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ORIGINAL PAPER

Crk-like adapter protein regulates CCL19/CCR7-mediated epithelial-to-mesenchymal transition via ERK signaling pathway in epithelial ovarian carcinomas

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Abstract Recent studies have suggested that Crk-like adapter protein (CrkL) and epithelial-to-mesenchymal transition (EMT) induced by CCL19/CCR7 play an important role in ovarian epithelial carcinogenesis. However, the regulatory mechanisms of CrkL on the CCL19/CCR7 signaling pathways in epithelial ovarian carcinomas (EOC) are not well characterized. Here, CCR7 and CrkL proteins were tested in 30 EOC tissues and cell lines. In vitro, the roles of CrkL in CCL19-stimulated SKOV-3 cell invasion and migration were investigated. In this work, CCR7 and CrkL over-expressed in EOC tissues and cell lines and correlated with FIGO stage and lymph node metastasis. Moreover, CCR7 and CrkL serve as an independent prognostic factor. In SKOV-3 cells, CrkL knockdown markedly suppressed the CCL19-stimulated expression of p-ERK and EMT biomarkers (N-cadherin, Snail and MMP9), compared with control. In contrast, p-AKT expression level did not change. On the other hand, functional analysis revealed CrkL knockdown could significantly decrease SKOV-3 cell invasion number of transwell invasion assay, and wound closure area of wound healing assay, compared to control. In conclusion, CrkL regulates CCL19/CCR7-induced EMT via ERK signaling pathway in EOC patients, which further suggested CrkL could be suggested as an efficient target in ovarian cancer treatment.

Keywords CrkL · CCL19/CCR7 · EMT · EOC

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Introduction

Epithelial ovarian carcinoma accounts for the gynecological cancer death in women [1]. Surgery and platinum/taxane regimens are recommended as first-line therapy in recent years, but more than 3/4 of patients relapse into metastatic and essentially incurable chemoresistant condition [1, 2]. Pathologically, EOC is broadly classified as serous or non-serous histology. Each subtype has distinct clinical characteristics and is associated with different genetic risk factors and molecular events [3, 4]. Therefore, understanding of molecular mechanisms of EOC is crucial to the treatment of ovarian cancer.

CrkL is a member of the human Crk adapter protein family, which is comprised of two alternatively spliced isoforms of CRK, the human homologue of the avian sarcoma retroviral v-crk oncogene [5, 6]. CrkL is best known as a key substrate and effective downstream molecule of the BCR-ABL1 oncogenic tyrosine kinase in breast cancer [7] and bladder cancer [8]. CrkL contains the domain structure SH2–SH3–SH3 and has been reported to bind through its N-terminal SH3 domain with various signaling molecules such as Sos1 and C3G [9], two guanine nucleotide exchange factors for the Ras family of small GTP-

binding proteins. Recent studies have shown that CrkL mediates activation of the Ras family's small GTPases, Ras and Rap1, as well as the Rho family's [10–12].

Reportedly, EOC tumorigenesis become very dependent on CCR7 pathways [13], partly because CCL19/CCR7 deregulation activates proliferative signaling and promotes angiogenesis and metastasis [14, 15]. However, the regulatory mechanisms of CrkL on CCL19/CCR7 signaling pathways in EOC were not unraveled. In this work, our findings implicated that CrkL plays a regulatory role in the CCL19-induced EMT process, and the blockade of CrkL maybe benefit EOC patients.

Materials and methods

Cell culture and reagents

The human EOC cell lines H08910, A2780, SKOV-3, ES-2 and HEY were purchased from ATCC (American Type Culture Collection, Manassas, VA). All cells were maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5 % CO_2 at 37 °C. Recombinant human CCL19 was purchased from Sigma (St. Louis, USA). Antibodies were purchased from the same resources: anti-CCR7, anti-CrkL, anti-p-CrkL, anti-p-AKT, anti-p-ERK, anti-E-cadherin, anti-N-cadherin, anti-Snail and anti-MMP9 antibody (Santa Cruz Biotechnology, Santa Cruz, USA), and anti-β-actin antibody (Santa Cruz Biotechnology).

