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Original Article

Activation of the IL-17 signalling pathway by the CXCL17-GPR35 axis affects drug resistance and colorectal cancer tumorigenesis

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Abstract: Colorectal cancer (CRC) is one of the most commonly diagnosed cancers, and drug resistance following prolonged treatment leads to downregulation of the efficacy of chemotherapy against CRC. CXCL17 is an inflammatory factor that plays a crucial role in tumorigenesis. However, the function of the CXCL17-GPR35 axis in CRC and resistance to chemotherapy is not entirely clear. Bioinformatic analysis was used to identify differentially expressed genes (DEGs) in oxaliplatin (OXA)-resistant CRC tumour tissues compared to OXA-sensitive counterparts. To further determine the function of CXCL17 in taxol-resistant CRC cells (HCT15), proliferation, migration, invasion, cell cycle, and apoptosis were analysed by CCK-8, wound healing, Transwell®, and flow cytometry assays, respectively. In addition, RNA sequencing, western blotting, CCK-8, wound healing, and Transwell® assays were used to further identify and confirm the downstream effects of CXCL17 regulation on taxol resistance. Our study found that CXCL17 and GPR35 were upregulated in OXA-resistant tumour tissues compared to in OXA-sensitive tissues. CXCL17 silencing significantly decreased the viability, migration, and invasion of taxol-resistant CRC cells. CXCL17 silencing arrested taxol-resistant CRC cells in the G2/M phase and promoted apoptosis. The IL-17 signalling pathway is involved in regulation of the CXCL17-GPR35 biological axis in HCT15 cells, and the addition of IL-17A distinctly reversed the decreased proliferation, migration, and the enhanced apoptosis of HCT15 cells upon CXCL17 deletion. In summary, these findings demonstrate that the CXCL17-GPR35 axis and IL-17 signalling pathway are involved in mediating CRC tumorigenesis and drug-resistance. Inhibition of the CXCL17-GPR35 axis and IL-17 may hence be promising therapeutic targets for CRC resistance to OXA.

Keywords: Colorectal cancer, drug-resistance, CXCL17, GPR35, IL-17 signaling pathway

Introduction

Colorectal cancer (CRC) is a common malignant disease of the intestinal tract, with the third highest incidence rate among all tumour-causing diseases [1]. According to the World Health Organization's International Agency for Research on Cancer, 935000 cases of CRC were reported worldwide in 2020 [2]. Chemo- and targeted therapies generally result in a limited increase in overall survival of CRC patients, and the main reason for this is therapy resistance [3]. Currently, oxaliplatin (OXA) is a common antitumour drug used to treat CRC because it can inhibit DNA replication and transcription by binding to DNA and forming platinum-DNA adducts [4]. However, chemoresistance following prolonged chemotherapy leads to a decrease in the efficacy of the chemotherapy [5, 6] and drug resistance remains a major barrier to the long-term clinical efficacy of CRC chemotherapy. Hence, there is an urgent need to reduce or eliminate chemoresistance to further improve the efficacy of CRC therapy.

Various inflammatory cells and cytokines are present in the tumour microenvironment and are closely associated with CRC development [7]. It is widely thought that chemokines are involved in numerous processes, such as angiogenesis and immune diseases, thereby further affecting tumourigenesis. For example, the

