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Original Article

CCR7 high expression leads to cetuximab resistance by cross-talking with EGFR pathway in PI3K/AKT signals in colorectal cancer

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Abstract: Cetuximab (C225), an anti-Epidermal Growth Factor receptor (EGFR) monoclonal antibody, has been widely used as a routine treatment for patients with metastatic colorectal cancer (mCRC); However, many patients who initially respond to cetuximab acquire resistance. The purpose of this study was to characterize new mechanism of acquired Cetuximab resistance. Firstly, tissue microarrays (TMA) comprising 191 CRC patients was constructed to evaluate the expression of chemokine receptor 7 (CCR7) by using immunohistochemistry (IHC). In CRC tumor tissues, CCR7 was significantly over-expressed compared with paired normal tissues ($P < 0.001$), and correlated with the infiltration depth ($P = 0.03$) and the regional lymph node metastasis ($P = 0.006$). Significant differences were also found in forms of overall survival (OS) and disease-free survival (DFS) between normal and tumor tissues ($P < 0.001$). More interestingly, EGFR was also highly expressed and co-localized with CCR7 in the tumor tissues from the patients who were insensitive to Cetuximab treatment. Secondly, we further explored the relationship between CCR7 expression and Cetuximab resistance by two CCR7 positive CRC cell lines, Caco-2 with wild-type *KRAS* ($KRAS^{wt}$) and HCT116 with mutated *KRAS* ($KRAS^{G13D}$). By the treatment of secondary lymphoid tissue chemokine (SLC, an exogenous high-affinity legend of CCR7), the inhibition rate of Cetuximab significantly decreased in both cells. Furthermore, the activation of SLC/CCR7 axis promoted epithelial mesenchymal transformation (EMT) in CRC tumor cells by increasing the expression of Twist and β-catenin. By using of CCR7 neutralizing antibody and p-AKT inhibitor rescued the above effects. These findings suggested that CCR7 was a key factor in those CRC patients, who have poorer reaction to Cetuximab. So combined inhibition of CCR7 and p-AKT will represent a rational therapeutic strategy for Cetuximab resistance patients.

Keywords: CCR7, colorectal carcinoma, Cetuximab resistance

Introduction

Colorectal cancer (CRC) is one of the most frequently diagnosed malignant and leading causes of cancer-related death worldwide [1, 2]. Apart from conventional strategies (e.g. surgery, radiotherapy and chemotherapy), novel molecule-targeted therapy has received considerable attention and become the focal point of treatment methods for metastatic colorectal cancer (mCRC) [3].

One promising targeted therapy agents for mCRC patients is Cetuximab (also known as Erbitux, C225), a blocking monoclonal antibody of EGFR [4]. As previously reported, high expression of epidermal growth factor receptor (EGFR) was associated with poor overall survival (OS), and activation of EGFR downstream PI3K/AKT and MAPK pathways contributed to tumor progression [5, 6]. Therefore, this anti-EGFR drug has been used in routine combining with standard chemotherapy regimens, especially for the treatment of mCRC patients harboring wild-type *K-RAS* and EGFR gene expressions [4]. As our previous study showed, Cetuximab treatment is effective; even increased the late stage patients' operation opportunity [6, 7]. However,

recently, many clinical reports showed only a 10-20% overall tumor response to Cetuximab treatment because of various levels of drug resistance [8]. The drug resistance to Cetuximab can be explained by high mutational burden, such as *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* [9, 10]. However, even among the patients with all the above genes wild-type, Cetuximab is not effective as expected, suggesting there are other mechanisms of resistance [11, 12]. Our previous study gave an indication on this aspect where we found additional biomarkers beyond *RAS* that affect the efficacy of C225 [13].

Chemokine receptor 7 (CCR7) is a seven transmembrane G protein-coupled chemokine receptor that is mainly expressed in the surface of naive T cells and DCs (Dendritic Cells). SLC (secondary lymphoid chemokine, also named as CCL21) is its high-affinity ligand. SLC/CCR7 can make T cells and DCs migrate and relocate into the surrounding lymphoid tissue or the site of immune response by binding to its ligands [14]. However, tumors with a high level of CCR7 expression are more likely to exhibit angiolymphatic invasion and lymph node metastasis, consistent with our previous studies [15, 16]. A recent report provided direct evidence that the SLC/CCR7 axis plays a key role in mediating the epithelial mesenchymal transition (EMT) process in tumor cells [17]. The relationship between CCR7 and lymph node metastasis in CRC was still controversial [18, 19]. Despite the important function CCR7 has in regulating the EMT in tumor cells, the downstream pathway of SLC/CCR7 shares the same signaling pathways as EGFR in mediating cancer cell metastasis, for example, the PI3K/AKT pathway. Furthermore, a study in head and neck cancer displayed that CCR7 activates the PI3K/AKT pathway to induce metastasis and therefore, participates in EGFR-targeted resistance [20]. However, whether CCR7 plays regulatory functions in CRC EMT and the relationship to Cetuximab treatment have not been fully elucidated.

In the present study, by using CRC specimens and cell lines, for the first time, we revealed the relationship between CCR7 expression level and clinic prognostic and Cetuximab curative effect of CRC. SLC/CCR7 pathway blocked significantly sensitizes colorectal cancer cells to Cetuximab. Our study not only elucidated the mechanisms behind the Cetuximab resistance in CRC but also provided a potential therapeutic strategy for overcoming Cetuximab resistance in patients with mCRC.

Materials and methods

Patients and follow-up

One hundred and ninety patients with colorectal cancer (CRC) were enrolled in our study, who had received surgical treatment at Zhongshan Hospital (Shanghai, China) from June 2008 to September 2015. Tumor tissue and paired normal tissue samples that had been formalin-fixed and paraffin-embedded and clinicopathologic data were retrieved from our prospectively constructed CRC database. The tumor stage was determined according to the seventh edition of the International Union Against Cancer (UICC)/American Joint Committee on Cancer (AJCC) TNM classification. The patients were followed every 3 months in the first year after surgery. The overall survival (OS) was calculated from the day of surgery to the date of death due to CRC or last follow-up. Disease-free survival (DFS) was measured from the date of surgery to the date of documented recurrence or metastasis.

Samples for evaluating the therapeutic effect of Cetuximab were from 14 patients with wild-type *KRAS* synchronous non-resectable liver-limited metastases from colorectal cancer, which had demonstrated EGFR expression. After resection of their primary tumors, the patients received chemotherapy (mFOLFOX6 [modified fluorouracil, leucovorin, and oxaliplatin]) plus Cetuximab. After 8 weeks, tumor response was assessed by a multidisciplinary team with the use of contrast-enhanced computed tomography or magnetic resonance imaging (and optional positron emission tomography scan), according to RECIST criteria [21]. Ethical approval was obtained from the Clinical Research Ethics Committee of Zhongshan Hospital of Fudan University (Shanghai, China). Signed informed consent was obtained from all of the patients.

Cell lines

The human CRC cell lines HCT116, Caco-2, DLD-1, SW620, SW480, HT-29 and LoVo were all from the Institute of Biochemistry and Cell

Biology (SIBS, CAS) and maintained in a 5% CO_2-humidified atmosphere at 37°C. HCT116, DLD-1, SW620, SW480 and LoVo cells were maintained in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA). Caco-2 cells were maintained in MEM medium (Gibco, USA) supplemented with 10% FBS. HT-29 cells were maintained in McCoy's 5A medium (Gibco, USA) supplemented with 10% FBS.

