C-C motif chemokine 14 as a novel potential biomarker for predicting the prognosis of epithelial ovarian cancer

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Abstract. Previous studies have demonstrated that C-C motif chemokine 14 (CCL14) plays an important role in the occurrence and development of cancer. However, the significance of CCL14 in the progression and prognosis of epithelial ovarian cancer (EOC) has not yet been reported. The standard EnVision procedure for tissue microarrays was used to evaluate the immunohistochemical expression of CCL14 protein in 154 patients with EOC who underwent tumor-debulking operations at the Central Cancer Department of Sun Yat-Sen University (Guangzhou, China) or Jiangmen Central Hospital (Jiangmen, China). The association between CCL14 expression and clinicopathological variables was assessed using the χ^2 test. For survival status of patients with EOC, Kaplan-Meier survival analysis and a Cox multivariate regression model was used. Expression of CCL14 protein was significantly associated with International Federation of Gynecology and Obstetric stage (P=0.014) and pN status(P=0.005). Kaplan-Meier survival analysis revealed that the survival time of patients with high expression of CCL14 was 136.1 months and that of patients with low expression of CCL14 was 98.9 months (P=0.026). Multivariate analysis demonstrated that CCL14 upregulation was associated with overall survival time (HR, 0.48; 95% CI, 0.261-0.896; P=0.021) and progression-free survival time (HR,0.437; 95% CI, 0.228-0.839; P=0.013). In conclusion, CCL14 is an independent prognostic factor for EOC and upregulation of CCL14 is associated with a more favorable prognosis in patients with EOC.

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Introduction

In 2018, 22,240 cases of epithelial ovarian cancer (EOC) were reported in the United States of America, with the associated death toll being 14,070 individuals (1). EOC is the primary cause of death from gynecological cancer in the US, with the morbidity and mortality being 11.5/100,000 and 6.7/100,000, respectively (2). The findings of EOC are usually advanced, and the 5-year survival rates for EOC diagnosed at stages III and IV are only 41% and 20%, respectively, in the United States (2). Histologically, EOC can be divided into 4 types: Serous carcinoma (68-71%), endometrioid carcinoma (9-11%), clear cell carcinoma (12-13%) and mucinous carcinoma (3%) (3,4). Studies have revealed that EOC is a heterogeneous tumor (5,6). The current standard treatment for EOC consists of cytoreductive surgery and platinum-based chemotherapy (7). Despite aggressive treatment, overall survival rate (OS) has not significantly improved in recent decades (8,9). Therefore, novel therapeutic targets are required to improve the current prognosis of patients with EOC.

In recent years, numerous studies have demonstrated that the tumor microenvironment plays an important role in the progression and metastasis of cancer (10,11). Chemokines are important members of the tumor microenvironment and mediate the recruitment of immune cells to the tumor microenvironment (12). Thus, chemokines are directly and indirectly involved in the formation of the tumor environment and immune milieu (13). C-C motif chemokine 14 (CCL14) is an important member of the chemokine family and was originally isolated from blood filters of patients with chronic renal failure. The protein is composed of 74 amino acids, of which 4 cysteine residues are linked by disulfide bonds (14). The relative molecular weight of CCL14 is 8,673 kDa and it has 46% sequence homology to macrophage inflammatory protein (14). CCL14 specifically binds to chemokine receptor 1 (CCR1), CCR3 and chemokine receptor 5 (CCR5) to exert biological effects (15,16). A previous study in patients with breast cancer have demonstrated that CCL14 promotes angiogenesis and metastasis (17). It has also been reported that CCL14 is involved in the occurrence and development of oral cancer (18). Therefore, the biological functions of CCL14 are diverse. The

involvement of CCL14 in the formation of the immune microenvironment indicates that the CCL14/CCR1/CCR5 axis can be used as a potential target for immunotherapy. The underlying mechanism of action of CCL14 in EOC is unclear and the significance of CCL14 in the progression and prognosis of EOC has not been reported. The present study aimed to elucidate the role of CCL14 in EOC.