CXCL12-CXCR4/CXCR7 biological axis has been reported to be closely related to proliferation, invasion, and metastasis in a variety of tumours (small-cell lung cancer, breast cancer, pancreatic cancer, and prostate cancer) [8-11]. CXC chemokine ligand 17 (CXCL17) is a novel 119 amino acid CXC chemokine [12] that has received considerable attention since its discovery and is closely associated with the development of numerous cancers. CXCL17 plays a role in HCC cell migration and invasion in hepatocellular carcinoma [13]. It has been shown that primary tumours express high levels of CXCL17 relative to normal colon [12]. The receptor for CXCL17 is the G protein-coupled receptor GPR35 (CXCR8) [14]. GPR35 is involved in the development of asthma, cardiovascular disease, enteritis, diabetes, and several other diseases [14]; however, its specific functions and mechanisms have not been studied. Activation of the GPR35 pathway promotes colorectal tumour growth by directly augmenting the proliferation of epithelial cells expressing the receptor [15]. CXCL17 and GPR35 are involved in the development of multiple cancer types, and they are significantly and positively correlated in endometrial cancer and are involved in lymphocyte and immune regulation [16]. The development and progression of gastric cancer are regulated by CXCL17 and GPR35 [17]. CXCL17 and GPR35 expression are positively correlated with colon cancer [18]. However, the relationship between CXCL17 and GPR35 in CRC has not been investigated much to date. In addition, although studies have shown that chemosensitivity to OXA can be enhanced by several molecular mechanisms, such as the ELFN1-AS1/EZH2/DNMT3a axis-mediated MEIS1 [19] and the KIF20A/NUAK1/Nrf2/GPX4 signalling pathway [20], OXA resistance is highly complex and not completely overcome, thus warranting considerable further research. Therefore, it is important to study the CXCL17-GPR35 biological axis in normal and drug-resistant CRC. In recent years, the role of interleukin 17 (IL-17) in the development and progression of CRC has been increasingly recognised. IL-17 belongs to a family of proinflammatory cytokines, including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F [21], which are secreted by T cells. IL-17 recruits neutrophils, promotes the secretion of inflammatory factors, enhances cell proliferation and angiogenesis, and participates in the development of many diseases, including chronic inflammation and tumours [22, 23]. IL-17A is an important member of the IL-17 family and is associated with multiple biological processes such as the proliferation, growth, and apoptosis of malignant tumours. The expression of IL-17 signalling-related genes is elevated in numerous human malignant cancers, including cervical cancer, oesophageal cancer, gastric cancer, hepatocellular carcinoma, and CRC [25, 26]. IL-17A increases PD-L1 expression and promotes resistance to anti-PD-1 therapy in patients with CRC [24]. Previous studies have confirmed that IL-17 levels are high in the serum and tumour tissues of patients with CRC and that IL-17 plays an important role in the metastasis and tumourigenesis of CRC [27, 28]. CRC development is inextricably linked to the proliferation and metastasis of cancer cells, and IL-17 increases the viability and migration of CRC-initiating cells [29]. Furthermore, it has been shown that IL-17R regulates CXCL1 and CXCL2 through activation of NF-κB, thereby promoting distal CRC development [30]. However, the role of IL-17 and the correlation between CXL17 and IL-7 in OXA resistance in CRC have not been fully elucidated.

In our study, we found upregulated DEG CXCL17 and its receptor GPR35 in OXA-resistant CRC tissues and explored their effects on taxol-resistant cell proliferation, migration, and invasion in CRC. The downstream pathway of CXCL17-GPR35 was determined using transcriptome analysis. We further demonstrated the influence of CXCL17-GPR35 on proliferation, migration, and invasion through the IL-17 pathway in mouse and cell models with the aim of elucidating its regulatory mechanism in drug resistance and CRC progression. This may provide a new therapeutic target for this disease.

Materials and methods

Cell culture

Normal human colorectal mucosal cells (FHC) were grown in RPMI 1640 medium supplemented with 10% foetal bovine serum. Drug-resistant human colorectal cancer cells (HCT15) were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, 1% penicillin and streptomycin, and 500 ng/ml taxol (a

microtubule-stabilising drug used to treat cancer) (Med Chem Express, Monmouth Junction, NJ, USA). The cell culture conditions involved a humid environment at 37°C and 5% CO_2.