Tissue sections

Thirty paraffin-embedded EOC tissue sections were selected and underwent paraffin sections, which had been clinically and histopathologically diagnosed and were retrieved from pathology archives in Qilu Hospital of Shandong University, Affiliated Hospital of Shandong Academy of Medical Sciences. The mean age of patients at diagnosis was 50 ± 12. According to FIGO stage, all cases were divided into stage I (7), stage II (5), stage III (10) and stage IV (8). All tissue samples were obtained with the informed consent of the patient according to protocols and procedures approved. All patients were followed up regularly, with the mean follow-up period of 32 months, ranging from 3 to 80 months.

Immunohistochemistry

The slides were placed in 3 % hydrogen peroxide for 5 min to block endogenous peroxidase activity. Antigen retrieval was achieved using treatment in EDTA buffer at 99 °C for 30 min. After blocking with goat serum for 15 min, the sections were incubated in primary antibody overnight at 4 °C and washed twice in a PBS solution. The sections were then incubated in biotin-conjugated secondary antibody (Thermo Fisher Scientific Inc., USA) for 30 min and then in streptavidin peroxidase (Invitrogen) for 30 min. A DAB kit (Sigma Diagnostics, USA) was used for chromogen detection. The primary antibodies were replaced by rabbit serum as a control. The staining intensity in epithelial cells was evaluated on the following scale: 0 for a negative stain, 1 for weak positivity, 2 for median positivity and 3 for strong positivity. The area containing positive cells was scored as 0 to 100 %. Next, the score was calculated as the intensity of positivity multiplied by the positive area. When the score was <4, it was negative or low expression; positive or high expression when the score was equal to or more than 4.

CrkL siRNA transfection

Cells were plated in six-well plates. Twenty-four hours later, the cells were transfected with control siRNA or with CrkL siRNA (Santa Cruz, CA, USA) using siRNA transfection reagent (Santa Cruz Biotechnology, Inc.) according to the manufacturer's instructions.

Western blotting analysis

Total protein from cultured cells was extracted in cell lysis buffer (PIERCE, Rockford, IL) and quantified using the Bradford method. Fifty micrograms of protein were loaded and separated on SDS-PAGE (12 %). After transferring to a polyvinylidene fluoride membrane (Millipore, Billerica, MA), the membrane was incubated overnight at 48 °C with primary antibody. After incubation with peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology) at 37 °C for 2 h, bound proteins were visualized using ECL (Pierce) and detected using BioImaging Systems (UVP Inc., Upland, CA). The relative protein levels were calculated by normalizing to β-actin protein as a loading reference.

Transwell invasion assay

The chamber of a non-type I-coated 24-well culture insert (Millipore, Billerica, MA, USA) was used, and the upper chamber of the insert was coated with extracellular matrix gel (Sigma, St. Louis, MO, USA). The cells were seeded into the upper chamber with serum-free medium and the lower chamber with medium containing 10 % FBS. After 24 h, the cells migrating through the filter were dried for 10 min, fixed in absolute alcohol and stained with hematoxylin and eosin. Cells on the lower chamber were counted under a microscope. The experiment was repeated three times.

