chemerin treatment, cells were lysed with RIPA buffer, and total protein concentration was determined using the BCA Protein Assay Kit (23227, Thermofisher). Samples (20–40 µg) were separated over a 10% SDS -polyacrylamide gel and proteins were then transferred to nitrocellulose membranes (0.45 µm), which were then blocked with 5% bovine serum albumin for 60 minutes at room temperature and incubated overnight at 4°C with the following primary antibodies: anti-Stat3 (1:1000, sc8019, Santa cruz), anti-pStat3 (1:1000, 9145s, Cell signalling), and anti-GAPDH (1:5000, 5174, Cell signalling). Membranes were incubated with horseradish peroxidase-linked secondary antibodies for an additional 60 minutes at room temperature. Blots were developed using Clarity Western ECL Substrate (1705061, Biorad) and viewed using Luminescent image Analyzer, Fusion FX (Vilber).

2.6 Statistical analysis

All data were presented as mean ± standard deviation (s.d.). Differences between groups were analysed by one-way analysis ANOVA. The value of P < 0.05 was considered statistically significant. The Spearman coefficient was calculated to assess the correlations between chemerin expression level and cytokines expression levels. All statistical analyses were performed with the SPSS 17.0 software (SPSS, Chicago, IL).
3. Results

3.1 Serum inflammatory factors expression in OSCC patients

Serum cytokine levels in OSCC patients with or without positive cervical lymph node metastasis were measured using the Luminex liquid suspension assay. As shown in Table 1, serum levels of IL-6, IL-15, GM-CSF, RANTES, TNF-α and VEGF were significantly higher in OSCC patients with lymph node metastases than in patients without lymph node metastases. Serum concentrations of selected factors were validated in OSCC patients by ELISA, and we confirmed that OSCC patients with lymph node metastases had significantly higher blood levels IL-6, IL-15, GM-CSF, RANTES, TNF-α (Fig. 1A-F). We also investigated the correlation between serum chemerin and inflammatory factors. Serum chemerin level was determined by ELISA, and we found that serum chemerin was positively correlated with serum concentrations of IL-6, GM-CSF, TNF-α and VEGF (Table 2). The results suggest that chemerin positively regulates inflammatory factors in OSCC patients.
Table 1
The serum inflammatory factor levels in OSCC patients with or without cervical lymph node metastasis