Cell transfection

Three siRNAs targeting CXCL17 (si-CXCL17-1, 2, and 3) were used to knockdown CXCL17 in HCT15 cells, and non-targeting siRNAs (siNCs) were used as controls (the siRNA sequences are shown in Supplementary Table 1). Cells were inoculated into 24-well plates the day before transfection so that the cell density reached approximately 30%-50%. After 24 h, the medium was replaced with fresh medium (containing serum and without antibiotics) per well prior to transfection. Then, 1 μL of Lipo6000™ Transfection Reagent and 20 pmol of the various siRNAs or reference buffer were added to 25 μL aliquots of DMEM medium. The mixtures were incubated at room temperature for 5 min and then added to wells with cells, and the medium was replaced with fresh medium after 6 h of transfection to maximise the efficiency.

Bioinformatics analysis

The GEO database GSE76092 was used to analyse differentially expressed genes (DEGs) between OXA-sensitive and OXA-resistant tumour tissues. To identify the DEGs, the ggplots2 package was used to plot volcano mappings of the DEGs.

Western blotting

Cells were lysed with protein lysis buffer (RIPA; Roche, Shanghai, China), and total protein was measured using a BCA protein analysis kit (Solarbio, Beijing, China). Equal amounts of protein were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Roche) and incubated overnight at 4°C with the following primary antibodies: anti-CXCL17 (Abcam, ab177612), anti-GPR35 (Abcam, ab76217), anti-IL-17A (Abcam, ab79056), and anti-IL-17F (Abcam, ab187059), all diluted to 1:1000, and actin was used as a loading control. The membranes were then incubated with secondary antibodies for 1 h at room temperature. Protein levels were assessed using the ECL Plus western blot detection system with a luminescent image analyser.

RT-qPCR

Total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A total of 1 μg RNA was used for cDNA synthesis using HiScript® II QRT SuperMix (Vazyme, Nanjing, China). qRT-PCR was performed on a CFX384 Real-Time PCR Detection System (Bio-Rad) using TB Green® Premix Ex Taq™ II (Takara, Beijing, China) following the manufacturer's instructions. *GAPDH* was used as an internal control, and the primers used for qRT-PCR are listed in Supplementary Table 2.

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were collected and the expression of CXCL17 and GPR35 was detected using an ELISA kit (Esebio, Shanghai, China). The standard and sample wells were set up as required, and all wells were incubated with horseradish peroxidase (HRP)-labelled antibody for 1 h at 37°C. Substrates A and B were then mixed and added to the wells to measure OD values after incubation for 15 min.

CCK-8 assay

CCK-8 assay was used to assess the viability of HCT15 cells transfected with si-CXCL17 or siNC. HCT15 cells (4×10^4) were seeded into a 96-well plate and incubated at 37°C until 80% cell confluency. Standard and blank wells were used to eliminate errors. CCK-8 dilution buffer (100 μL of 1:10) was added and the samples incubated for 2 h, and the absorbance at 450 nm was then measured every hour to determine the most suitable concentration, and the cell viability was determined by statistical analysis.

Wound-healing assay

Cells transfected with si-CXCL17 or siNC were inoculated in 6-well plates at a density of 1×10^5. After creating a monolayer wound with a pipette tip, the cells were washed and then cultured in serum-free medium at 37°C with 5% CO_2. The cell monolayer wound was imaged at the beginning and after 24 h of incubation. The migration ability was analysed using ImageJ software.

Transwell® assay

The invasive ability of HCT15 cells was determined by Transwell® assay. Matrigel™ was diluted (1:8) and coated onto the upper surface of the bottom membrane of the Transwell®. Cells were seeded at a density of 5×10^4 on the bottom surface. After 24 h of incubation, the cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal purple for 20 min. The invasion rate was determined by counting the number of cells that had migrated using a microscope.

Cell cycle assay

Cells (1×10^6) were fixed with 70% ethanol and then stored at 4°C overnight. The cells were stained with PI for 30 min in the dark at room temperature, followed by flow cytometry (BD FACSCalibur™, USA). The percentages of G0-G1, S, and G2-M/phase cells were calculated using ModFit™ software (Verity Software House, USA).