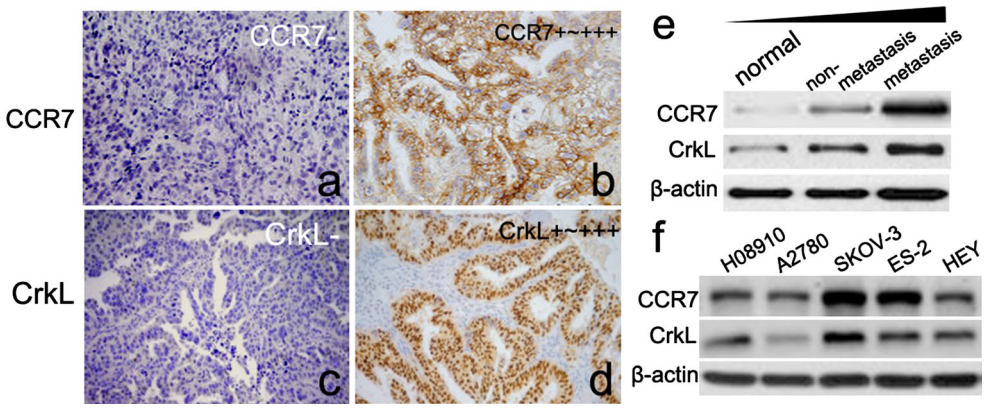


Fig. 1 Expression of CCR7 and CrkL protein in EOC sections and cell *lines*. Immunohistochemical staining and Western blotting were conducted in all 30 EOC sections and all cell *lines* using anti-CCR7 or anti-CrkL antibodies. Representative images of CCR7 (**a, b**) and CrkL (**c, d**) protein expression in paraffin-embedded tissues from 30 EOC patients (−, +, ++, +++). Western blotting analysis determined the relative protein expression levels of CCR7 and CrkL (**e, f**) in all cell *lines*, normal tissues and EOC cancer tissues with or without metastasis

Wound healing assay

For analysis of cell migration and proliferation, cells were seeded onto 10-cm-diameter plates in RPMI 1640 with 10 % FBS overnight. The injury line was created in confluent cells by cell scraping using a pipette tip and was washed with medium to remove free-floating cells and debris. Wound healing within the scrape line was recorded every day.

Statistical analysis

SPSS (version 13.0) was available for data analyses. Either a Student's *t* test or an ANOVA was utilized to compare the differences in the mean among groups when the data displayed approximately normal distribution and homogeneity in variance. The Spearman correlation was utilized to analyze the tendency between CCR7 and CrkL expression. The general association test was performed using either the Pearson Chi-squared test or Fisher's exact test for categorical data. The survival curves were estimated using the Kaplan–Meier method, and the comparison of the survival curves was performed using either the log-rank test or the Cox regression model. A p value <0.05 (two-sided test) was considered significant.

Results

Expression of CCR7 and CrkL in epithelial ovarian carcinomas tissues

To investigate the potential roles of CrkL in CCR7-induced EMT in EOC, we first explored the expression status of CCR7 and CrkL in EOC tissues and cell lines using the immunohistochemistry and Western blotting. As shown in Fig. 1, CCR7 protein was detected in 22 cases, of which weak staining was detected in 4 cases, moderate staining in 6 cases and strong staining in 12 cases. In normal human ovarian normal epithelial cells, CCR7 protein expression was very low and scarcely observed, and in ovarian cancer cells, CCR7 protein expression was mainly focused in the cytoplasm of ovarian cancer cells (Fig. 1b, d). Statistically, CCR7 expression was higher in carcinoma tissues than that in normal ovarian tissues based on raw data ($p < 0.05$). Interestingly, CrkL showed the same expression patterns with CCR7.

On the other hand, Western blotting assay indeed showed that both CCR7 and CrkL were highly expressed in EOC cell lines: H08910, A2780, SKOV-3, ES-2 and HEY (Fig. 1h). It should be noted that SKOV-3 cells showed the highest CCR7 and CrkL expression level. In the next study, we used SKOV-3 cells to carry out in vitro studies. What is more, Spearman's rank correlation analysis revealed that CCR7 expression was significantly associated with CrkL expression ($p < 0.01$).