Patients and methods

Patient specimens. The Medical Ethics Committees of the Cancer Center of Sun Yat-Sen University (Guangzhou, China) and Jiangmen Central Hospital (Jiangmen, China) approved the present study. The requirement for patient consent was waived by the ethics committees. A total of 154 patients with EOC were enrolled between January 2008 and December 2015. The mean age of enrolled patients was 48.5 years, ranging from 17-86 years. Among these cases, 82 patients had undergone ovariectomy at the Central Cancer Department of Sun Yat-Sen University and 72 patients had undergone ovariectomy at Jiangmen Central Hospital. The inclusion criteria for these patients in the present study were: Tissues could undergo immunohistochemical examination; no history of chemotherapy, radiotherapy and surgery prior to ovariectomy; complete immune function; and no other malignant tumors or secondary primary tumors. The follow-up period was censored to December 2018. All cases were classified on the basis of the World Health Organization Classification of Tumors of Female Reproductive Organs (19), the Tumor Node Metastasis Classification System of the US Joint Commission (20). The stage of tumors was assessed according to the International Federation of Gynecology and Obstetrics (FIGO) (21). The positive control tissues in the present study were derived from adjacent renal tissue from the surgical specimens of patients with renal cancer, which were paraffin-embedded tissue specimens archived following pathological diagnosis.

Tissue microarray (TMA) and immunohistochemistry (IHC). The standard EnVision procedure for tissue microarrays was used to evaluate the immunohistochemical expression of the CCL14 protein (22). To avoid potential bias caused by the heterogeneity of tumors, the TMA included 154 patient samples and each sample had 3 selected points with a diameter of 1.5 mm. IHC was performed on all points for each sample. The results of IHC were calculated by averaging the 3 points. All samples were fixed with 10% neutral buffered formalin (NBF) at room temperature for 48 h. IHC sections were prepared using 3-μm TMA paraffin-embedded sections. Xylene was used to de-wax the sections and then the sections were rehydrated in a descending alcohol series (100, 95, 85, 75 and 65%) and distilled water at room temperature. Subsequently, the sections were pressure-cooked at 100˚C with citric acid buffer solution (pH 6.0) for 3 min to repair antigen, then sections were placed in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. After the pre-processing in the aforementioned steps, TMA sections were incubated with CCL14 primary antibody (polyclonal antibody; cat. no. PA5-28819; Invitrogen; Thermo Fisher Scientific, Inc.) at a dilution ratio of 1:500 for 50 min at 37˚C in the incubator. Following this, TMA sections were incubated with CCL14 secondary antibody (undiluted; cat. no. K5007; Dako; Agilent Technologies, Inc.) for 30 min at 37˚C in the incubator. The sections were then stained with 3,3-diaminobenzidine. The last step involved using hematoxylin to counterstain the sections, which were finally fixed using dehydration. Human kidney tissue was used as the positive control (14) and in the negative control, human kidney tissue were incubated with 0.02 mol/l PBS instead of the primary antibody against CCL14.

IHC evaluation. Expression levels of CCL14 was evaluated by 2 independent pathologists using a light microscope (cat. no. BX51; Olympus Corp.). Percentages (0-100%) were used to define the number of positive tumor cells and a scoring system was used to evaluate dye strength as follows: Negative expression, -; low positive expression, 1+; moderate positive expression, 2+; and strong positive expression, 3+. Scoring of each sample was performed using the following formula: Percentage of positive cells x dye strength and the total score of each sample ranged from 0-300 (22).

Statistical analysis. SPSS v16.0 (SPSS, Inc.) was used to perform the statistical analysis of the present study. Data are presented as mean standard deviation. The χ^2 test was used to analyze the association between CCL14 protein expression levels and clinicopathological parameters in patients with EOC. Survival analysis of patients with EOC was performed using the Kaplan-Meier method with the log-rank test for evaluation. A Cox proportional hazard model was used to perform multivariate analyses. All P-values were analyzed bilaterally. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Immunohistochemical analysis of CCL14 in tissues from patients with EOC. IHC results revealed that CCL14 was expressed in most cases, mainly in the cytoplasm of cancer cells. Representative image of expression levels are presented in Fig. 1, from negative to strong positive. IHC scoring showed that 17 cases were scored between 0-50 (11.04%), 39 cases were scored between ≥50-100 (25.32%); 22 cases were scored between ≥100-150 (14.29%), 27 cases were scored between ≥150-200 (17.53%), 20 cases were scored between ≥200-250 (12.99%) and 29 cases were scored ≥250 (18.83%) (data not shown).