Drugs

Cetuximab (Erbitux, C225), an anti-EGFR human-mouse commercial mAb, was kindly provided by Merck Serono (USA). LY29004, a selective PI3K/AKT signaling pathway inhibitor, was purchased from Cell Signaling Technology (CST, USA). LY29004 was dissolved in sterile dimethyl sulfoxide, and a 10 mM working solution was prepared. All solutions were stored in aliquots at -20°C and diluted to the working solution before use.

Bioinformatics analysis

The network composed of CCR7 and its downstream genes was constructed using Toppgene and Exploratory Gene Association Networks (EGAN) software [22]. Pathway enrichment was based on the Kyoto Encyclopedia of Genes and Genomes pathways(KEGG, http://www.genome.jp/kegg/) database using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/home.jsp) [23].

Western blotting

Isolated proteins were analyzed by immunoblotting with primary antibodies against CCR7 (1:2000), p-EGFR, AKT, p-AKT (Ser473) (1:1000) (all from CST, USA); EGFR (1:1000) (Pierce, USA); and GAPDH (1:10000; KangCheng Biotech Inc., China); E-cadherin (1:1000), Snail (1:500), β-catenin (1:4000), and Twist (1:100) (all from Abcam, USA); N-cadherin (1:4000) (Epitomics, USA).

Tissue microarrays and immunohistochemistry

Tissue microarrays were constructed as previously described [24], Immunohistocytochemistry of the chip was performed using a two-step protocol (Novolink Polymer Detection System, Novocastra, Newcastle, UK). Briefly, sections were deparaffinized with xylene and rehydrated with graded ethanol solutions. After the endogenous peroxide activity in the slides was blocked by incubation in 0.3% H_2O_2, retrieval was performed by placing the sections in boiling citrate or ethylenediamine tetraacetic acid (EDTA) buffer for 10 min, and nonspecific binding sites were blocked with 5% goat serum (Beyotime, China) in PBS. The slides were incubated with a primary antibody against CCR7 (1:200), horseradish peroxidase (HRP)-conjugated secondary antibodies (1:400) (KPL, MD, USA), and 3'-diaminobenzidine (DAB) solution (Sigma-Aldrich, MO, USA). Negative control slides without primary antibodies were included in all assays.

Evaluation of immunohistochemical staining

All specimens were counterstained with hematoxylin and examined under light microscopy (TCS-SP5; Leica Camera AG; Germany). Staining intensity was referred as the standard of results judgment. The intensity of peroxidase deposits, ranging from light beige to dark brown, was assessed visually in the tumor cell membrane and scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). Two pathologists in gastroenterology who are blinded to the clinical data pathologically assessed all specimens independently.

Cell cycle analysis

PI staining was used to analyze DNA content. Cells were plated in 6-well culture plate at concentrations determined to yield 40-50% confluence within 24 h. Cells were pre-treated with SLC (200 ng/ml) or Ne-CCR7 (4 μg/ml) for 12 h, and then treated with Cetuximab 48 h. Cells were collected and resuspended in PBS, fixed with 70% ethanol, labeled with PI (0.05 mg/ml), incubated at room temperature in the dark for 30 min. DNA content was then analyzed by BD flow cytometry.

Immunofluorescent staining

For immunofluorescent staining, cells were seeded in 24-well plates with coverslips. After the cells attached, the medium was changed to medium with or without SLC (Peprotech, USA), and the cells were cultured for 8 h for HCT116 cells and 12 h for Caco-2 cells. Then, the cells were fixed, permeabilized, blocked and incubated with primary antibodies overnight at 4°C. Cell monolayers were then incubated with fluo-

Table 1. The Relationship between the clinical characteristics and CCR7 expression in 190 patients with CRC

	CCR7 Negative (n = 77)	CCR7 Positive (n = 113)	*P*
	No. (%)	No. (%)	
Age (years)			0.97
≤ 60	35 (45.5)	51 (45.1)	
> 60	42 (54.5)	62 (54.9)	
Gender			0.33
Male	51 (66.2)	67 (59.3)	
Female	26 (33.8)	46 (40.7)	
Stage			0.06
I-III	59 (76.6)	72 (63.7)	
IV	18 (23.4)	41 (36.3)	
Tumor size(cm)			0.66
< 3	18 (23.4)	22 (19.5)	
3-5	33 (42.9)	46 (40.7)	
≥ 5	26 (33.7)	45 (39.8)	
Gross appearance			0.90
Exophytic	19 (24.7)	25 (22.1)	
Ulcerative	48 (62.3)	74 (65.5)	
Others	10 (13.0)	14 (12.4)	
Histological type			0.21
Adenocarcinoma	8 (10.4)	19 (16.8)	
Mucinous adenocarcinom	69 (89.6)	94 (83.2)	
Tumor Differentiation			0.76
I-II (Well)	64 (83.1)	92 (81.4)	
III-IV (Poor)	13 (16.9)	21 (18.6)	
Depth of invasion			0.03*
T1-T2	18 (23.4)	13 (11.5)	
T3-T4	59 (76.6)	100 (88.5)	
Lymph node Metastasis			0.006**
Negative	58 (75.3)	63 (55.8)	
Positive	19 (24.7)	50 (44.2)	
Liver Metastasis			0.218
Negative	55 (71.4)	71 (62.8)	
Positive	22 (28.6)	42 (37.2)	

*$P < 0.05$, **$P < 0.01$.

rescein isothiocyanate 594-labeled secondary antibody (1:1000) (KPL, USA) for 2 h and stained with 1 μg/μL 4',6-diamidino-2-phenylindole (DAPI, Sigma, USA). Slides were mounted, covered with coverslips, and analyzed using a laser-scanning confocal microscope (magnification 400 ×, TCS-SP5, Leica Camera AG, Germany).

Cell viability assay

Cell proliferation was quantitatively evaluated using the Cell Count kit-8 (CCK-8, Dojindo, Japan). Cells were plated in 96-well plates and incubated for 72 h. CCK-8 solution (10 μL) was added to each well. The absorbance at 450 nm (OD450) was determined using a microplate reader (Model 680; Bio-Rad, CA, USA). The inhibition rate was calculated using the formula: (OD of control group-OD of test group)/(OD of control group-OD of blank group) × 100%.

Migration assays

The migration capability of cells was assessed using a scratch-wound assay. The cells were seeded into 6-well tissue culture dishes at a concentration of 1×10^6 cells/mL and cultured in medium containing 10% FBS to achieve confluent cell monolayers. Then, a cross-shaped linear wound was generated in the monolayer with a sterile 200 μL plastic pipette tip. Any cellular debris was removed by washing the coverslips with phosphate buffer saline (PBS). Serum-free medium with 100 ng/mL SLC was added to the experimental group, while the control group was covered with serum-free medium only and incubated at 37°C with 5% CO_2. Images of cell migration into the wounds were captured with bright field microscopy at 24 h and 48 h. The results were analyzed with ImagePro Plus (USA). An independent T-test was conducted to determine the significance of the migratory effect. The wound healing effect was calculated as the percentage of the remaining cell-free area compared with the area of the initial wound. The experiments were performed at least in duplicate.

