CCL20 and CXCL8 synergize to promote progression and poor survival outcome in patients with colorectal cancer by collaborative induction of the epithelial–mesenchymal transition

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CCL20
CXCL8/interleukin-8
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Prognosis

Abstract
Liver metastases represent the major cause of death in patients with colorectal cancer (CRC). Recent studies have suggested that the chemotactic responses of tumor cells are necessary for metastatic spread to the liver, and CCL20 and CXCL8 have a strong association with CRC metastasis. The aim of our study was to identify the mechanisms by which CCL20 and CXCL8 synergize to promote metastatic progression and evaluated their potential as prognostic markers for CRC patients. The abilities of CCL20 and CXCL8 to promote CRC cell progression and epithelial–mesenchymal transition (EMT) phenotype were analyzed in vitro. Possible signaling pathways were investigated with specific pathway inhibitors and small interfering RNA (siRNA). 213 Patients with CRC who underwent surgery were enrolled for analysis of CCL20, CXCL8 and E-cadherin expressions in tumor tissues. Prognostic factors were then identified. CCL20 or CXCL8 alone was not sufficient to induce complete EMT in CRC cells, but both of them could coordinately induce EMT-like phenotype that was required to maintain CRC cell proliferation, migration and invasion. PI3K/AKT-ERK1/2 pathway crosstalk was demonstrated to be responsible for this process. Coexpression of CCL20 and CXCL8 was negatively correlated with E-cadherin expression in human CRC tissues. CRC patients with coexpression of CCL20 and CXCL8 were more likely to develop liver metastases and both coexpression was an independent high-risk factor for a most poor prognosis. CCL20 and CXCL8 synergize to promote CRC metastatic progression by coordinated induction of EMT via PI3K/AKT-ERK1/2 signaling axis. Detection of both coexpressions can be used to predict clinical outcomes in CRC patients.

Introduction
Colorectal cancer (CRC) is the third most frequent cancer worldwide and the second leading cause of cancer-related death in the developed countries [1]. Approximately 50% of CRC patients die from distant metastases within 6 years of the initial diagnosis, and the liver is the predominant and often the only organ for distant metastasis [2]. Recent studies in CRC have suggested that the chemotactic responses of tumor cells are necessary for metastatic spread to the liver, and chemokines as well as their receptors, in particular CCL20/CCR6 and CXCL8/CXCR1/2, play an important role in the metastatic process. CCL20 is the only chemokine known to interact with the chemokine receptor CCR6 [3–5], and expressed mainly in mucosa-associated and lymphoid tissues and the liver [5]. CCL20 and CCR6 are expressed predominantly

Abbreviations: AJCC, American Joint Committee; C, cysteine; CEA, carcinoembryonic antigen; CRC, colorectal cancer; E-cad, E-cadherin; EMT, epithelial–mesenchymal transition; FBS, phosphate-buffered saline; HCC, hepatocellular carcinoma; ICC, immunocytochemistry; IHC, immunohistochemistry; IRS, immunoreactive score; NCCN, National Comprehensive Cancer Network; PI3K, phosphatidylinositol 3-kinase; SD, standard deviation; TNM, tumor-node-metastasis; Vim, vimentin.

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in the microenvironment of CRC tumors [6–10]. A strong association has been found between CCL20/CXCR6 staining intensity in CRC samples and the development of synchronous liver metastases [6–8]. Stimulation of colon cancer cells by CCL20 has been shown to result in signaling events associated with cell proliferation, adhesion and migration [11]. In a murine model, stimulation of CCR6-expressing plasmacytoma cells with CCL20 before intravenous application resulted in a marked increase in their homing to the liver [12]. CXCL8, also referred to as interleukin-8 (IL-8), stimulated the migratory capacity of a distinct set of leukocytes [13]. Increased expression of CXCL8 has been detected in the tumor microenvironment and blood of CRC patients [14–22]. Overexpression of CXCL8 promotes tumor growth, progression, and metastasis [16–19], and is associated with tumor size, tumor grading, depth of infiltration, liver metastasis and survival in CRC [15–19,22].

To date, however, the precise mechanisms by which these chemokines determine tumor cell detachment and migration away from the primary tumor tissues remain to be established. Over the past decade, accumulating evidence has shown that the epithelial–mesenchymal transition (EMT) is a pathological process contributing to cancer progression, particularly to cancer invasion, dissemination, and metastasis [23]. While epithelial cells undergo EMT, loss of the epithelial marker E-cadherin (E-cad) and concomitant expression of distinct mesenchymal markers like vimentin (Vim) and beta-catenin play a vital role in this reversible transdifferentiation. Therefore, the EMT provides a mechanism for carcinoma cells to acquire a more aggressive phenotype [23].