Cut-off value for CCL14 expression levels. In order to select the appropriate cut-off point of CCL14 for further analysis, a receiver operating characteristic (ROC) curve was used to analyze each clinicopathological parameter. The point of infinite proximity (0.0, 1.0) on the ROC curve for each clinicopathological parameter had relevant specificity and sensitivity at the highest points (23), so these areas were defined as the cut-off points. The ROC curve analysis demonstrated that survival status, FIGO stage, pN status and relapse were taken as state variables for ROC analysis (Fig. 2) and that the classification efficiency of survival status (area under curve, 0.612; P=0.02) was the best (Fig. 2A). Therefore, survival status was taken as a state variable and 190 was defined as the cut-off point for CCL14 protein expression levels. High expression

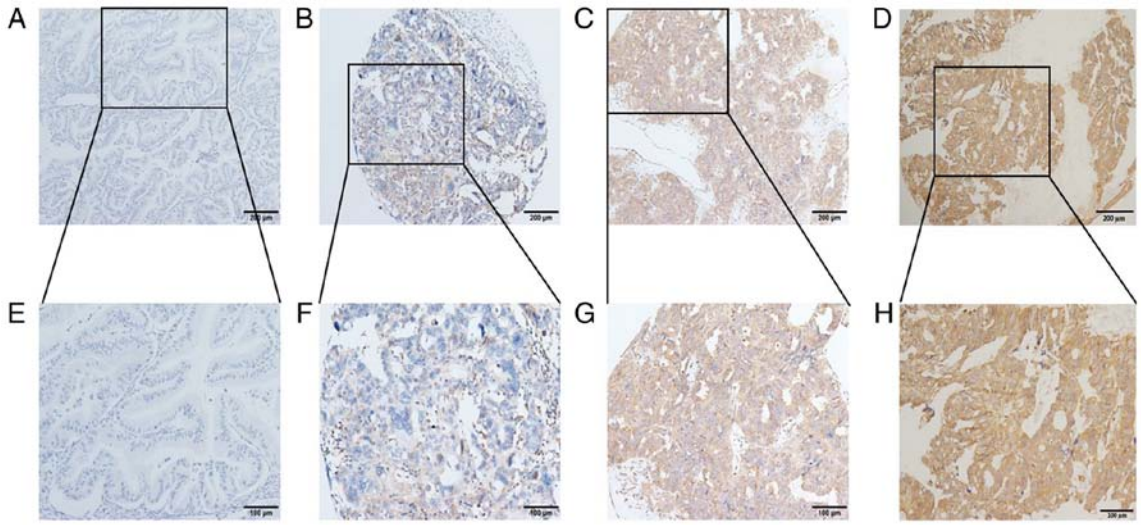

Figure 1. CCL14 protein expression levels in tissues from patients with epithelial ovarian cancer. (A) Negative expression (-), x10 magnification; (B) low positive (1+), x10 magnification; (C) moderate expression (2+), x10 magnification; and (D) strong expression (3+), x10 magnification. Images (E-G) and (H) represent the higher magnification (x20) from the area of the inset box in images (A-D), respectively. CCL14, C-C motif chemokine 14.