Statistical analysis

Experimental data were presented as the Mean ± SEM of at least three independent experiments performed in duplicate. The actual overall survival rates were calculated by the Kaplan-Meier method and analyzed by the log-rank

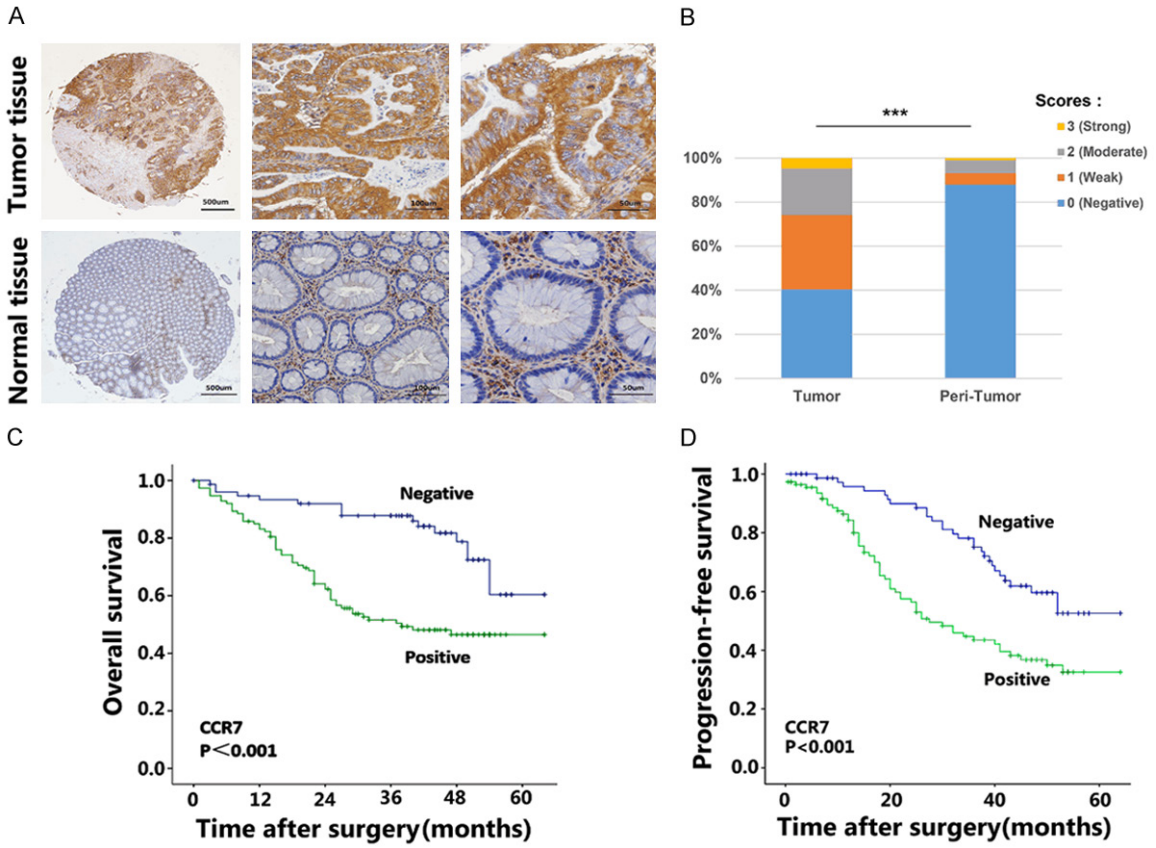

Figure 1. Strong CCR7 expression in CRC tissue correlated with poor prognostic. A. Representative images of CCR7 expression of paired normal and tumor tissues. (Original magnification: respectively × 40, 200, 400). B. Based on the intensity expression of CCR7, 190 patients were divided into 4 groups: strong, moderate, week and negative. The number of patients from each group among total patients were shown in different color. (***$P < 0.001$). C. The OS in CCR7 negative group was higher than CCR7 positive group. D. The DFS in CCR7 negative group was higher than CCR7 positive group.

test. A secondary analysis was performed to assess the relationship among the variables and clinicopathologic characteristics. Drug inhibition rate was analyzed by One-Way ANOVA. All analyses were performed using SPSS 20.0 software (SPSS, Chicago, IL) and GraphPad prism 5 (version 5.01).

Result

High expression of CCR7 in CRC tissue was correlated with poor prognostic and cetuximab reaction

The clinical and pathologic characteristics of the 190 patients with CRC were summarized in Supplementary Table 1. Paired normal and tumor tissues were taken from each patient and stained by IHC with CCR7 antibody. According to the expression intensity of CCR7 in tumor tissues, 190 patients were classified into CCR7-positive group (n = 113) and CCR7-negative group (n = 77). The relationship between the clinical characteristics and the level of CCR7 expression was summarized in **Table 1**. The data shown that CCR7 expression was significantly correlated with the infiltration depth (P = 0.03) and regional lymph node metastasis (P = 0.006). There was no significant correlation of CCR7 expression with the patients' age, gender, tumor differentiation, primary tumor parameters, distant metastasis, and TNM stage grouping.

At the same time, as shown in **Figure 1**, CCR7 was mainly expressed on the membrane of CRC cells (**Figure 1A**), and significantly higher in the paired tumor tissues ($P < 0.001$, **Figure 1B**). Furthermore, based on Kaplan-Meier method analysis, there was a significant differ-