Several chemokines for induction of EMT in epithelial tumor cells has been documented, such as CXCL12 for oral squamous cell carcinoma cells B88 and HN5 [24], CXCL16 for CRC cell HT29 [25], and CCL18 or CXCL8 for breast cancer cells [26,27], and CXCL8 for nasopharyngeal carcinoma cells [28]. However, CCL20 or CXCL8-induced EMT in CRC cells has not been reported. In the present study, we demonstrated for the first time that CCL20 or CXCL8 alone was not sufficient to induce complete EMT in human CRC cells, but both of them could jointly induce EMT-like phenotype that was required to maintain CRC cell proliferation, migration and invasion. We further found that the phosphatidylinositol 3-kinase (PI3K)/AKT-ERK1/2 pathway crosstalk mediated by CCL20 and CXCL8 appeared to be responsible for the EMT in the CRC cells. Furthermore, clinical evidence showed that there was a strong association of the coexpression of CCL20 and CXCL8 with the loss of E-cadherin expression in CRC tissues. Patients with CRC who had coexpression of CCL20 and CXCL8 were more likely to develop liver metastases and had a most poor prognosis. The present data indicate that CCL20 and CXCL8 synergize to promote CRC progression and metastasis by collaborative induction of EMT via PI3K/AKT-ERK1/2 crosstalk, therefore, detection of both coexpression can be used to predict patient outcomes, and targeting both CCL20 and CXCL8 or the PI3K/AKT pathway could be highly valuable for the therapy of recurrent or metastatic CRC.

Materials and methods

Patients and sample collection

Two hundred thirteen patients with CRC at the Colorectal Cancer Clinical Research Center, Third Affiliated Hospital, Kunming Medical University (Kunming, China) between February 2003 and May 2005 were enrolled onto this study. The median age of patients at the time of admission was 62 years, range 21–79 years. All the patients underwent radical resection of the primary tumor with regional lymph node dissection. All diagnoses of adenocarcinoma were confirmed histopathologically, and the paraffin blocks of all primary tumor specimens were available for analysis. Only patients with sporadic colorectal cancer were selected for our analysis, and patients with a positive medical history for hereditary non-polyposis colorectal cancer or familial adenomatous polyposis were excluded. No patients received preoperative chemotherapy. The patient demographics and clinicopathological factors were obtained from the patients’ medical and pathological records, which are summarized in Table 1. Primary tumor stage was defined according to the sixth edition of American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) staging system.

Patient follow-up consisted of physical examination, assessment of serum carcinoembryonic antigen (CEA) levels, and thoracoabdominal computed tomographic scan every 6 months for the first 5 years, then annually thereafter. The median duration of follow up was 61 months (range 3–105 months). Patients who developed recurrence were treated according to National Comprehensive Cancer Network (NCCN) guidelines.

Cell culture and treatment

Human CRC cell lines SW480 and Caco-2 (American Type Tissue Culture Collection) were maintained in RPMI 1640 and in DMEM (Invitrogen), respectively, supplemented with 10% FBS (Invitrogen), 100 IU/mL penicillin and 100 mg/mL streptomycin in a 5% CO2 incubator at 37 °C. For analysis of cell viability, adherent tumor cells were cultured in the presence of CCL20, CXCL8 (R&D Systems), AKT inhibitor MK2206 (Selleck Chemicals), sorafenib (Bayer Pharmaceuticals), or their combination. Partial media changes (50%) supplementing fresh chemokines were performed every other day.

Immunocytochemistry (ICC)

Cells were grown at the indicated concentrations of chemokines to confluence on coverslips in 6-well dishes. After washed twice, cells were fixed with 2% (v/v) paraformaldehyde and permeabilized with 1% (v/v) Triton X-100. Coverslips were blocked with 10% (w/v) normal goat serum in phosphate-buffered saline (PBS) at room temperature for 1 h and then incubated in primary antibody or isotype control immunoglobulins at 4 °C overnight. For immunofluorescence, cells were washed and incubated with Cy3-labeled secondary antibody (Beyotime) at room temperature for 1 h, and contained with DAPI (Sigma) to visualize nuclei. Images were obtained using a fluorescence microscope at 200× magnification. For non-fluorescent ICC staining, the ABC method and DAB substrate (Maixin-Bio) were used as described below.

Immunohistochemistry (IHC)

All specimens had been fixed in buffered formalin and embedded in paraffin. The embedded tissues were cut into 4 μm-thick serial sections for histological analyses by H&E staining, IHC to detect CCL8, CXCL12, E-cadherin and Vimentin (Abcam). The optimal antibody concentration was determined for each assay using a titration experiment, giving the best staining with minimum background/ non specific binding. Negative controls were performed by replacing the primary antibody with mouse or rabbit isotype control antibody. Counter-staining of the nucleus was performed using hematoxylin. Immunoreactive score (IRS, range 0–12) proposed by Remmele and Stegner was applied to immunoreactive evaluation [29]. A score of >4–6 was considered positive. The assessment of the staining was conducted by two investigators independently.

Western blot analysis

Total cellular proteins (30 μg/lane) prepared from cultured cells were electrophoresed on sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Blots were blocked in 5% skim milk and immunostained sequentially with primary and secondary horseradish peroxidase conjugated secondary antibodies at 4 °C overnight and at room temperature for 1 h, respectively. Primary antibodies were as follows: mouse anti-human E-cadherin, mouse anti-human vimentin, mouse anti-human beta-catenin (Abcam), rabbit anti-human p-AKT (Ser473), rabbit anti-human AKT (CST677), rabbit anti-human p-ERK1/2 (Thr202/Tyr204), and rabbit anti-human ERK1/2 (137F5) (Cell Signaling Technology). Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system with exposure of X-ray film. The images were analyzed by Quantity One (Bio-Rad).