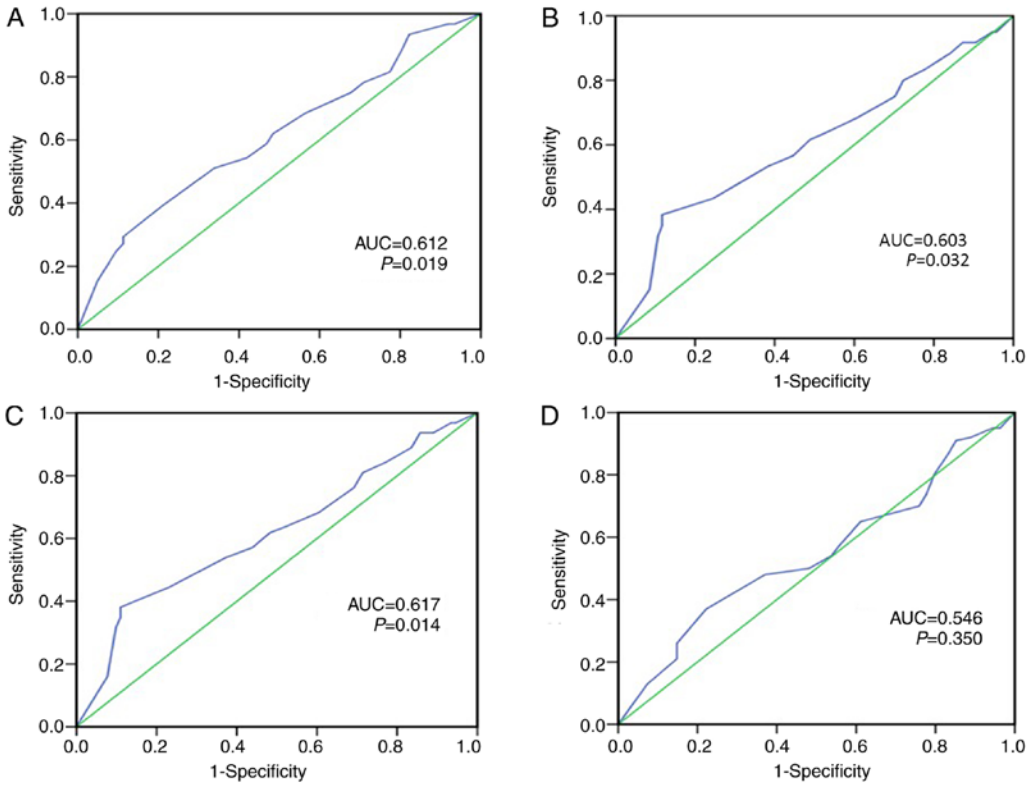

Figure 2. Receiver operating characteristic curve analysis to determine the cut-off value for upregulation of CCL14 protein in the tissues of patients with epithelial ovarian cancer. The sensitivity and specificity for each outcome were plotted: (A) survival status; (B) International Federation of Gynecology and Obstetrics stage; (C) pN status and (D) relapse. AUC, area under the curve; CCL14, C-C motif chemokine 14.

levels of CCL14 protein were defined as those with higher expression levels compared with the cut-off value and low expression levels of CCL14 was defined as those with expression levels which were below the cut-off value.

Association of CCL14 expression levels with clinicopathological features in patients with EOC. The expression levels of CCL14 is associated with a number of clinicopathological features. High expression levels of CCL14 protein were analyzed using the χ^2 test which revealed that it was significantly inversely associated with FIGO stage (P=0.014; Table I). Among 94 patients with advanced stage EOC (III+IV), 71 (75.5%) had low expression levels of CCL14 and 23 (24.5%) had high expression levels. Among the 60 patients with early stage EOC (I+II), 34 (56.7%) had low expression levels and 26 (43.3%) had high expression levels of CCL14. Patients with

Table I. Association between the clinicopathological variables and expression of CCL14 in patients with epithelial ovarian cancer.