Correlations between CCR7, CrkL expression and clinicopathological characteristics

The correlations of CCR7 expression in EOC with clinicopathological parameters were summarized in Table 1. Statistically, high CCR7 expression was not associated with age, histological type, grade and tumor diameter. Mucinous and endometrioid carcinomas did not exhibit significantly higher CCR7 expression levels than serous carcinomas ($p = 0.931$). The expression of CCR7 was remarkably lower in FIGO I + II stage than that in

Table 1 Correlations between CCR7, CrkL expression and clinicopathological characteristics in epithelial ovarian carcinomas

	N	CCR7		*p* value	CrkL		*p* value
		neg	pos		neg	pos	
Age							
≤50	16	5	11	0.544	5	11	0.544
>50	14	3	11		3	11	
Stage							
I + II	12	6	6	0.018	7	5	0.001
III + IV	18	2	16		1	17	
Grade							
G1	7	3	4	0.326	3	4	0.326
G2	9	3	6		3	6	
G3	14	2	12		2	12	
Type							
Serous	15	4	11	0.931	5	10	0.556
Mucinous	8	2	6		1	7	
Endometrioid	7	2	5		2	5	
Diameter							
≤2 cm	24	6	18	0.680	7	17	0.536
>2 cm	6	2	4		1	5	
Metastasis							
No	20	15	5	0.004	15	5	0.004
Yes	10	2	8		2	8	

III + IV stage ($p = 0.018$). Importantly, CCR7 expression showed a closely positive correlation with lymph node metastasis ($p = 0.004$). Similarly, CrkL expression also revealed that significant associations with the FIGO stage or lymph node metastasis ($p < 0.01$, Table 1). Additionally, Western blotting assay identified that CCR7 and CrkL expressions were obviously associated with lymph node metastasis (Fig. 1f).

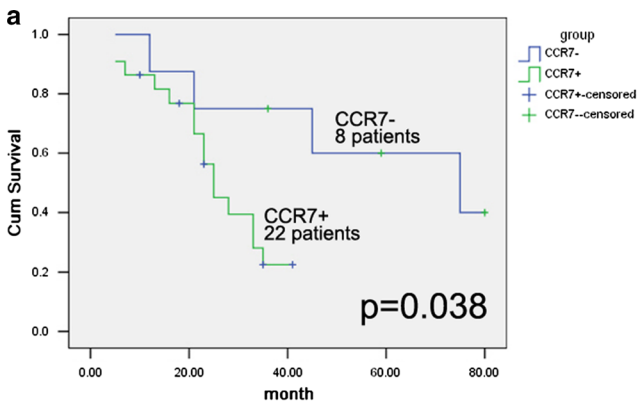
Both CCR7 and CrkL expressions serve as an independent prognostic factor

The potential covariables in the multivariate Cox regression model included age, FIGO stage, lymphatic metastasis, CCR7 and CrkL expression levels. In the 30 EOC tissue samples, using the Cox model, CCR7, CrkL expression levels, lymphatic metastasis and tumor stage were the important parameters for the overall survival (OS). The hazard ratio of the high CCR7 and CrkL expression group was 2.618 (95 % CI 1.422–4.820; $p = 0.002$) and 2.356 (95 % CI 1.414–3.925; $p = 0.001$), respectively, indicating that EOC patients with high CCR7 or CrkL expression are inclined to death, compared with those with low CCR7 or CrkL expression.

Kaplan–Meier curve for OS was analyzed in 22 patients with high CCR7 expression and 8 patients with low CCR7 expression. And then, the log-rank test was used to determine the difference between these two groups. Kaplan–Meier analysis revealed that patients with high CCR7 expression had significantly lower OS (31.8 %) compared with those with low CCR7 expression (50.0 %) ($p = 0.038$, Fig. 2a). Similarly, high CrkL expression also revealed poor five-year prognosis (36.4 vs. 62.5 %) ($p = 0.005$, Fig. 2b).