Apoptosis assay

Apoptosis was measured using an annexin V-FITC/propidium iodide (PI) kit (Procell, Wuhan, China) according to the manufacturer's instructions. After transfection, HCT15 cells were harvested and centrifuged at 300 × g for 5 min at 4°C after washing. The cells were then resuspended in cold binding buffer and gently mixed with annexin V-FITC and PI solutions. The cells were incubated for 15 min and then assayed by cytometry (BD FACSCalibur™).

Generation of animal model

Nude mice (BALB/c, female, 4-5 weeks old, 18-20 g) were subcutaneously inoculated with 5×10^6 cells in a volume of 0.1 mL. The mice were randomly divided into two groups (n=6/group) and injected with HCT15 cells transfected with si-CXCL17 or siNC. Four weeks after injection, the animals were euthanised, and the tumours were harvested and weighed. Vernier calipers were used to measure the longest and shortest dimensions of the tumours. The tumour volumes were calculated using the following formula: $v=1/2 \times a \times b^2$ (a is the long axis and b is the short axis).

Immunohistochemistry (IHC)

IHC was performed to measure the population of CXCL17-positive cells in the tumour tissues. The tumour tissues were fixed and embedded in paraffin. After dewaxing, hydration, washing, blocking, and antibody incubation (anti-CXCL17, 1:100), diaminobenzidine (DAB) was used as the chromogen to visualise positive staining, and haematoxylin was used to counterstain the nuclei. Differences in the ratio of positive cells between groups were detected using a microscope.

H&E staining

Histopathological changes in the tumour were observed by H&E staining. First, tumour tissues were fixed in acetone at -20°C and then stained with haematoxylin, followed by eosin. Finally, the slides were dehydrated with different concentrations of alcohol and dimethylbenzene and the stained cells observed under a light microscope and photographed.

RNA-sequencing

For RNA sequencing, we collected HCT15 cells transfected with siNC or si-CXCL17 and extracted total RNA for library construction. The cDNA library preparation and sequencing steps included RNA fragmentation, cDNA reverse transcription, cDNA repair, adapter ligation, and qPCR quality control. Sequencing libraries were paired-end sequenced on an Illumina NovaSeq 6000 platform (Gene Denovo Biotechnology Co., Guangzhou, China). Gene expression was quantified using StringTie software. DEGs were analysed using DESeq2. FDR < 0.05 and |Log2FC| > 1 were taken to indicate that the gene was differentially expressed between the two groups. Gene Ontology (GO) enrichment analysis of the DEGs was performed using the GOseq R package. KOBAS software was used to test the statistical enrichment of DEGs in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The top ten upregulated and downregulated DEGs are listed in Supplementary Table 3.

Statistical analysis

All of the data were analysed using GraphPad Prism 7.04 software (GraphPad Inc., USA) and