Sensing of AKT and ERK1/2 with siRNA

siRNAs (AKT siRNA, ERK1/2 siRNA and scrambled control siRNA) (Cell Signaling Technology) and lipofectamine 2000 (Invitrogen) were separately diluted according manufacturer’s protocol, mixed gently, and incubated for 20 min at room temperature. The siRNA-lipofectamine 2000 mixture was added to CRC cells grown in 6-well plates to 80–90% confluence. The transfection reagent was replaced after 6 h, and cells were then treated with chemokines as described above. Scrambled control siRNA was used as blank control.

Statistical analyses

Comparison of cell proliferative rate and migratory cell number with different treatments were performed using Student’s t test and a one-way analysis of variance (ANOVA). All experiments for cell cultures were performed independently at
least three times and in triplicate for each time. Data are expressed as median ± standard deviation (SD). Relationships (potential associations) between chemokine expression and EMT marker expression as well as correlations between chemokine expression and the clinicopathological characteristics were evaluated using the chi-square test. Patient survival was calculated using the Kaplan–Meier method. Variables with a P value of <0.2 in the univariate analyses were further tested using multivariate Cox proportional hazards model with a stepwise procedure to evaluate prognostic factors for survival. SPSS software (version 12.0 for Windows; SPSS Inc., Chicago, IL) was used for all statistical analyses. A P value <0.05 was considered statistically significant.

Results

Both CCL20 and CXCL8 coordinately induced EMT-like phenotype in colon cancer cells

CCL20 or CXCL8 alone at a concentration of 100 ng/mL for a period of 48 h was not enough to induce an EMT-like phenotype in SW480 cells, but after cotreatment with both chemokines at the same concentration, most cells not only changed to a spindle-shaped, elongated morphology, but also exhibited a loose association and occasional misorientation compared to the closely contacted monolayer, polygon cobblestone-like cells (Fig. 1A). Concomitant expression of epithelial and mesenchymal markers is often used to identify cells that are undergoing EMT [23]. The results of immunofluorescence staining and Western blot showed that there was unchanged expression of E-cadherin but increased expression of vimentin and beta-catenin in SW480 cells on CXCL8 alone stimulation for 48 h, but not on CCL20 alone stimulation (Figs. 1B and 2B; *P < 0.01). However, decreased expression of E-cadherin and increased expression of vimentin and/or beta-catenin were obvious in both chemokine-costimulated cells (Figs. 1B and 2B; *P < 0.01), which were in accordance with the observed morphological changes. This evidence makes it reasonable to suggest that both CCL20 and CXCL8 coordinately induced EMT-like phenotype in CRC cells. The effect of CCL20 and/or CXCL8 on biological behavior of CRC cells (SW480 and Caco-2) were examined in parallel experiments, and the results showed that CCL20 or CXCL8 alone could promote CRC cell proliferation, migration, and invasion in vitro, but the combination of both had a stronger effect (Supplementary Fig. S1).

CCL20 and CXCL8 combine to strengthen activation of both PI3K/AKT and MEK/ERK1/2 pathways

PI3K/AKT and Raf/MEK/ERK1/2 signaling pathways have been reported to be hyperactivated in many models of EMT [30–33]. In our study, SW480 cells displayed a slight upregulation of both p-AKT and p-ERK1/2 expression compared to normal human colon cells FHC (Fig. 2A). To examine the involvement of CCL20 or CXCL8 in the two pathways, SW480 cells were treated with or without CCL20 or CXCL8 for 48 h before their p-AKT and p-ERK1/2 levels were analyzed. As indicated in Fig. 2B, CCL20 or CXCL8 alone could result in simultaneous upregulation of p-AKT and p-ERK1/2 expressions but unchanged expressions of T-AKT and T-ERK1/2, and cotreatment appeared to have the same effect (Fig. 2B; *P < 0.01). In contrast, a much stronger phosphorylation of AKT and ERK1/2 was observed after costimulation, the expressed protein bands increasing more than 1.60–1.73-fold in relative amount compared to a single chemokine stimulation (Fig. 2B; **P < 0.05).

Table 1
Correlation of CCL20 or CXCL8 expressions in CRC tissues with clinicopathological status in 213 CRC patients.