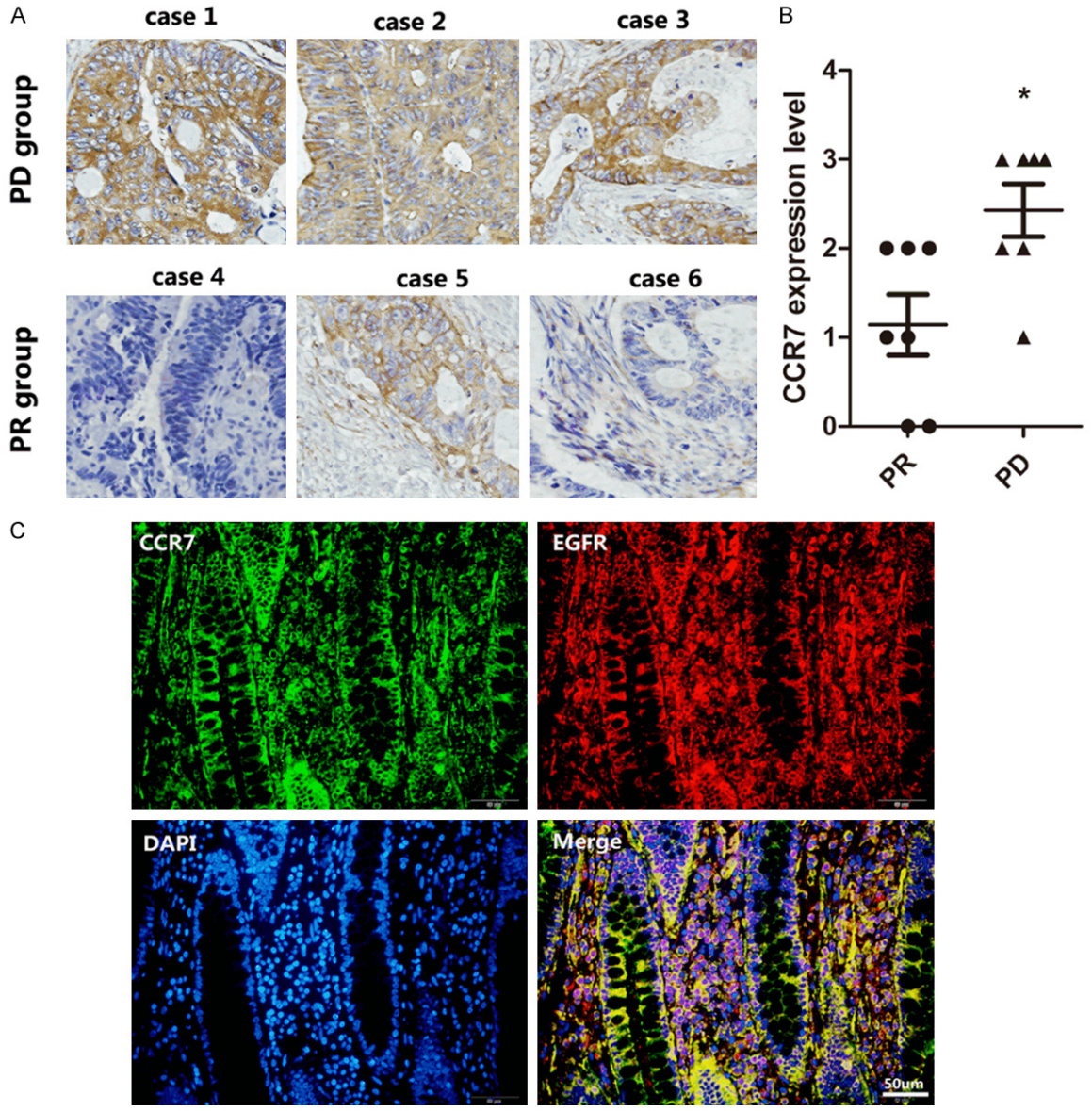

Figure 2. Cetuximab insensitive patients expressed higher CCR7 in tumor tissue. A. Representative images of CCR7 expression of CRC tissues from patients received Cetuximab therapy, Case 1, case 2, case 3 were from PD (progressive disease) group, and case 4, case 5, case 6 was from PR (partial remission) group. (Magnification: × 400). B. 14 samples was analyzed in this part, compared to PR group, the CCR7 expression level was significantly higher in the PD group, data was showed as mean expression intensity ± SEM (*P = 0.014). C. Double immunofluorescent staining of CCR7 and EGFR in CRC tissues from PD group (Magnification: × 400).

ence both in overall survival (OS) (P < 0.001, **Figure 1C**) and Disease-free survival (DFS) (P < 0.001, **Figure 1D**) between the patients of CCR7-positive group and CCR7-negative group.

To explore whether CCR7 expression involves in Cetuximab (C225) reaction, CRC patients with Cetuximab treatment were taken into consideration and classified into PD (progressive disease) group (n = 7) and PR (partial remission) group (n = 7), based on their reactions to Cetuximab treatment. As shown in **Figure 2**, we found that the expression of CCR7 was significantly higher in the patients of PD group compared with the patients of PR group (**Figure 2A** and **2B**, P = 0.014). More interesting, we found that EGFR also highly expressed and co-localized with CCR7 in PD group by double immunofluorescent staining (**Figure 2C**). These data implied CCR7 expression closely related to

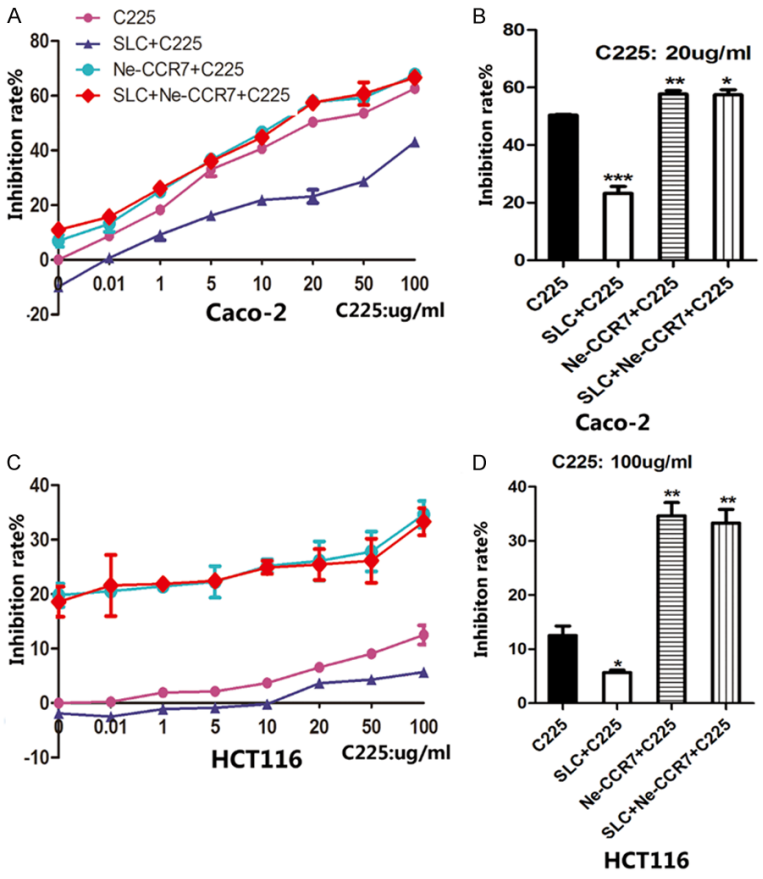

Figure 3. Pre-block CCR7 pathway with Ne-CCR7 restores sensitivity to Cetuximab. (A and C) Pre-blocking CCR7 group, the inhibition rate was significantly increased either the presence or absence of SLC. The inhibition rate was measured by applying different treatments in Caco-2 cells (B) and HCT116 (D). Significance was represented as *P < 0.05; **P < 0.01; ***P < 0.001.

EGFR expression in the CRC patients who are resistant to Cetuximab treatment.

SLC/CCR7 axis lead to cetuximab drug resistance in CRC cells

In order to further explore the relationship of CCR7 high expression and Cetuximab drug resistance, we explored Cetuximab inhibition rates and CCR7 expression in seven different CRC cell lines. Seven CRC cell lines displayed various levels of CCR7 expression (Supplementary Figure 1A) and higher CCR7 expression lead to lower sensitive to Cetuximab treatment (Supplementary Figure 1B). HCTT-116 harbors a mutated *KRAS* gene while Caco-2 has a wide-type one, we chose CCR7-high expression cell lines HCT116 ($KRAS^{G13D}$) and Caco-2 ($KRAS^{wt}$) to do further research. As shown in **Figure 3**, after SLC stimulation, the Cetuximab inhibition rate was significantly decreased. For Caco-2 cells, the inhibition rate decreased from 50.37% to 28.7% at 20 ug/ml node (**Figure 3A** and **3B**, $^{**}P$ = 0.005), and 14% to 6% in HCT116 cells at 100 ug/ml node (*P = 0.01, **Figure 3C** and **3D**). However, pre-treating cells with Ne-CCR7 reverse SLC induced Cetuximab resistance, and the inhibition rate in HCT116 cells increased more than one-fold compare Ne-CCR7 pre-treating group to control group. Meanwhile, cell cycle analysis of HCT116 showed that SLC-stimulated cells re-accumulate in S phase in response to treatment with Cetuximab and Ne-CCR7 (Supplementary Figure 2).

PI3K/AKT pathway was activated by SLC/CCR7 axis in CRC cells

To explore the underlying mechanisms of SLC induced drug resistance, CCR7, EGFR and theirs downstream genes were firstly screened by bioinformatics analysis. The data showed that PIK3CA and PIK3CB were involved in the signaling cascades of both CCR7 and drug resistance (Supplementary Figure 3). More importantly, PI3K/AKT signaling not only was the key downstream pathway of CCR7, but also the activated signaling during the process of drug resistance. Therefore, the expression of AKT was checked. SLC treatment induced phosphorylation of AKT (p-AKT) (**Figure 4A**) while Ne-CCR7 down-regulated p-AKT (Ser473) in the both groups with or without SLC treatment. We next treated CRC cells with Cetuximab. The data shown p-AKT was up-regulated by SLC and SLC *plus* Cetumimab treatment groups, respectively (**Figure 4B**). Surprisingly, EGFR were also activated in these two groups (Supplementary Figure 4).