<table>
<thead>
<tr>
<th>inflammatory factors</th>
<th>LN- (n = 10)</th>
<th>LN+ (n = 10)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>347.14 ± 117.18</td>
<td>317.41 ± 78.24</td>
<td>0.513</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.07 ± 0.15</td>
<td>1.00 ± 2.04</td>
<td>0.383</td>
</tr>
<tr>
<td>IL-2</td>
<td>10.45 ± 3.97</td>
<td>10.49 ± 3.02</td>
<td>0.982</td>
</tr>
<tr>
<td>IL-4</td>
<td>7.34 ± 0.92</td>
<td>7.72 ± 2.26</td>
<td>0.627</td>
</tr>
<tr>
<td>IL-5</td>
<td>29.53 ± 5.37</td>
<td>27.57 ± 12.96</td>
<td>0.664</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.73 ± 1.11</td>
<td>7.46 ± 4.14</td>
<td>0.006**</td>
</tr>
<tr>
<td>IL-7</td>
<td>24.27 ± 4.94</td>
<td>23.88 ± 3.96</td>
<td>0.849</td>
</tr>
<tr>
<td>IL-8</td>
<td>15.06 ± 4.13</td>
<td>17.84 ± 6.62</td>
<td>0.274</td>
</tr>
<tr>
<td>IL-9</td>
<td>631.95 ± 32.27</td>
<td>647.83 ± 89.90</td>
<td>0.606</td>
</tr>
<tr>
<td>IL-10</td>
<td>7.30 ± 1.63</td>
<td>6.87 ± 1.82</td>
<td>0.584</td>
</tr>
<tr>
<td>IL-12</td>
<td>3.74 ± 1.69</td>
<td>3.96 ± 1.22</td>
<td>0.740</td>
</tr>
<tr>
<td>IL-13</td>
<td>10.44 ± 1.02</td>
<td>10.40 ± 1.58</td>
<td>0.950</td>
</tr>
<tr>
<td>IL-15</td>
<td>255.69 ± 57.27</td>
<td>308.10 ± 23.78</td>
<td>0.020*</td>
</tr>
<tr>
<td>IL-17A</td>
<td>10.38 ± 1.40</td>
<td>11.68 ± 2.08</td>
<td>0.120</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>175.45 ± 49.51</td>
<td>198.19 ± 123.38</td>
<td>0.595</td>
</tr>
<tr>
<td>bFGF</td>
<td>49.16 ± 6.83</td>
<td>45.75 ± 5.46</td>
<td>0.233</td>
</tr>
<tr>
<td>G-CSF</td>
<td>310.22 ± 98.89</td>
<td>291.14 ± 38.39</td>
<td>0.600</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>5.33 ± 2.57</td>
<td>7.58 ± 1.81</td>
<td>0.036*</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>24.54 ± 5.44</td>
<td>25.11 ± 11.74</td>
<td>0.891</td>
</tr>
<tr>
<td>IP-10</td>
<td>2034.58 ± 906.68</td>
<td>2014.60 ± 975.26</td>
<td>0.963</td>
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<tr>
<td>MCP-1</td>
<td>76.99 ± 27.47</td>
<td>64.64 ± 13.14</td>
<td>0.216</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>5.44 ± 3.05</td>
<td>4.16 ± 1.70</td>
<td>0.263</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>190.51 ± 40.01</td>
<td>179.40 ± 13.59</td>
<td>0.423</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>18201.16 ± 11676.95</td>
<td>24817.09 ± 8822.57</td>
<td>0.170</td>
</tr>
<tr>
<td>RANTES</td>
<td>11887.71 ± 3915.49</td>
<td>15300.22 ± 2359.26</td>
<td>0.032*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>22.80 ± 3.89</td>
<td>30.13 ± 6.21</td>
<td>0.005**</td>
</tr>
</tbody>
</table>
### Table 2
Correlation analysis between serum chemerin and inflammatory factors in OSCC patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chemerin</th>
<th>r</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td></td>
<td>0.976</td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>IL-15</td>
<td></td>
<td>0.282</td>
<td>0.430</td>
</tr>
<tr>
<td>GM-CSF</td>
<td></td>
<td>0.820</td>
<td><strong>0.004</strong></td>
</tr>
<tr>
<td>RANTES</td>
<td></td>
<td>0.291</td>
<td>0.414</td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td>0.768</td>
<td><strong>0.009</strong></td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
<td>0.664</td>
<td><strong>0.036</strong></td>
</tr>
</tbody>
</table>

Unit: pg/ml, LN: Lymphatic node; *P < 0.05, **P < 0.01 compared with control.

3.2 Chemerin induces IL-6 and TNF-α production of OSCC cells

Considering that several inflammatory factors are involved in cancer development, we wondered whether chemerin promotes invasion by regulating the expression of key cytokines. To test this hypothesis, SCC25 cells were treated with chemerin (100ng/ml) for 24 hours. We found that chemerin treatment resulted in a significant increase in IL-6 and TNF-α production in the culture supernatant of SCC25 cells (Fig. 2A-B). In contrast, the production of GM-CSF and VEGF was not significantly affected by chemerin treatment (Fig. 2C-D). These results suggest that chemerin may be an upstream activator of IL-6 and TNF-α production. The findings suggested that chemerin may contribute to the progression of OSCC by promoting an inflammatory microenvironment.

3.3 Chemerin triggers IL-6 and TNF-α secretion via STAT3-dependent pathway

The STAT3 pathway has been reported to be involved in the regulation of IL-6 production[11]. We therefore investigated the possible mechanism that chemerin mediates IL-6/TNF-α secretion via activation of STAT3 in OSCC cells. To this end, we cultured SCC25 and SCC9 cells treated with or without
human recombinant chemerin at different concentrations and found that chemerin stimulated increased phosphorylation of STAT3 in vitro in a dose-dependent manner (Fig. 3A-D).