<table>
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<tr>
<th>Items</th>
<th>N</th>
<th>CCL20 Positive (n = 136)</th>
<th>CCL20 Negative (n = 77)</th>
<th>P</th>
<th>CXCL8 Positive (n = 119)</th>
<th>CXCL8 Negative (n = 94)</th>
<th>P</th>
<th>CCL20 and CXCL8 Both positive (n = 79)</th>
<th>CCL20 and CXCL8 Others (n = 134)</th>
<th>P</th>
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use with several types of cancer [34]. Sorafenib, a multiple kinase inhibitor, is the first and only drug that is clinically approved for patients with advanced hepatocellular carcinoma (HCC) [35]. The major target of sorafenib is the serine/threonine kinase Raf-1, which is involved in the Raf/MEK/ERK1/2 signaling cascade. In our study, treatment of the cells with 1 μM MK2206 and 2 μM sorafenib for 48 h led to effective blockade of the activation of AKT and ERK1/2 in SW480 cells, respectively (Fig. 2A). To test the involvement of the two pathways in EMT induced by both CCL20 and CXCL8, an alteration in the expression of epithelial and mesenchymal marker was examined in MK2206 or sorafenib-treated SW480 cells. Interestingly, after cells were pretreated with sorafenib for 1 h and then coincubated with both chemokines for 48 h, the expressions of E-cadherin, vimentin and beta-catenin maintained changed, relative to levels in parallel experiments without sorafenib treatment. However, when sorafenib was replaced by MK2206, their expressions remained unchanged, indistinguishable from those without both chemokine treatments (Fig. 2B). We then analyzed the reason why MK2206 could, but not sorafenib, prevent the both chemokine-induced EMT process, and rather surprisingly found that in the presence of both chemokines, sorafenib showed no inhibitory effect on ERK1/2 and AKT kinase activity, whereas MK2206 not only reduced p-AKT expression, but also simultaneously inhibited ERK1/2 activity (Fig. 2B). AKT inhibitor abrogated ERK1/2 activation and its associated EMT process in response to both chemokines, suggesting that ERK1/2 activation mediated by both chemokines is dependent on AKT in CRC cells, and a signaling crosstalk relays between these two pathways.

To further confirm the involvement of PI3K/AKT-ERK1/2 pathway crosstalk, we tested the two signaling pathways using AKT-specific siRNA and ERK1/2-specific siRNA. The efficacy of silencing AKT and ERK1/2 was determined by Western blot analysis, and was evident from 48 to 96 h after transfection and reached 70–80% reduction (Fig. 3A). As expected, the simultaneous expression of AKT siRNA significantly inhibited both chemokine-induced AKT and ERK1/2 activities compared with cells transfected with non-specific siRNA (Fig. 3B). Accordingly, AKT- or ERK1/2-specific siRNA did not change the expression of EMT markers in both chemokine-mediated cells (Fig. 3B), confirming that the collaborative effects of both chemokines on EMT induction were AKT-specific in CRC cells. In addition to the above, treatment of both chemokine-exposed cells with ERK1/2 siRNA suppressed limited ERK1/2 activity, but had no effect on AKT activity (Fig. 3B). Parallel experiments with Caco-2 cells revealed changes in kinase activities similar to those in SW480 (data not shown). Taken together with the findings from our experiments treating CRC cells with MK2206 and sorafenib, our results suggest that PI3K/AKT could transduce a signal to the ERK1/2 pathway in both chemokine-stimulated CRC cells but not vice versa.

**Simultaneous interruption of the PI3K/AKT and Raf/MEK/ERK1/2 pathways significantly inhibited the proliferation, migration, and invasion of both chemokine-stimulated CRC cells in vitro**

To validate the potential role of the PI3K/AKT and Raf/MEK/ERK1/2 pathways on the cell proliferation, we then investigated the effect of MK2206 and sorafenib in killing both chemokine-treated CRC cells. Our data showed that treatment of the cells with 1 μM MK2206 or 2 μM sorafenib led to effective inhibition of the proliferation and migration of SW480 cells (Fig. 4A and B), however, treatment with 1 μM MK2206 for 48 h led to more effective inhibition of the proliferation of both CCL20 and CXCL8-stimulated SW480 cells than did treatment with 2 μM sorafenib, and cotreatment with MK2206 and sorafenib did not significantly decrease growth of both CCL20 and CXCL8-stimulated cells compared to treatment with MK2206 alone (Fig. 4A). These results were coincident with the findings described above that PI3K/AKT-ERK1/2 crosstalk is implicated in both CCL20 and CXCL8-induced proliferation. Similar inhibitory effects on cell migration were observed in parallel cell migration assays performed with MK2206 and sorafenib (Fig. 4B).

**Concomitant expression of CCL20 and CXCL8 is associated with changes of EMT markers in human CRC specimens**

To find distinctive CCL20 and CXCL8 expression patterns, CCL20 and CXCL8 expressions were determined on 213 human CRC tissue samples by immunohistochemistry (IHC) staining. The results revealed that CCL20 and CXCL8 were located in the cytoplasm and apparently excluded from the nucleus [Supplementary Fig. S2A]; 63.9% (136/213) and 55.9% (119/213) of the CRC specimens were positively stained with CCL20 and CXCL8 antibodies, and the corresponding values for their nontumor counterparts were 12.7% (27/213) and 18.8% (40/213), respectively ($P < 0.001$ and $P < 0.001$, respectively). Furthermore, positive double-staining for CCL20 and CXCL8 was found in 37.1% (79/213) of the CRC specimens, whereas the corresponding values for their nontumor counterparts was 5.2% (11/213) ($P < 0.001$). These results indicate that CCL20 and CXCL8 expressions in the cancerous tissues of CRC patients are significantly higher than those in adjacent normal tissues, and both chemokines are coexpressed in a subset of CRC tissues.