Selective PI3K/AKT inhibitor sensitizes colorectal cancer cells to cetuximab

To further confirm SLC/CCR7 induced Cetuximab resistance via PI3K/AKT pathway, we next treated cells with Cetuximab and/or

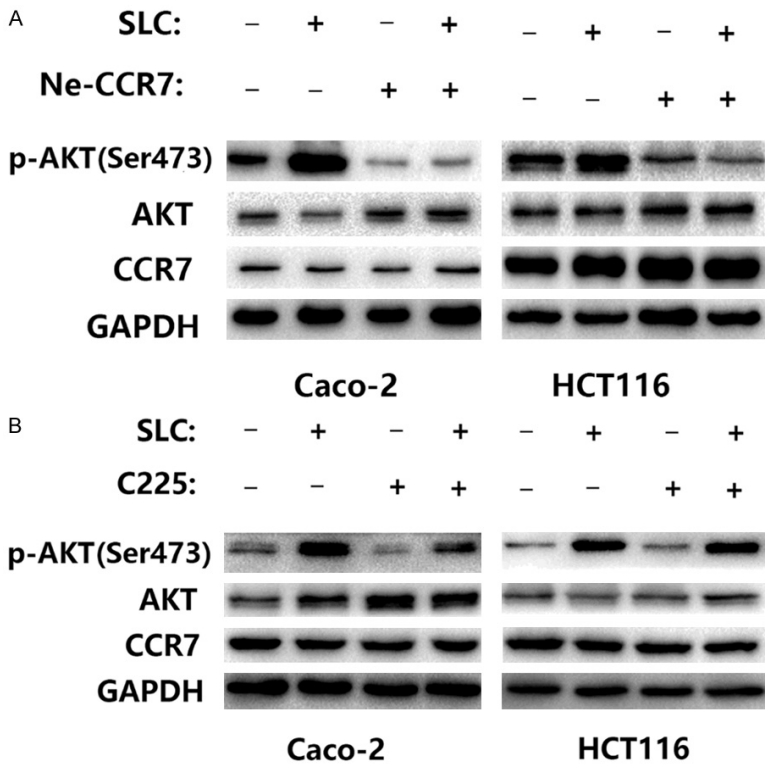

Figure 4. PI3K/AKT pathway was activated by SLC/CCR7 axis. Two CRC cell lines were differently treated with SLC stimulations or/and Ne-CCR7 or/and C225. Western blot analysis for PI3K/AKT signals. A. p-AKT was up-regulated with the stimulation of SLC. Pre-treating CRC cells with 4 ug/ml Ne-CCR7 for 12 h, p-AKT could not be effectively up-regulated. B. p-AKT was up-regulated with SLC stimulation and C225 treatment did not rescue the effect.

LY294002. As shown in **Figure 5**, compared with Cetuximab *plus* SLC group, Cetuximab *plus* LY294002 combined treatment significantly reversed Cetuximab resistance that was induced by SLC (**Figure 5A-D**). The Ne-CCR7 treatment also resulted in an increase of inhibition rate compared with Cetuximab *plus* LY294002 in CRC cells.

Activation of SLC/CCR7 axis facilitated CRC cell lines undergoing EMT

As long as PI3K/AKT, the downstream pathway of CCR7 and EGFR was activated after SLC treatment, how they facilitated tumor cells metastasis seems to be interesting. Our bioinformatic analysis identified CCR7 and EGFR were closely related to EMT (Supplementary Figure 3). It showed that the migration ability of the two cell lines were significantly promoted by the treatment of SLC after 48 hours (***P < 0.001) (**Figure 6A-D**). And the EMT transcription factor Twist1 and β-catenin was up regulated after treated with SLC (**Figure 6E** and **6F**). We next applied Ne-CCR7 to see whether blocking the pathways would prevent EMT of tumor cells. As expected, the expressions of Twist1 and β-catenin significantly decreased in the circumstance with SLC or not (**Figure 6E** and **6F**). These results indicated that activation of SLC/CCR7 downstream pathway PI3K/AKT contributes to the tumor cells undergoing EMT.

Discussion

SLC/CCR7 axis plays a role in immunoregulation and is widely applied in the research of cancer immunotherapy [25]. However, there was a debate on the the relationship between CCR7 and lymph node metastasis in CRC. Gunther *et al*. revealed an association between the CCR7 expression level and lymph node metastasis in CRC [18]. And some other researches did not find any significant correlation [19, 26]. In this study, we found that CCR7 expression was significantly correlated with the infiltration depth (P = 0.03) and regional lymph node metastasis (P = 0.006). In this fashion, we confirm that CCR7 played an important role in CRC. More importantly, we found CCR7 was significantly higher in the patients of Cetuximab-induced resistant group.

Cetuximab has proven to be effective in combination with chemotherapy or as a single agent for the treatment of patients with wild-type *KRAS* metastatic colorectal cancer (mCRC). Clinical data indicate that even the best responses are transient, and eventually all patients develop acquired resistance [11]. Elucidating the mechanisms of Cetuximab resistance is critical for the development of effective therapies of mCRC. Although several retrospective studies provided evidence that primary resistance to cetuximab could be correlated with mutations of kinds of genes, such as *KRAS*, *BRAF*, *NRAS*, and *PIK3CA*, but 25%

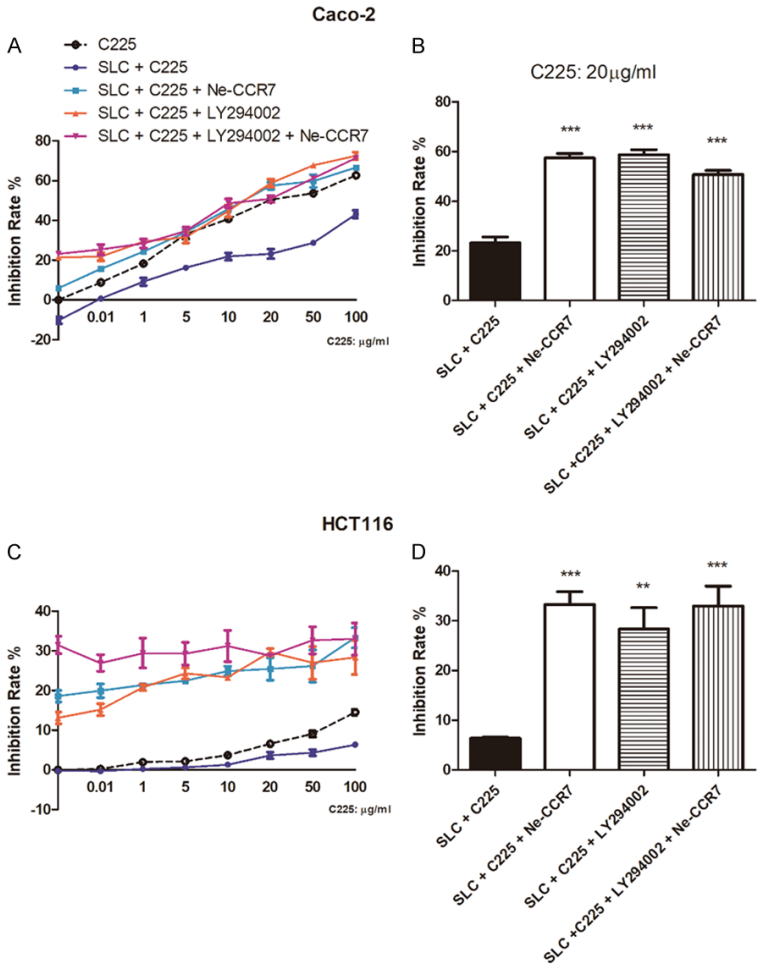

Figure 5. PI3K/AKT inhibitor sensitizes colorectal cancer cells to Cetuximab. Pre-treating the Caco-2 and HCT116 with LY29004 for 2 h, the inhibition rate were increased. In Caco-2 cells (A and B), compared with SLC *plus* C225 group, Ne-CCR7 or/and LY29004 treatment significant increased the inhibition rate (***P<0.001); In HCT116 cells (C and D), the Cetuximab inhibition was significantly increased in groups of SLC *plus* C225 *plus* Ne-CCR7 (***P<0.001), SLC *plus* C225 *plus* LY294002 (**P<0.01) and SLC *plus* C225 *plus* Ne-CCR7 *plus* LY294002 (***P<0.001).