Next, we investigated whether chemerin affected IL-6/TNF-a secretion via the STAT3 pathway. For this purpose, a STAT3 inhibitor was used to inhibit STAT3 signalling. Inhibition of STAT3 signalling attenuated IL-6/TNF-a production in chemerin-treated cells. Taken together, these results suggest that the STAT3 signalling pathway may be involved in chemerin-induced secretion of IL-6 and TNF-a in OSCC cells (Fig. 3E-F).

### 3.4 Blockade of IL-6 and TNF-a reduces the migration and invasion induced by chemerin

Previous studies have shown that IL-6 and TNF-α in the tumour microenvironment can promote tumour progression\[12, 13\]. To further investigate whether IL-6 and TNF-α are involved in the prometastatic effects induced by chemerin, SCC9 and SCC25 cells were treated with chemerin and IL-6, TNF-α neutralising antibodies. Consistent with previous report\[8\], chemerin promoted cell invasion and migration in OSCC cells (Fig. 4A-D), and IL-6 and TNF-α blocking antibodies significantly abolished chemerin-induced migration and invasion of OSCC cells. These results suggest that chemerin can trigger IL-6 and TNF-α to promote OSCC cell migration and invasion.

### 4. Discussion

We previously shown that chemerin contributed to metastasis of OSCC\[8\]. In this study, we discovered that a tight relationship between plasma levels of chemerin and inflammatory markers in patients with OSCC. Importantly, chemerin augments the secretion of pro-inflammatory cytokines like IL-6 and TNF-a by via the STAT3 pathway and promotes OSCC cell invasion.

Many studies have shown that chemerin and its receptor ChemR23 play an important role in regulating inflammatory responses and adipocyte differentiation via autocrine or paracrine pathways\[14, 15\]. High concentrations of chemerin have been detected in a variety of inflammatory fluids, including synovial fluid from patients with arthritis and ascites from patients with white follicular sacs\[16\]. The expression level of chemerin is closely related to obesity, type II diabetes, coronary heart disease, leukoplakia and other diseases\[17–19\]. The mechanisms by which chemerin contributes to cancer progression remain to be elucidated. In general, it is associated with inflammation, immune metabolism, and oxidation-reduction status. In the study by Shang et al\[20\], it was found that chemerin and inflammatory cytokines were highly expressed in the kidney tissue and blood of patients with diabetic nephropathy, and that a p38 MAPK inhibitor attenuated chemerin-induced kidney damage and inflammation in a mouse model of diabetic nephropathy. Wang et al\[21\]. found that chemerin inhibits the expression of Sirt1 by binding to the ChemR23 receptor, activates NF-κB, and acetylates P65, inducing a pro-inflammatory effect on keratinocytes.
Chemerin is known to support the recruitment of immune cells to sites of inflammation and promote the release of inflammatory factors that enhance the inflammatory response and contribute to the progression of cancer[22]. Therefore, in this study, we investigated whether high serum concentration of chemerin lead to changes in the concentration of inflammatory factors of OSCC patients, which sets the stage for promoting cancer cell metastasis. We compared the inflammatory factors in the serum of 10 pairs of OSCC patients with or without cervical lymph node metastases by liquid suspension array. The results confirmed that the expression levels of inflammatory factors, IL-6, IL-15, GM-CSF, RANTES, TNF-a, and VEGF were significantly elevated in OSCC patients, which was further validated by ELISA. Since chemerin was upregulated in OSCC patients and cell lines as our previously reported, we were interested in finding out if there was a relationship between chemerin and these upregulated inflammatory factors. Correlation analysis demonstrated that serum chemerin level was positively correlated with IL-6, GM-CSF, TNF-a and VEGF, suggesting serum chemerin may be associated with these inflammatory factors. Mechanistic investigation further revealed that recombinant chemerin stimulation promoted production of IL-6 and TNF-a in OSCC cells in vitro, but not GM-CSF and VEGF. We then explored the potential signal pathways, and results demonstrated that STAT3 pathway probably involved in this process. Dysregulation of the STAT3 signalling pathway is common in several types of cancer, including OSCC[23]. This pathway has been shown to play a role in facilitating tumour invasion, promoting angiogenesis and enabling immune evasion. Once activated, STAT3 translocates to the nucleus where it assumes the role of regulating the transcription of specific genes associated with cell survival, proliferation and inflammation. The mechanistic link between chemerin signalling and OSCC invasion is established through the activation of STAT3, which leads to the production of IL-6 and TNF-a induced by chemerin. On the other hand, we observed that in OSCC cells, the ability of chemerin mediated invasion and migration was diminished after neutralization of IL-6 and TNF-a. These findings suggested that IL-6 and TNF-a may be the main inflammatory factors mediating the malignant progression of OSCC promoted by chemerin.