Microscopic evaluation of IHC staining revealed membranous expression of E-cadherin [Supplementary Fig. S2B]. E-cadherin staining was mostly found in cancer cells where vimentin expression was almost undetectable. In contrast, 55.4% (118/213) of the cancerous tissues of CRC patients were positive for E-cadherin. The correlations of CCL20 and CXCL8 expression with E-cadherin expression are detailed in Table 2. The results showed that either CCL20 expression or CXCL8 expression had no statistical correlation with E-cadherin expression ($P = 0.702$; $P = 0.419$), but coexpressions of CCL20 and CXCL8 had a high negative correlation with the loss of E-cadherin expression ($P = 0.001$).

**Correlations of CCL20 or CXCL8 or both expressions in CRC with clinicopathological factors**

The clinicopathological variables between different CRC subtypes with different expression of CCL20 or CXCL8 or both were compared (Table 1). The chi-square test showed that the positive expression rate of CCL20 was significantly higher in patients with tumor size 5 cm, with stage T3 or T4, and with liver metastasis than in the corresponding subtypes; the positive expression rate of CXCL8 was significantly higher in patients with stage T3 or T4, lymph node metastasis and liver metastasis; coexpression of CCL20 and CXCL8 was significantly correlated with advanced T staging (deeper wall infiltration), lymph node metastasis, and further development of liver metastasis.

**CRC subtypes with CCL20 and CXCL8 concomitant expression had a very poor prognosis**

To investigate the clinical outcomes of each CRC subtypes identified above, we carried out Kaplan–Meier survival analysis of CRC cases. As shown in Fig. 5, CCL20 or CXCL8 expression was associated with poor overall survival ($log rank = 14.31$, $P < 0.001$; log rank = 13.89, $P < 0.001$) and disease-free survival ($log rank = 13.84$, $P < 0.001$; log rank = 13.96, $P < 0.001$) in CRC patients, and importantly, CRC subtypes with coexpression of both chemokines had significantly shorter overall survival, disease-free survival than did CRC subtypes with CCL20 expression alone ($log rank = 10.21$, $P = 0.001$; log rank = 10.24, $P = 0.001$; respectively) or CXCL8...
expression alone (log rank = 9.02, \(P = 0.003\); log rank = 9.22, \(P = 0.002\); respectively).

Multivariate analysis identified four factors (CCL20 and CXCL8 coexpression, CEA, lymph node metastasis and liver metastasis) significantly influencing the overall survival rate and disease-free survival rate (Table 3). Coexpression of both CCL20 and CXCL8 was confirmed to be an independent factor for overall and disease-free survival.

Fig. 1. Costimulation with CCL20 and CXCL8 induced EMT-like phenotype in colorectal cancer cells. (A) The morphology changes were observed in SW480 cells under invert microscope (200\(\times\)). No changes in cell morphology was occurred after treatment with CCL20 or CXCL8 at the concentration of 100 ng/ml for 48 h, but most cells exhibited an elongated, spindle-shape mesenchymal morphology and a loose association and occasional misorientation after costimulation. (B) EMT-like cell morphological changes were accompanied by the altered expression of epithelial and mesenchymal markers (200\(\times\)). E-cadherin and vimentin were detected by immunofluorescence. Morphological changes associated with loss of E-cadherin and acquisition of vimentin in SW480 cells were observed after costimulation. Exposure to CXCL8 resulted also in higher expression of vimentin but unchanges in cell morphology and E-cadherin expression.
Chemokines have been demonstrated to be involved in the pathogenesis and metastasis of tumors [36]. In CRC, the tumor cells selectively upregulate expression of some chemokines and chemokine receptors, providing a basis for efficient autocrine and paracrine loops [37,38]. Multiple clinical studies implicate chemokines in the development, progression, and metastasis of CRC [39]. However, almost all of these studies focused respectively on a single chemokine involvement and did not address the precise mechanisms underlying the promoting effects of these chemokines on CRC cell invasion and metastasis. In the present study, we found that CCL20 and CXCL8 costimulation had a stronger effect (Supplementary Fig. S1). Interestingly, CXCL8 alone, but not CCL20 alone, could induce increased expression of mesenchymal markers vimentin and beta-catenin but without being accompanied by changes in the expression of E-cadherin and cell morphology in CRC cells (Fig. 1). Klymkowsky and Savagner referred to this dual nature as the “metastable phenotype” [40], which has also been called “partial EMT” [41–43]. Cells undergoing “partial EMT” are in the midst of EMT, sometimes described as “transitioning cells” [44]. Noticeably, CCL20 and CXCL8 coexposure to CRC cells could be sufficient to induce a complete EMT phenotype, concomitant changes in typical EMT-like morphology, and expression of epithelial and mesenchymal markers (Fig. 1), which enhanced cell proliferation, motility, and invasion in parallel (Supplementary Fig. S1). These results extend the role of chemokines in CRC to the context of chemokine-mediated EMT. Recently, several chemokines for induction of EMT in epithelial tumor cells has been documented, such as CXCL12 for oral squamous cell carcinoma cells B88 and HNT [24], CXCL16 for CRC cell HT29 [25], and CCL18 or CXCL8 for breast cancer cells [26,27], and CXCL8 for nasopharyngeal carcinoma cells [28]. Our research not only provide another example of chemokine-mediated EMT in cancer cells, but also defined a novel mode of action of multiple chemokines jointly induced-EMT. Such an action mode may be probably common in a variety of tumor types, and may have a significant role in tumor progression and metastasis.