patients who with poor reaction to Cetuximab harbor all the wild-type gene above [27], this suggested there are other mechanism of resistance. Therefore, efforts have been made to understand the molecular mechanisms of resistance to Cetuximab [11, 12, 23, 28], but still unclear now.

Based on our clinical data, we further assessed the possibility of CCR7 mediating Cetuximab resistance. We found that activation of CCR7 pathway can prevent tumor cells from being damaged by cetuximab. This phenomenon can be reversed by application of CCR7 neutralizing antibody. Previous study in head and neck cancer cells revealed that CCR7 downstream pathway activation was involved in anti-EGFR therapy. By blocking of CCR7 pathway can enhance the effects of EGFR inhibition [20]. Although in our study, there was no obvious difference of the inhibition rate between the none-treated and Ne-CCR7 groups, which might due to the activation of other pathways under CCR7 such as NF-kB pathway [29], still, Ne-CCR7 indeed decreased the inhibitory effects of SLC treatment. Similar to previous study [30], we also found Cetuximab can promote HCT116 accumulated in S phase. However, SLC stimulation significantly decreased Cetuximab-induced cycle arrest and inhibition rate. And this might be caused by SLC up-regulated cyclin A, which is essential for cycle progression through the S phase [31, 32].

To understand the signaling cascades of SLC/CCR7 axis induced drug resistance, bioinformatics analysis was performed. *PIK3CA* and *PIK3CB* were predicted as potential genes which was known as *PI3K*. PI3K signaling pathway play a critical role in tumor development and could be regulated by EGFR [33]. Especially, PIK3CA mutation has been regarded as a factor of Cetuximab therapy criteria [34]. To confirm the effect of CCR7 on decreasing Cetuximab-induced resistance was associated with PI3K pathway; we detected the protein expression of colorectal cancer cell after SLC and/or Ne-CCR7 treatment. We illustrated that activation of CCR7 by exogenous SLC can effectively activate PI3K/AKT in CRC cell lines. In addition, previous studies demonstrated that chemokine receptor 9 and C-X-C chemokine receptor 4 participated in regulating the drug sensitivity [35, 36]. So, this finding gave us hints on the

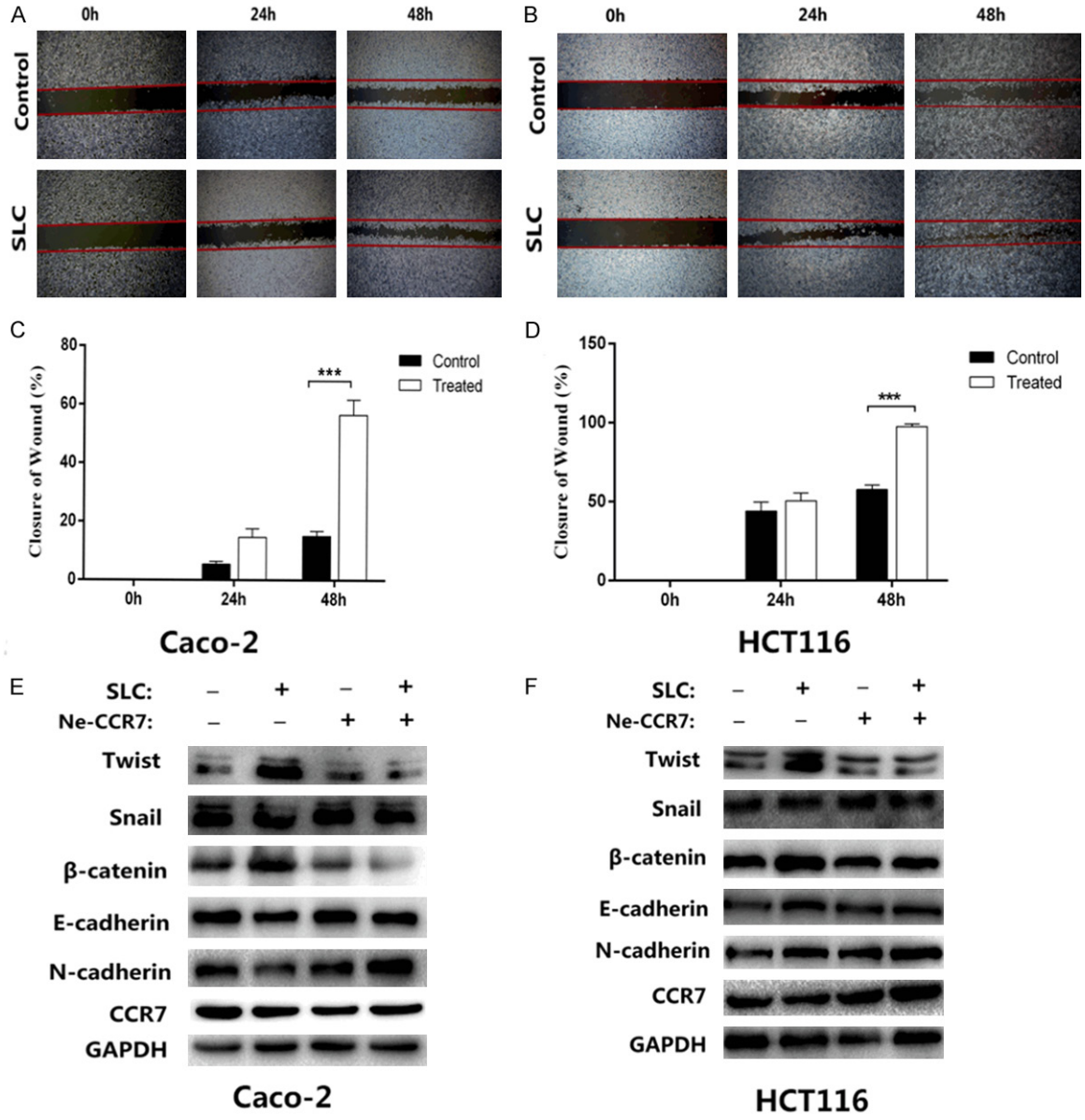

Figure 6. Pre-block CCR7 rescued cells from SLC-induced EMT. A-D. Scratch assay was used to detect cell migration ability. The cells were treated with 200 ng/ml SLC for 24 hours and 48 hours. The migration ability was calculated as the percentage of remaining cell-free area compared with the area of the initial wound. Results showed that SLC stimulation could significantly improve the healing ability of both HCT116 (****P < 0.001*) and Caco-2 (****P < 0.001*) cell lines at 48 h. E and F. Pre-treat cells with 4 ug/ml Ne-CCR7 for 12 h can neutralize the upregulation of Twist and β-catenin treated with SLC in both cell lines.