CCL19 activates CrkL signaling and EMT process in SKOV-3 cells

To investigate the possible roles of CCR7 signaling on the EMT process, we treated SKOV-3 with CCL19 (5, 10, 15, 20, 30 ng/ml) and conducted Western blot to detect the expression of p-CrkL, p-AKT, p-ERK and EMT biomarkers: E-cadherin, N-cadherin, Snail and MMP9. Our data showed that p-CrkL, p-AKT, p-ERK, N-cadherin, Snail and MMP9 were highly expressed in SKOV-3 cells in response



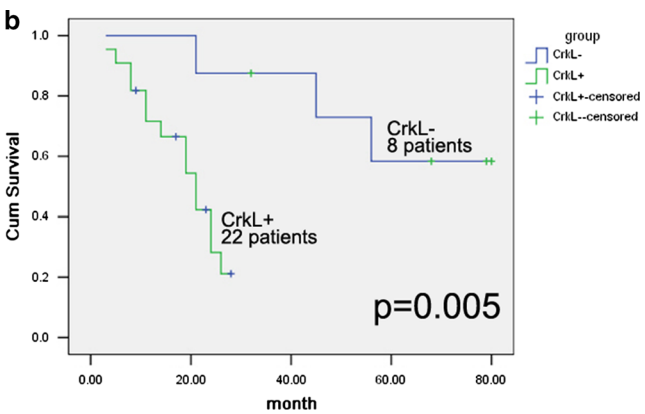


Fig. 2 The overall survival *curves* are shown for 30 EOC patients with different levels of CCR7 and CrkL expression. **a** Kaplan–Meier *curves* for overall survival in 22 patients with high CCR7 expression and 8 patients with low CCR7 expression. The difference between these two groups is determined by the log-rank test. **b** Kaplan–Meier *curves* for overall survival in 22 patient with high CrkL expression and 8 patients with low CrkL expression. High CCR7 and CrkL expression was obviously associated with 5-year prognosis

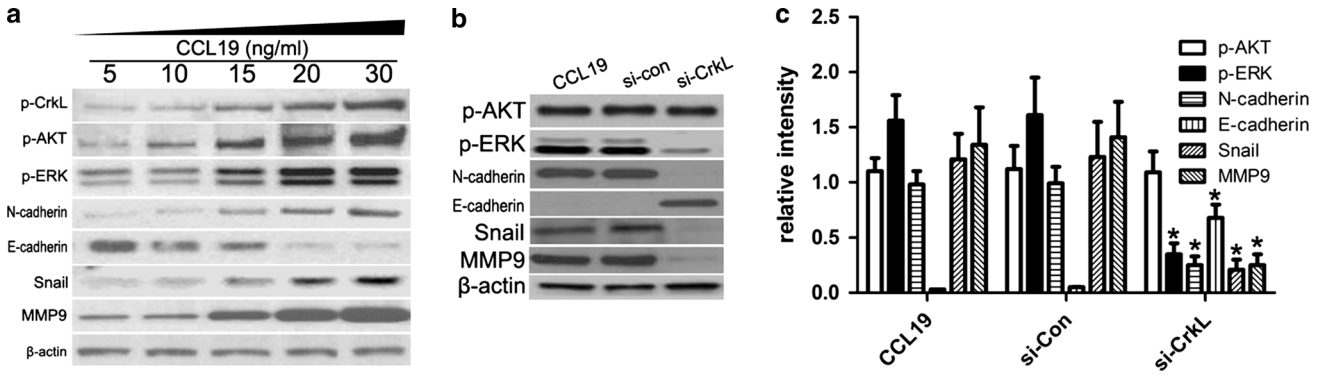


Fig. 3 The effects of CrkL protein on CCL19-stimulated signaling pathways and EMT. **a** Cells maintained in serum-free medium were treated with human recombinant CCL19 at different concentrations. After 30-min treatment, cells lysates were subjected to Western blotting. **b** Cells were transfected with si-control or si-CrkL. After 48 h, cells were subjected to 30 ng/ml of CCL19 treatment. And then, cells lysates were subjected to Western blotting. Cells transfected with CrkL siRNA obviously abrogated CCL19-stimulated signaling pathways and EMT biomarkers. Protein expression level was measured with Image J. β-actin was used as a normalization control. Each *bar* represents the mean ± SEM of three independent experiments; $*p < 0.001$, compared with si-control

to CCL19 stimulation in a dose-dependent manner. (Figure 3a). According to raw data, 30 ng/ml of CCL19 enhanced the higher p-CrkL, p-AKT, p-ERK, N-cadherin, Snail and MMP9 expression as much as possible. In addition, the results also showed that CCL19 stimulation decreased the expression of E-cadherin in a dose-dependent manner. These results indicated that CCL19 activated CrkL signaling and EMT process in SKOV-3 cells.