A critical molecular feature of EMT is the downregulation of E-cadherin and upregulation of mesenchymal markers such as vimentin and beta-catenin [45]. Beta-catenin is also a chief protein in AKT/beta-catenin or Wnt/beta-catenin signaling pathway [46,47]. A variety of signal transduction pathways impinge on the regulation of E-cadherin levels or subcellular distribution. The oncogenic serine/threonine kinase AKT (also known as PKB), a downstream effector of the PI3K, has been shown to repress transcription of the E-cadherin gene, which initiates cellular responses leading to the conversion of epithelial cells into invasive mesenchymal cells [48]. Moreover, the PI3K/AKT pathway has been shown to be activated by a variety of chemokines, consequently stimulating cell migration and invasion in various human cancers. Chen et al. found that PI3K/AKT/mTOR pathway played an important role in CXCL12/CXCR4-mediated cell migration in human gastric carcinoma cells [49]. Wang et al. showed that CXCL12 induced downregulation of E-cadherin/beta-catenin complex expression via phosphorylation of PI3K/AKT, promoting cell migration in HT29 colon cancer cells [50]. Huang et al. reported that CCL5 acted through PI3K/AKT, resulting in the activation of alpha v beta 3 integrin and contributing to the migration of human lung cancer cells [51]. In the current study, the results were consistent with findings of previous studies that PI3K/AKT and Raf/MEK/ERK1/2 pathways contributed to CCL20- or CXCL8-mediated proliferation, migration and invasion in CRC cells or other types of cancer cells [13,52,53], and importantly, our results indicated that CCL20 and CXCL8 combine to strengthen activation of both PI3K/AKT and Raf/MEK/ERK1/2 pathways, which may responsible for the process of EMT in CRC cells. (A) Western blot was performed for p-AKT/AKT and p-ERK1/2/ERK1/2 in normal human colon cells FHC and CRC cells SW480, the latter cells were treated with MK2206 or sorafenib. SW480 cells displayed a slight upregulation of p-AKT and p-ERK1/2 compared to FHC cells. Treatment of the cells with 1 μM MK2206 or 2 μM sorafenib led to effective blockade of the activation of AKT and ERK1/2. (B) Western blot analysis of p-AKT/AKT, p-ERK1/2/ERK1/2, and EMT markers in SW480 cells that were pretreated with or without inhibitors before stimulated with chemokines. CCL20 or CXCL8 alone resulted in simultaneous upregulation of p-AKT and p-ERK1/2 expressions but unchanged expressions of T-AKT and T-ERK1/2. In contrast, costimulation with two chemokines induced a much stronger phosphorylation of AKT and ERK1/2. After cells were pretreated with sorafenib, and then coincubated with both chemokines, the expressions of EMT markers maintained unchanged, relative to levels in parallel experiments without sorafenib treatment. After stimulation with both chemokines, sorafenib showed little inhibitory effect on ERK1/2 activity, and no effect on AKT activity, whereas MK2206 not only reduced p-AKT expression, but also simultaneously inhibited ERK1/2 activity. Results are representative of three independent experiments (*P < 0.01; **P < 0.05).

Fig. 2. CCL20 and CXCL8 combined to strengthen activation of both PI3K/AKT and Raf/MEK/ERK1/2 pathways, which may responsible for the process of EMT in CRC cells. (A) Western blot was performed for p-AKT/AKT and p-ERK1/2/ERK1/2 in normal human colon cells FHC and CRC cells SW480, the latter cells were treated with MK2206 or sorafenib. SW480 cells displayed a slight upregulation of p-AKT and p-ERK1/2 compared to FHC cells. Treatment of the cells with 1 μM MK2206 or 2 μM sorafenib led to effective blockade of the activation of AKT and ERK1/2. (B) Western blot analysis of p-AKT/AKT, p-ERK1/2/ERK1/2, and EMT markers in SW480 cells that were pretreated with or without inhibitors before stimulated with chemokines. CCL20 or CXCL8 alone resulted in simultaneous upregulation of p-AKT and p-ERK1/2 expressions but unchanged expressions of T-AKT and T-ERK1/2. In contrast, costimulation with two chemokines induced a much stronger phosphorylation of AKT and ERK1/2. After cells were pretreated with sorafenib, and then coincubated with both chemokines, the expressions of EMT markers maintained unchanged, relative to levels in parallel experiments without sorafenib treatment. After stimulation with both chemokines, sorafenib showed little inhibitory effect on ERK1/2 activity, and no effect on AKT activity, whereas MK2206 not only reduced p-AKT expression, but also simultaneously inhibited ERK1/2 activity. Results are representative of three independent experiments (*P < 0.01; **P < 0.05).
2 signaling pathways. In vitro, SW480 cell line was reported to be sensitive to Raf inhibitor sorafenib independent of mutation status of K-Ras [54]. However, sorafenib exhibited no inhibitory effect on Raf/MEK/ERK1/2 pathway in both chemokine-treated cells. In contrast, AKT-specific inhibitor MK2206 not only inhibited PI3K/AKT pathway, but also simultaneously inhibited Raf/MEK/ERK1/2 pathway (Fig. 2B). The results imply the existence of a signaling crosstalk between these two pathways. Accordingly, sorafenib