"oncogenic shift", which indicated that although cetuximab might block activation of EGFR downstream pathway PI3K/AKT, PI3K/AKT pathway could be still possible activated by CCR7, following providing the tumor cells continuously metastasis.

Furthermore, Ne-CCR7 reversed the SLC induced AKT phosphorylation and Cetuximab resistance. We even found Ne-CCR7 also inhibited AKT activation and rescue cell from Cetuximab resistance in the group without SLC stimulation, indicating that Cetuximab-induced resistance of colorectal cancer cell was driven by SLC binding to CCR7. We next treated cell with PI3K inhibitor, and similar to the pervious results, LY294002 sensitized colorectal cancer cells to Cetuximab. It has implied that SLC/

CCR7 axis promoted Cetuximab resistance was via PI3K/AKT pathway. In addition, up-regulated Twist1 has been provided as a driver of resistance to EGFR inhibitor [37], that means SLC/CCR7 axis induced EMT might also contributes to the Cetuximab resistance.

Besides PI3K/AKT pathway, we also found p-EGFR was up-regulated by SLC stimulation, but the mechanism is still need to be explored. A recent study demonstrated that chemokine can induce reactive oxygen species production leading to EGFR activation [38]. Therefore, chemokine induced reactive oxygen species might be an explanation of EGFR phosphorylation.

Taken together, our findings suggested that CCR7 played a key role in the process of cetuximab resistance in colorectal cancer cells, which in the end undergone EMT. Therefore, the inhibition of CCR7 or combined inhibition targeting to PI3K could represent a rational therapeutic strategy for preventing cetuximab resistance and EMT in patients with mCRC.

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Disclosure of conflict of interest

None.

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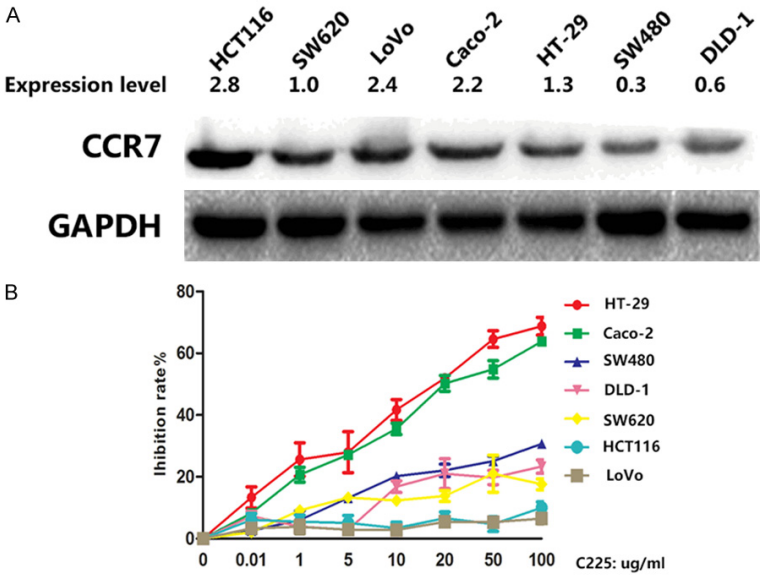
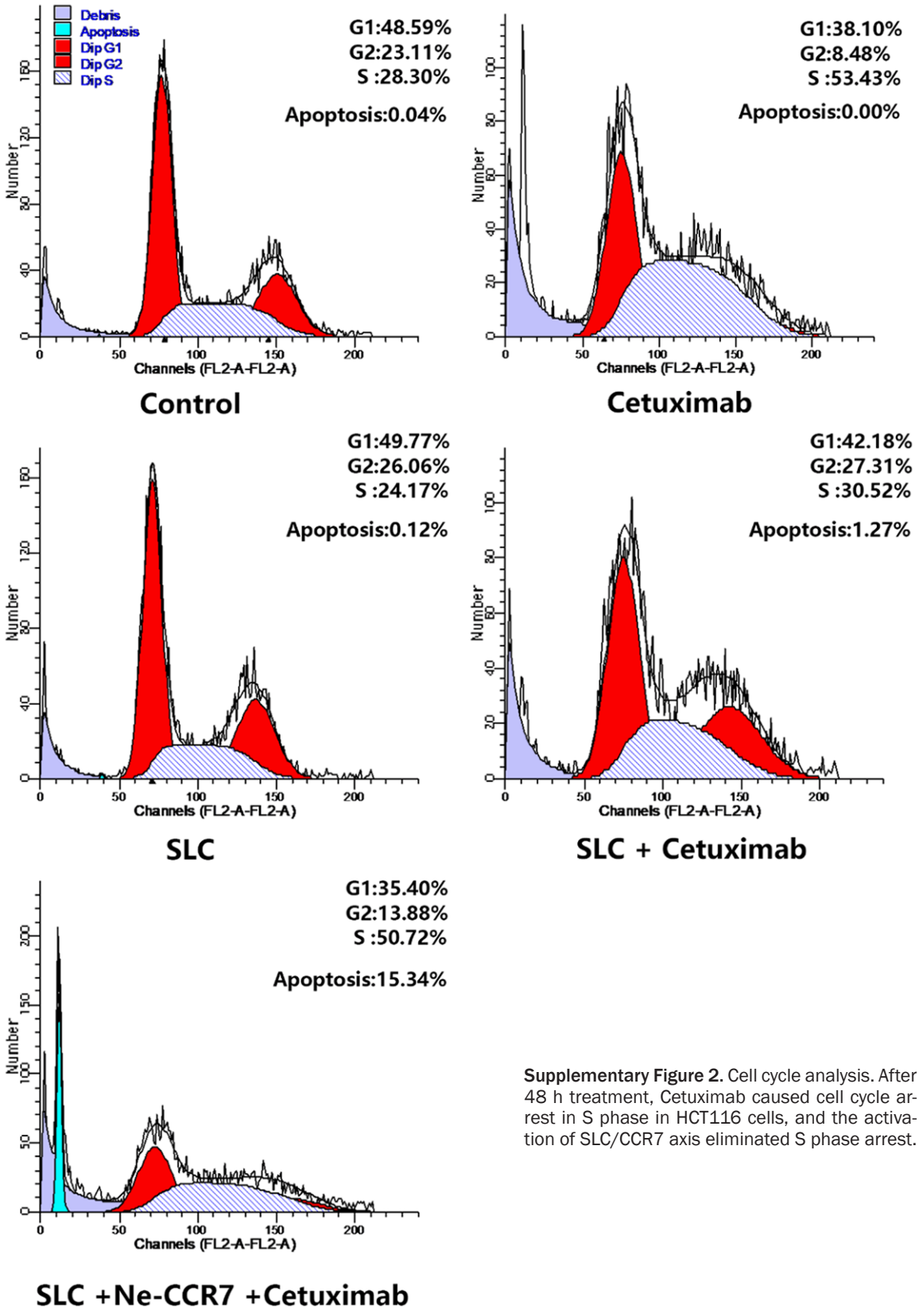
Supplementary Table 1. Clinical characteristics of the 190 colorectal cancer patients