The blockade of CrkL diminishes CCL19-stimulated ERK signaling and EMT

In order to knockdown endogenous CrkL protein, we used CrkL siRNA to inhibit the expression of CrkL. To elucidate the effects of silencing of CrkL on CCL19-stimulated CrkL signaling and EMT, we conducted Western blotting to detect CCL19 pathway signaling molecules and EMT biomarkers as mentioned above. As shown in Fig. 3b, we observed that p-AKT, p-ERK, N-cadherin, Snail and MMP9 were up-regulated in SKOV-3 with non-transfection. However, when SKOV-3 was transfected with si-CrkL, the expression of p-ERK, N-cadherin, Snail and MMP9 was decreased in comparison with non-transfection. However, p-AKT expression level was not affected. Next, we investigated the effects of CrkL on E-cadherin expression and observed E-cadherin protein expression was potently enhanced in comparison with non-transfection. These indicated that CrkL acts as a key mediator in CCL19-stimulated ERK signaling and EMT process.

Knockdown of CrkL attenuates CCL19-induced SKOV-3 cell invasion and migration

Next, we carried out transwell invasion and wound healing assay to figure out the effects of the knockdown of CrkL on the invasion and migration of SKOV-3 cells. In the present study, transwell invasion assay demonstrated that the knockdown of CrkL indeed abrogated the SKOV-3 cell invasion number (132 ± 55) in comparison with non-transfection, indicating CrkL dominated the invasion capacity of SKOV-3 cells (Fig. 4a). Alternatively, wound closure of SKOV-3 cells with si-CrkL was remarkably affected ($p < 0.001$). As shown in Fig. 4b, our data showed that the wound closure area of SKOV-3 cells with si-CrkL was decreased by 48 %. These results indicated that knockdown of CrkL attenuated CCL19-induced SKOV-3 cell invasion and migration of SKOV-3 cells.

Discussions

The Crk family of adaptor proteins includes CrkI, CrkII, CrkL and v-Crk, each of which contains predominantly Src homology 2 (SH2) and SH3 domains [16]. It is reported that identification of regulatory pathways of CrkL will promote the rational drugs design for advanced malignancies. In the present study, we have attempted to unravel how CrkL activates CCR7-induced EMT pathway. Firstly, high CCR7 and CrkL expressions were identified in EOC tissues and all cell lines, including H08910, A2780, SKOV-3, ES-2 and HEY. Importantly, IHC and Western blotting indeed validated that CCR7 and CrkL were associated with FIGO stage or lymph node metastasis of EOC patients, and high CCR7 expression was also associated with high CrkL expression. In addition, Kaplan–Meier analysis revealed that patients with high CCR7/CrkL expression had significantly lower survival rate compared with those with low CCR7/CrkL expression, and at the same time, the multivariate Cox regression model demonstrated that CCR7/CrkL expression serves as an