Fig. 3. PI3K/AKT-ERK1/2 crosstalk was implicated in both CCL20 and CXCL8 coordinately induced-EMT. (A) SW480 cells were transfected with siRNA targeting AKT, ERK1/2 or control siRNA for 48 h. Equal amounts of total protein prepared from the transfected cells were subjected to Western blot analyses with specific antibodies against p-AKT, p-ERK1/2 or GAPDH. (B) Total lysates were prepared from SW480 cells which were transfected with AKT siRNA or ERK1/2 siRNA before stimulated with chemokines for 48 h. Western blot was carried out for the detection of p-AKT, p-ERK1/2, EMT markers and GAPDH. The images were analyzed by Quantity One (Bio-Rad). The simultaneous expression of AKT siRNA significantly inhibited both chemokine-induced AKT and ERK1/2 activities compared with cells transfected with nonspecific siRNA. Accordingly, AKT or ERK1/2 specific siRNA did not change the expression of EMT markers in both chemokine-stimulated cells. Treatment of both chemokine-exposed cells with ERK1/2 siRNA suppressed limited ERK1/2 activity, but had no effect on AKT activity. Results are representative of three independent experiments (*P < 0.01, **P < 0.05).
treatment had no inhibitory effect on EMT induction after stimulation with both chemokines, but MK2206 treatment reversed both chemokine-induced alterations in the expression of epithelial and mesenchymal markers to the level present in chemokine-un treated cells (Fig. 2B). Similar results were also obtained in the same cells with AKT-specific siRNA and ERK1/2-specific siRNA (Fig. 3). These observations convince us that CCL20 and CXCL8 combine to strengthen activation of both PI3K/AKT and Raf/MEK/ERK1/2 pathways, and consequently induce EMT process; Raf/MEK/ERK1/2 pathway activation is dependent on PI3K/AKT in CRC cells, which transducers a signal to the ERK1/2 pathway in both chemokine-stimulated CRC cells but not vice versa.

To establish whether overactivated p-AKT and p-ERK1/2 contribute to the proliferation and migration of the both chemokine-treated CRC cells, we then performed the cell proliferation and cell migration assays, while inhibiting the AKT pathway with

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**Table 2**

Correlation of chemokine expressions with E-cadherin expression in 213 patients with CRC.

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>E-cadherin</th>
<th>( \chi^2 )</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>CCL20</td>
<td>74</td>
<td>62</td>
<td>0.148</td>
</tr>
<tr>
<td>CXCL8</td>
<td>63</td>
<td>56</td>
<td>0.659</td>
</tr>
<tr>
<td>CCL20 + CXCL8</td>
<td>32</td>
<td>47</td>
<td>11.272</td>
</tr>
</tbody>
</table>

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**Fig. 4.** Interruption of the PI3K/AKT and MEK/ERK1/2 pathways significantly inhibited the proliferation, migration, and invasion of both chemokine-stimulated CRC cells. (A) Cell proliferation was analyzed by MTT. SW480 cells were pretreated with 1 \( \mu \)M MK2206, 2 \( \mu \)M sorafenib, or both of them for 1 h (DMSO was used as control), and then 100 ng/ml CCL20 and CXCL8 were added into the medium. Treatment with MK2206 or sorafenib led to significant inhibition of cell proliferation, and treatment with both of CCL20 and CXCL8 led to significant increase in cell proliferation. However, MK2206 and sorafenib led to more effective inhibition of cell proliferation than did sorafenib (\( ^* P < 0.01 \)). (B) Wound-healing assays revealed inhibitory effect on cell migration similar to proliferative inhibition (\( ^* P < 0.01 \) (100×).
MK2206 or the ERK1/2 pathway with sorafenib. Sorafenib alone was ineffective against both chemokine-treated cells, in contrast, these cells were sensitive to MK2206 treatment. As expected, dual-agent combination treatment displayed no synergistic effect on growth and migration of both chemokine-treated cells relative to MK2206 alone (Fig. 4). These results further implicate the critical role of PI3K/AKT-ERK1/2 crosstalk in the determination of the aforementioned phenotypes, and lead to the hope that PI3K/AKT pathway inhibitors such as MK2206 will be a novel therapeutic strategy for the metastasis of CRC, and offer therapeutic benefit for CRC patients.