		CCL14 expression levels		
Variable	All cases, n	Low expression, n (%)	High expression, n (%)	P-value
Age, years				0.614
≤49	80	56 (70.0)	24 (30.0)	
>49	74	49 (66.2)	25 (33.8)	
FIGO stage				0.014[a]
I+II	60	34 (56.7)	26 (43.3)	
III+IV	94	71 (75.5)	23 (24.5)	
Histological type				0.132
Serous	101	73 (72.3)	28 (27.7)	
Mucinous	53	32 (60.4)	21 (39.6)	
Pathological grade				0.207
G1	27	20 (74.1)	7 (25.9)	
G2	48	28 (58.3)	20 (41.7)	
G3	79	57 (72.2)	22 (27.8)	
Median tumor size, cm				0.271
≤10.05	76	55 (72.4)	21 (27.6)	
>10.05	78	50 (64.1)	28 (35.9)	
Relapse				0.060
No	100	63 (63.0)	37 (37.0)	
Yes	54	42 (77.8)	12 (22.2)	
pN status				0.005[b]
0	63	35 (55.6)	28 (44.4)	
1	91	70 (76.9)	21 (23.1)	
CA125, U/ml				0.821
≤33	17	12 (70.6)	5 (29.4)	
>33	137	93 (67.9)	44 (32.1)	
CA19-9, U/ml				0.753
≤35	101	68 (67.3)	33 (32.7)	
>35	53	37 (69.8)	16 (30.2)	
CEA, μg/ml				0.953
≤5	123	84 (68.3)	39 (31.7)	
>5	31	21 (67.7)	10 (32.3)	

[a]P<0.05, [b]P<0.01. CCL14, C-C motif chemokine 14; FIGO, International Federation of Gynecology and Obstetrics.

pN 1 status had similar results (P=0.005; Table I), 70 (76.9%) had low expression levels and 21 (23.1%) had high expression levels of CCL14. In those patients with pN 0 status, 35 (55.6%) had low expression levels of CCL14 and 28 (44.4%) had high expression levels. However, the other clinical and demographic parameters of age, histological type, pathological grade, tumor size, relapse, CA125, CA19-9 and CEA, were not significantly associated with the expression levels of CCL14.

Association between clinicopathological characteristics, CCL14 status and patient survival. To elucidate the best clinicopathological factors for the prognosis of EOC, a univariate analysis of each clinicopathological parameter was performed. The results demonstrated that patients with high expression levels of CCL14 had a high OS time (mean, 136.1 months); this was significantly higher compared with the mean of 98.9 months in patients with low CCL14 expression levels (P=0.026; Table II; Fig. 3A). A similar trend was observed for progression-free survival (PFS) (Fig. 3B). For FIGO stage, the mean OS time of patients with stage I+II cancer was 155.4 months, which was significantly higher compared with that of patients with stage III+IV cancer (mean, 86.2 months) (P<0.001; Table II; Fig. 3C). PFS of patients with stage I+II EOC was significantly higher compared with that of patients with stage III+IV cancer. (P<0.001; Fig. 3D). For pathological grade, the mean OS time for patients with a high differentiation grade was longer compared