The established role of chemerin in facilitating OSCC invasion through the IL-6/TNF-α-STAT3 pathway raises several important questions and potential avenues for future research. First, the precise mechanisms of Chemerin-induced IL-6 and TNF-production in OSCC cells need to be clarified. It is essential to understand the crosstalk between Chemerin signaling and other pathways involved in OSCC invasion in order to gain a deeper understanding of how OSCC progresses. Furthermore, identifying the downstream effectors regulated by the chemerin-STAT3-IL-6/TNF-a axis will help us to better understand the precise processes that drive OSCC invasion. Targeting these effectors may provide viable treatments to slow the progression of OSCC and improve patient outcomes.

In conclusion, our results suggest that chemerin-mediated tumor angiogenesis involves activation of the STAT3 signaling pathway and subsequent up-regulation of IL-6 and TNF-a production in OSCC patients. Therapies that target IL-6 and TNF-a may be developed as potential therapeutic approaches to inhibit OSCC.
Declarations

Conflict of interest

No potential conflict of interest was reported by the authors.

Funding

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Ethical Approval

The ethical committee of the First Affiliated Hospital of Sun Yat-sen University approved this study. Written informed consent was obtained from all subjects included in the study.

Consent to participate

Written informed consent was obtained from all subjects included in the study or from patients’ family member.

Consent to publish

All participants provide informed consent for the publication of the study.

Authors’ contributions

Z. L. designed the studies, performed experiments, acquired data and analyzed the data. J.L. and Q.W. conducted experiments, acquired data and assisted analysis of data. Y.W. and W.W. contributed to experimental design and manuscript drafting. Y.C. guided conception of the study design, wrote the manuscript and finalization of the manuscript. All authors reviewed the manuscript and agreed to the published version of the manuscript.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References


**Figures**

**Figure 1**

**Serum inflammatory factors levels in OSCC patients with or without cervical lymph node metastasis.** (A-F): The serum IL-6, TNF-α, VEGF, IL-15, GM-CSF and RANTES levels in patients with cervical lymph nodes metastasis (n=10) and without metastasis (n=10). *p 0.05 **p 0.01
Figure 2

Chemerin induced IL-6 and TNF-α production in OSCC cells.

(A-D) SCC25 cells were treated with chemerin (100 ng/ml) for 24h and expression levels of IL-6, TNF-α, GM-CSF and VEGF from cell culture supernatant were examined by ELISA. n=3, **p<0.01.
Chemerin triggered IL-6 and TNF-α secretion in OSCC cells via STAT3 pathway. (A, C) Western blotting was examined to detect the expression of p-STAT3 and STAT3 in SCC9 and SCC25 cells treated with chemerin for different concentration for 24h (n = 3). (B, D) quantification of pSTAT3 to STAT3. (E, F) The concentrations of IL-6 and TNF-α in supernatants of SCC9 and SCC25 cells were measured by ELISA after chemerin and STAT3 inhibitor, cryptotanshinone (10uM) treatment. Cry: cryptotanshinone. ***p<0.01.
Figure 4

Chemerin promoted migration and invasion through the IL-6/TNF-α production in OSCC cells.

(A) The SCC9 and (C) SCC25 cells were treated with neutralizing monoclonal antibodies against IL-6 and TNF-α and chemerin for 24h. The migration and invasion of SCC9 and SCC25 cells were assessed in a transwell system. Quantification of the migrated cells in the migration and invasion assay of SCC9 (B) and SCC25 (D) cells. ***P < 0.001.