To our knowledge, no data have been published before on CCL20 expression in human CRC tissues. Several studies have been performed to examine CXCL8 expression in CRC tissues and report a wide range of CXCL8 expression in CRC tissues (19.2–100%) [55,56]. In the present study, we detected CCL20 and CXCL8 concomitant expression in human CRC specimens and showed that the coexpressions of CCL20 and CXCL8 were significantly and inversely associated with E-cadherin expression in CRC tissues. These results further support that both CCL20 and CXCL8 coordinately induce EMT. To confirm the potential effects of the CCL20 and CXCL8 expressions on clinical consequences of CRC, we then compared the various clinicopathological features according to the expression status of CCL20 and CXCL8. The results revealed that the clinicopathological characteristics of CCL20- or CXCL8-expressing CRC were similar, CCL20 expression was correlated with tumor size, lymph node metastasis and liver metastasis, while CXCL8 expression as well as coexpression of both, the latter to a significant degree, was correlated with deeper wall infiltration, lymph node metastasis and liver metastasis. These data imply a critical role of CCL20 and CXCL8 coexpression in the promotion of tumor progression, thereby leading to poor prognosis in CRC patients.

Indeed, subsequent Kaplan–Meier survival analysis of CRC cases demonstrated that CRC subtypes with coexpression of both chemokines had significantly shorter overall survival and disease-free survival than did CRC subtypes with CCL20 or CXCL8 alone (Fig. 5). These results further support that both CCL20 and CXCL8 coordinately induce EMT. To confirm the potential effects of the CCL20 and CXCL8 expressions on clinical consequences of CRC, we then compared the various clinicopathological features according to the expression status of CCL20 and CXCL8. The results revealed that the clinicopathological characteristics of CCL20- or CXCL8-expressing CRC were similar, CCL20 expression was correlated with tumor size, lymph node metastasis and liver metastasis, while CXCL8 expression as well as coexpression of both, the latter to a significant degree, was correlated with deeper wall infiltration, lymph node metastasis and liver metastasis. These data imply a critical role of CCL20 and CXCL8 coexpression in the promotion of tumor progression, thereby leading to poor prognosis in CRC patients.

Table 3
Multivariate Cox proportional hazards analysis for the candidate variables.

<table>
<thead>
<tr>
<th>Prognostic factors</th>
<th>Overall survival</th>
<th>Recurrence-free survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95%CI</td>
</tr>
<tr>
<td>Double CCL20 and CXCL8 (positive vs. others)</td>
<td>1.956</td>
<td>1.162–3.290</td>
</tr>
<tr>
<td>CEA (ng/mL) (&gt;5 vs. &lt;5)</td>
<td>1.697</td>
<td>1.028–2.800</td>
</tr>
<tr>
<td>Lymph node metastasis (yes vs. no)</td>
<td>2.451</td>
<td>1.457–4.124</td>
</tr>
<tr>
<td>Liver metastasis (yes vs. no)</td>
<td>8.246</td>
<td>4.687–14.510</td>
</tr>
</tbody>
</table>

Fig. 5. The impact of chemokine expressions on the survival of CRC patients. Survival rates were estimated by Kaplan–Meier method according to CCL20 or CXCL8 or both. (A) CCL20-associated overall survival rate and disease-free survival rate. (B) CXCL8-associated overall survival rate and disease-free survival rate. (C) CCL20 and CXCL8-associated overall survival rate and disease-free survival rate.
expression alone, respectively. Furthermore, multivariate Cox analysis identified four factors significantly influencing the overall survival rate and disease-free survival rate, among them coexpression of CCL20 and CXCL8 was an independent favorable factor for overall and disease-free survival. As for CXCL8, there is a discrepancy in previous reports. Olaipoe et al. showed that CRC patients with positive CXCL8 staining in the tumor infiltrate had a significantly improved recurrence-free survival compared with patients with negative staining [57], while Doll et al. [15] and Terada et al. [58] reported that CXCL8 expression in CRC tissues was significantly associated to a shorter overall post-operative survival time. The latter observations accord with our present study indicating that CRC subtypes with CXCL8 expression had shorter survival times. This discrepancy probably resulted from characterization of CXCL8 expression in different cells within tumor tissues, the former for the tumor-infiltrating cells, and the latter for the tumor cells. Importantly, our study suggests that CRC patients who have coexpression of CCL20 and CXCL8 are more likely to develop liver metastases and have most poor prognosis, and coexpression can be used to predict patient outcomes.

It is also worth mentioning that based on what is known currently about a paramount role of chemokines in pathogenesis of tumors, removal or blocking the accumulation of the chemokines has becoming a promising strategy in the treatment of cancer, and different chemokine antagonists are currently under investigation. Our finding that CCL20 and CXCL8 synergistically promote CRC progression and metastasis by coordinated induction of EMT may lead to a dual–antagonist combination treatment strategy for simultaneous inhibition of both CCL20 and CXCL8. Further experiments are necessary to determine whether effective blockade of them can have a more positive effect on CRC therapy, especially on the prevention of liver metastasis.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2014.03.008.

References