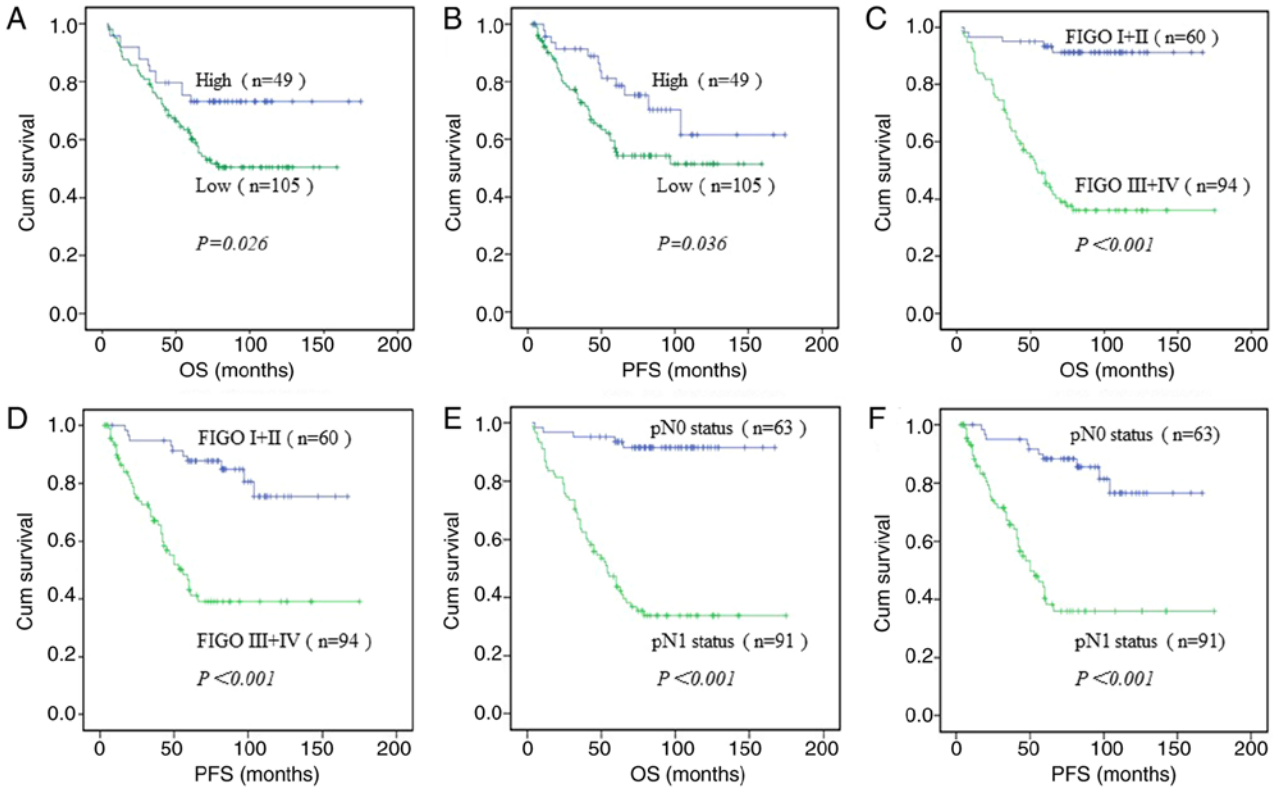

Figure 3. Different prognostic factors affecting OS and PFS time in 154 patients with epithelial ovarian cancer. Each outcome was plotted: (A and B) CCL14 expression levels; (C and D) FIGO stage and (E and F) pN status. OS, overall survival; PFS, progression-free survival; FIGO, International Federation of Gynecology and Obstetrics; cum, cumulative.

with that for patients with a low differentiation grade (G1 mean, 143.3 months; G2 mean, 130.9 months; G3 mean, 90.4 months) (P<0.001; Table II). The mean OS time for relapse was shorter (mean, 73.8 months) compared with that for no relapse (mean, 130.9 months) (P=0.001; Table II). The mean OS time for patients with EOC who had pN 1 status (mean, 83.1 months) was shorter compared with that for patients with pN 0 status (mean, 156 months) (P<0.001; Table II; Fig. 3E). A similar trend was observed for PFS (P<0.001; Fig. 3F). The results also revealed that CA125 is a relevant factor in survival (P=0.006; Table II). However, age, histological type, tumor size, CA19-9 and CEA did not impact the OS time of patients with EOC (Table II).

Independent prognostic factors for patients with EOC. Multivariable analysis using a Cox risk regression model demonstrated that upregulation of CCL14 protein in patients with EOC was significantly associated with OS (HR, 0.483; 95% CI, 0.261-0.896; P=0.021; Table III) and was an independent prognostic factor. Upregulation of CCL14 was also associated with a favorable PFS (HR, 0.437; 95% CI: 0.228-0.839, P=0.013; Table III). In addition, two interesting phenomena were observed: Pathological grade was an independent prognostic factor in OS (HR, 1.865; 95% CI, 1.179-2.948; P=0.008; Table III) and PFS (HR, 1.774; 95% CI, 1.128-2.791; P=0.013; Table III) in the patients with EOC.