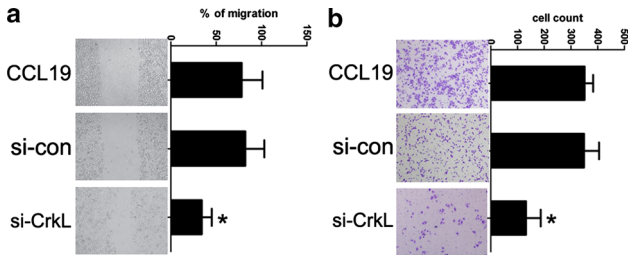


Fig. 4 The effects of CrkL on the invasion and migration of SKOV-3 cells. **a** Wound healing within the scrape *line* was recorded every day. Representative scrape lines are shown at day 2; *dashed line* indicates the margin of the scratch at 24 h. The average initial wound width was measured and considered 100 %. Compared with CCL19 and si-control, wound closure was significantly affected in cells transfected with si-CrkL at 48 h following creation of wounds in confluent cell monolayers. **b** Transwell invasion assay showed that the blockade of CrkL inhibited the invasion number of SKOV-3 cells. All experiments were performed in triplicate. Data were expressed as the mean ± SEM. $*p < 0.001$, versus si-control or CCL19 treatment

independent prognostic factor for EOC patients. Consistent with our data, some reports demonstrated the chemical receptor CCR7 and CrkL highly expressed in esophageal squamous cell carcinoma [17], squamous cell carcinoma of head and neck [18] and lung cancer [19] and correlated with metastasis. Based on all mentioned above, our team assumed that CrkL plays a potential role in the CCL19/CCR7 signaling pathway in EOC development.

Previously, we found CCR7 can constitutively express in epithelial ovarian carcinomas (EOC) and be induced rapidly in response to hypoxia, which indeed participates in EMT development and prompts the cell migration and invasion [20]. In the present study, we knocked down the CrkL protein in SKOV-3 cells to observe the effects of CrkL on CCR7-induced EMT. Of particular interest, we found that CCL19 stimulation activates p-CrkL expression and initiates the CCR7 signaling pathway to induce EMT process. Conversely, when SKOV-3 was transfected with si-CrkL, the expression of p-ERK, N-cadherin, Snail and MMP9 was affected, and on the other hand, the expression of E-cadherin protein was potently enhanced in comparison with non-transfection. Besides, CrkL is also reported to regulate the mitogenic ERK-dependent pathways in transformed cells [21]. These findings suggested that CrkL mediates the signaling transduction of CCR7 pathway and acts as a key component in regulating ERK signaling, further leading to the regulation of EMT development.

Mechanical analysis was conducted to test the biological impact of CrkL on EOC cancer cells. In comparison with non-transfection, transwell invasion assay demonstrated that the knockdown of CrkL indeed abrogated the SKOV-3 cell invasion number, indicating CrkL dominated the invasion capacity of SKOV-3 cells. At the same time, wound closure capacity of SKOV-3 cells transfected with si-CrkL was obviously abrogated. Both assays indicated that the blockade of CrkL attenuated CCL19-induced invasion and migration of SKOV-3 cells, and these results were consistent with our IHC conclusion.

In cancer cells, the EMT process may promote their metastatic potential, during which transcription factors, including Snail and TWIST, have been demonstrated to be master regulators [22]; meanwhile, these proteins are transcriptional repressors of E-cadherin and promoters of MMP9. E-cadherin is one of the most important adhesion molecules and is essential for the maintenance of intact cellular functionality. The deregulation of E-cadherin and up-regulation of MMP9 have been also identified as the most important events in tumorigenesis [23, 24]. Published reports had demonstrated that CCR7/JAK2/STAT3 regulating SCCHN metastasis can also occur via E-cadherin. In recent years, various molecules, such as growth factors and cytokines, have been revealed to drive the progress of cancer cells migration, invasion and metastasis [25]. Thus, we provided more key pathways involved in EMT process, highlighting the target value of EMT signaling molecules, such as CrkL molecules.

In conclusion, our results suggest CCR7 and CrkL expressions were altered in EOC development. CrkL regulates CCL19/CCR7-induced EMT via ERK signaling pathway in EOC, which can mediate the development of EOC. This study also suggested the dysfunction of CrkL may be indicative of a key factor of human cancer, and targeting of CrkL will benefit more EOC patients.

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Conflict of interest The authors indicated no potential conflicts of interest.

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