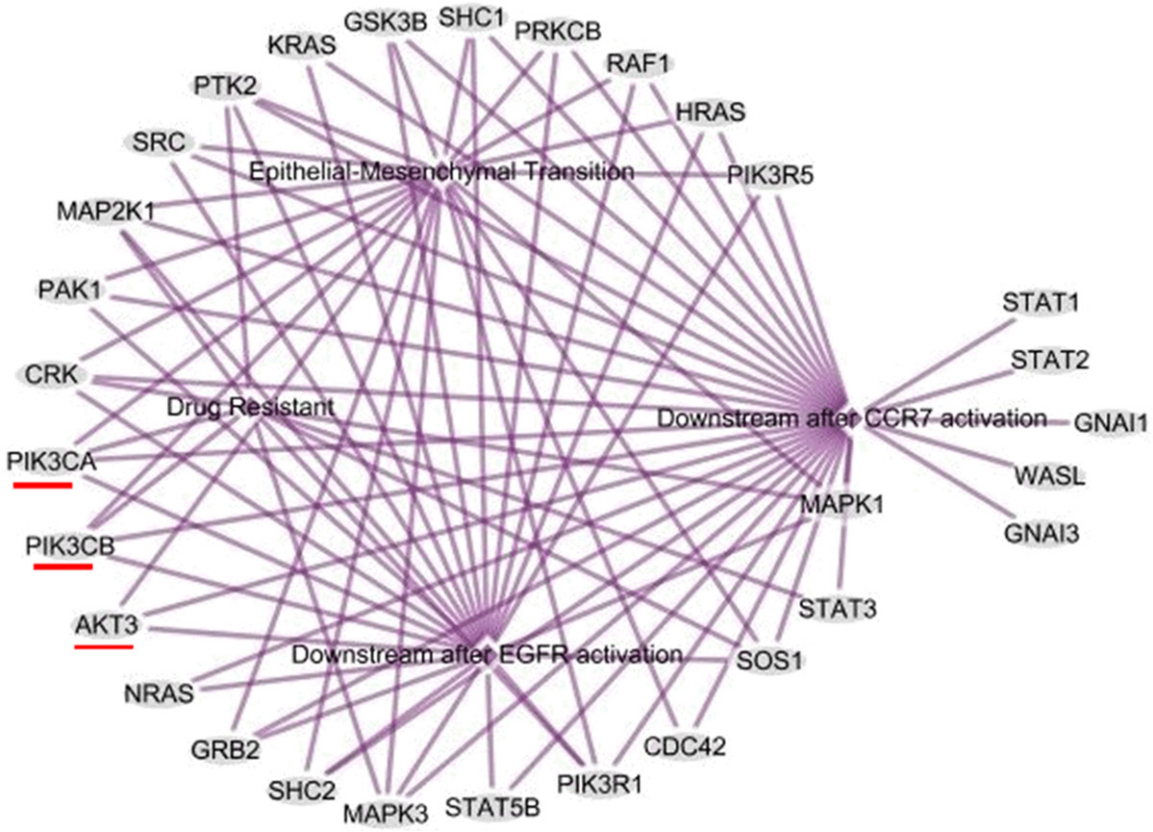
Variables	Number	Percentage (%)
Age(years)		
< 60	86	45.3
≥ 60	104	54.7
Gender		
Male	118	62.1
Female	72	37.9
Tumor Size (cm)		
< 3	40	20
3-5	79	41.6
≥ 5	71	37.4
Gross appearance		
Exophytic	44	23.1
Ulcerative	122	64.2
Infiltrative	24	12.7
Tumor Differentiation		
I-II	141	74.2
III	49	25.8
Depth of invasion		
T1-T2	31	16.3
T3-T4	159	83.7
Lymph node Metastasis		
Negative	106	55.8
Positive	84	44.2
Liver Metastasis		
Negative	131	68.9
Positive	59	31.1
Stage		
I-II	131	68.9
III-IV	59	31.1



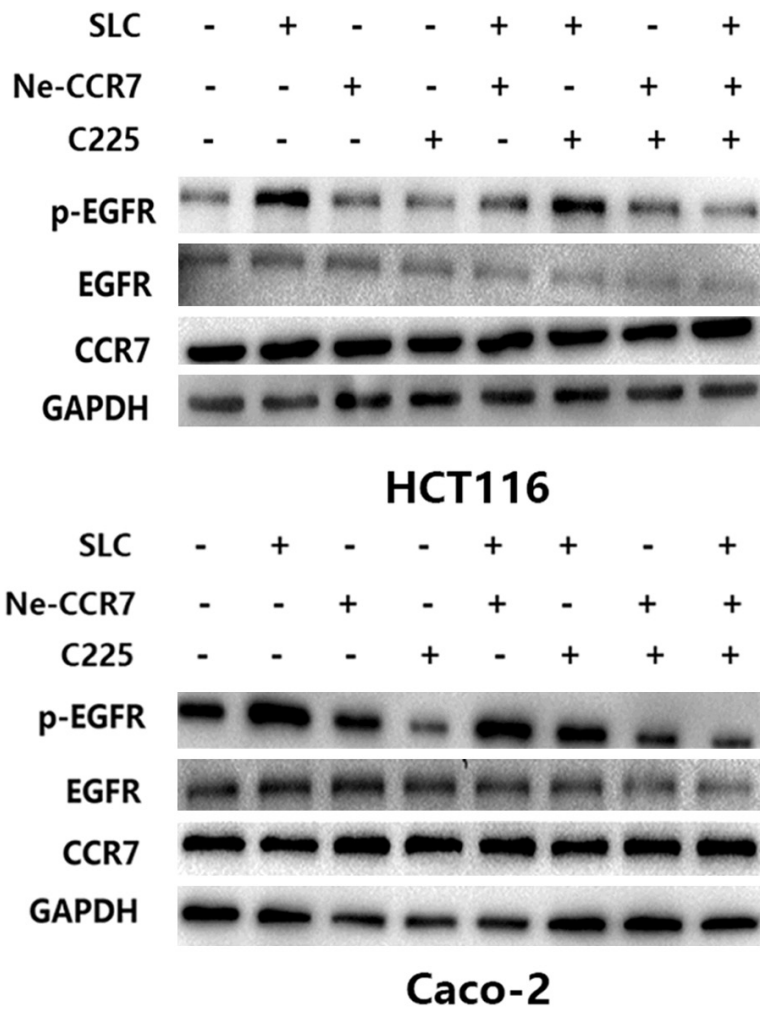
Supplementary Figure 1. Cetuximab inhibition rate and EGFR expression of CRC cell lines. A. Western blotting was used to detect CCR7 expression of seven CRC cell lines, expression level was normalized to SW620 cells. B. Detection of 72 h inhibition rate of seven CRC cell lines at different Cetuximab concentrations: from 0 ug/ml to 100 ug/ml, 72 hours, data analysis was shown mean ± SEM.



Supplementary Figure 2. Cell cycle analysis. After 48 h treatment, Cetuximab caused cell cycle arrest in S phase in HCT116 cells, and the activation of SLC/CCR7 axis eliminated S phase arrest.



Supplementary Figure 3. Bioinformatic analysis potential genes involved in the SLC/CCR7 mediated drug resistance. Bioinformatic analysis with tumor related proteins under the CCR7 pathway showed 25 proteins were involved in crosstalk with EMT, drug resistance and EGFR signal pathways.



Supplementary Figure 4. p-EGFR was up-regulated after SLC treatment. Pre-treat cells with 4 ug/ml Ne-CCR7 for 12 h, protein was extract at 24 h after C225 treatment (100 ug/ml for HCT116 and 20 ug/ml for Caco-2). Western Blotting results showed that compaired to SLC-induced team, p-EGFR was down-regulated in Ne-CCR7 group and was up-regulated with the stimulation of SLC and C225 treatment.