Discussion

Chemokines comprise a group of ~50 small secreted proteins (8-14 kDa). According to the position of the first 2 cysteine residues, chemokines can be divided into CC-chemokines, CXC-chemokines, C-chemokines and CX3C-chemokines. The role of these proteins is to interact with a family of ~20 7-transmembrane G-protein-coupled receptors (24). Some studies have demonstrated that chemokines are novel targets for cancer immunotherapy (25,26). CCL14 is a member of the chemokine family that has attracted considerable attention in recent years, as it has a common receptor (CCR5) against HIV, which may be used as a novel method of treating HIV (15,27). The gene for the CCL14 protein is located on human chromosome 17q11.2 and is the product of transcripts encoding single and double cis-trans and double cis-trans alignments of tandem genes (28). CCL14 is expressed in certain normal somatic tissues, including the spleen, liver, skeletal muscle, myocardium, intestinal tract and bone marrow and is a C-chemokine with unconventional biological activity (14). CCL14 can be converted into a monomer at physiological concentrations, which is able to activate the migration of different leukocytes by inducing Ca2+ flux (29). Studies have shown that the CCL14/CCR1/CCR5 axis is hypothesized to mediate the chemotaxis of monocytes, eosinophils and T lymphocytes, which is consistent with the high expression levels of CCR5/CCR1 in these cells (30,31).

In the present study, IHC was used to detect the expression levels of CCL14 protein. The results of the current study revealed that CCL14 protein was expressed in most patients with EOC. Upregulation of CCL14 accounted for 43.3% of those with early-stage EOC, which was a significantly higher percentage compared with that for late-stage patients with EOC (24.5%).

Table II. Univariate analysis of clinicopathological variables in 154 patients with ovarian cancer.

Variable	All cases, n	Mean OS time, months	P-value[d]
Age, years			0.057
≤49	80	127.19±7.75	
>49	74	90.77±6.76	
FIGO stage			<0.001[c]
I+II	60	155.37±5.02	
III+IV	94	86.20±7.34	
Histological type			0.413
Serous	101	112.85±7.08	
Mucinous	53	113.01±8.84	
Pathological grade			<0.001[c]
G1	27	143.26±8.60	
G2	48	130.91±9.96	
G3	79	90.38±7.52	
Tumor size, cm			0.897
≤10.05	76	116.81±8.07	
>10.05	78	96.35±6.42	
Relapse			0.001[b]
No	100	130.92±6.94	
Yes	54	73.77±6.12	
pN status			<0.001[c]
0	63	156.00±4.76	
1	91	83.13±7.38	
CA125, U/ml			0.006[b]
≤33	17	119.83±6.96	
>33	137	110.06±6.20	
CA19-9, U/ml			0.195
≤35	101	106.43±6.78	
>35	53	126.78±9.64	
CEA, μg/ml			0.407
≤5	123	109.15±5.67	
>5	31	107.49±13.37	
CCL14			0.026[a]
High expression	49	136.06±9.33	
Low expression	105	98.86±6.30	

[a]P<0.05, [b]P<0.01, [c]P<0.001. [d]Log-rank test. CCL14, C-C motif chemokine 14; FIGO, International Federation of Gynecology and Obstetrics.

The proportion of patients with high expression levels of CCL14 with pN 0 status was higher compared with that of patients with pN 1 status. The mean OS time of patients with high expression levels of CCL14 was significantly higher compared with that of the patients with low expression levels of CCL14. The results revealed that the upregulation of CCL14 was significantly associated with the OS time of patients with EOC. Furthermore, multivariate analysis revealed that the upregulation of CCL14 was significantly associated with OS and PFS time, which indicated that the upregulation of CCL14 was an independent prognostic factor for EOC.

Studies suggest that CCL14 is involved in the incidence and development of breast and oral cancer (17,18); however, there are no reports that investigate the role of CCL14 in EOC. Studies have demonstrated that CCL5/CCR5 and chemokine (C-X-C) motif ligand 12 (CXCL12) β promote tumor immune tolerance and tumor progression (32,33). CXCL12/CXCR4, CCL18 and CXCL16/CXCR6 can promote the progression and migration of OC (34-36). However, studies performed on CXCL9 and CXCL10 have revealed that it can promote an antitumor immune response (37,38). These studies have demonstrated that chemokines are directly involved in the formation of the immune microenvironment in OC. CCL14 appears to be significantly downregulated in 9 types of cancer, which implicates CCL14 as an important player in the pathogenesis of cancer (39). Previous studies have demonstrated that CCL14

Table III. Multivariate survival analyses of clinicopathological variables in patients with epithelial ovarian cancer.

	OS time			PFS time		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Age (>49 vs. ≤49 years)	1.310	0.758-2.263	0.333	1.027	0.579-1.821	0.927
Histological type (serous vs. mucinous)	1.068	0.603-1.892	0.821	1.239	0.670-2.289	0.494
Pathological grade (G3 vs. G1+2)	1.865	1.179-2.948	0.008	1.774	1.128-2.791	0.013[a]
Tumor size, cm (>10.05 vs. ≤10.05)	1.118	0.654-1.911	0.684	0.701	0.390-1.258	0.233
CA125, U/ml (>33 vs. ≤33)	6.511	0.868-48.846	0.068	7.126	0.946-53.693	0.057
CA19-9, U/ml (>35 vs. ≤35)	0.709	0.389-1.294	0.263	0.841	0.447-1.581	0.590
CEA, μg/ml (>5 vs. ≤5)	1.158	0.617-2.172	0.648	1.309	0.659-2.60	0.442
CCL14 expression (high vs. low)	0.483	0.261-0.896	0.021	0.437	0.228-0.839	0.013[a]

[a]P<0.05. CCL14, C-C motif chemokine 14; OS, overall survival; PFS, progression-free survival.

is a factor that can improve the prognosis of patients with hepatocellular carcinoma and human papilloma virus-related cervical intraepithelial neoplasia (40,41). Low expression levels of CCL14 is associated with poor immune function and disease promotion in these carcinomas (40,41). In multiple myeloma, CCL14 can recruit polarized macrophages to form an antitumor immune environment and improve the prognosis of patients with multiple myeloma (42). These studies have also demonstrated that CCL14 is a tumor suppressor gene.

In intestinal studies, CCL14 can recruit chemotactic immune cells and prevent pathogenic bacteria from affecting the intestine (43). This function of CCL14 plays an important anticancer role in OC, since a study demonstrated that there are a large number of macrophages and T lymphocytes in the OC microenvironment (44). Other studies reported that in OC, macrophages and T lymphocytes upregulate the expression levels of CCR1 and CCR5 receptors, which creates an antitumor immune environment leading to the necrosis of OC cells (45,46). Furthermore, another study investigating multiple myeloma demonstrated that when CCL14 binds to CCR1 and CCR5, it can lead to chemoattraction and recruitment of macrophages, which promotes proliferation by activating the PI3K-AKT, MAPK/ERK, JNK and p38MAPK pathways (42). These studies suggest that the CCL14 protein is involved in the generation of antitumor immunity. It is possible that CCL14 has a similar role and underlying mechanism in OC, although the mechanism by which it exerts antitumor activities requires further exploration.

In conclusion, CCL14 is an independent prognostic factor in EOC. Upregulation of CCL14 is associated with increased OS and PFS times for patients with EOC. Overall, the present study revealed that CCL14 may be used for the development of novel targeted therapies for EOC.

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Availability of data and materials

The data that support the findings of the present study are available from researchdata.org.cn of Sun Yat-sen University Cancer Center. However, restrictions apply to the availability of these data, which were used under license for the present study and are not publicly available. Data are however available from the authors upon reasonable request and with the permission of Research Data Deposit public platform of Sun Yat-sen University Cancer Center.

Authors' contributions

YC and JC designed the present study. HH, XC, YC and LH acquired and analyzed the data. YC, YL and LH performed all the experiments and drafted the manuscript. YX and ZZ collected and assembled the data and performed the experiments. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by The Institute Research Medical Ethics Committee of Sun Yat-Sen University Cancer Center (Guangzhou, China) and Jiangmen Central Hospital (Jiangmen, China). The requirement for informed consent (written or verbal) was waived for the use of retrospective data and paraffin tissue specimens from the patients in the present study, the majority of whom were deceased, since this was not deemed necessary by the Ethics Committee. All samples were anonymized.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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