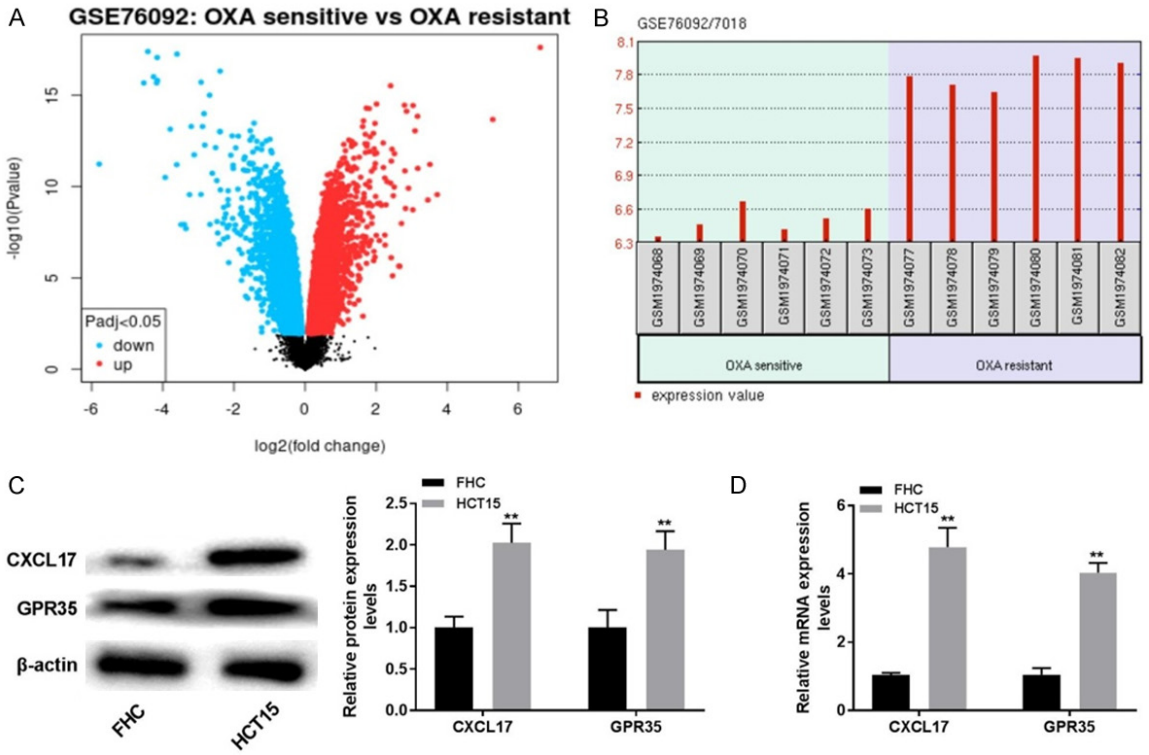


Figure 1. Chemokine CXCL17 and its receptor GPR35 exhibit higher levels in CRC drug-resistant tissues and cells. A. Differential gene volcano plot between OXA-sensitive and OXA-resistant groups in CRC tissues; B. Box plot of CXCL17 expression in OXA-sensitive and OXA-resistant tumor tissues; C. Western-blot detection of CXCL17 and GPR35 protein expression levels in FHC and HCT15 cells; D. RT-qPCR detection of mRNA expression of CXCL17 and GPR35. $^{**}P < 0.01$, compared with the FHC group.

presented as multiple groups of duplicate data or means ± SD. One- or two-way ANOVA was used to compare multiple groups. Tukey's multiple comparison test was used for pairwise comparisons after ANOVA, and comparisons between two groups were by t-test. Statistical significance was set at $P < 0.05$.

Results

Chemokine CXCL17 and its receptor GPR35 are upregulated in drug-resistant CRC cells

The GEO database GSE76092 was used to map the differential gene expression between the OXA-sensitive and OXA-resistant tumour tissues as volcano plots, and the results show that there were significantly up- and downregulated genes between the two groups (**Figure 1A**). Further analysis revealed that CXCL17 was the most differentially expressed chemokine and was highly expressed in OXA-resistant tissues compared to in OXA-sensitive counterparts (**Figure 1B**). The protein levels of CXCL17 and GPR35 were higher in HCT15 cells than in FHC cells (**Figure 1C**, $P < 0.01$), and consistent results were obtained after detection at the mRNA level using RT-qPCR (**Figure 1D**, $P < 0.01$). Collectively, these results demonstrate that CXCL17 and its receptor GPR35 are upregulated in drug-resistant CRC tissues and cells.

Verification of CXCL17 transfection efficiency and expression of its receptor-GPR35

HCT15 cells were transfected with si-CXCL17 to silence CXCL17, and the transfection efficiency was determined by RT-qPCR. The expression levels of CXCL17 in cells transfected with si-CXCL17-1, 2, and 3 were significantly decreased compared to those in cells transfected with siNC, and transfection with si-CXCL17-3 had the greatest effect (**Figure 2A**, $P < 0.01$). ELISA showed that the levels of CXCL17 and its receptor GPR35 in cell supernatants were reduced upon CXCL17 silencing (**Figure 2B**, $P < 0.01$). In addition, the protein levels of CXCL17 and its receptor GPR35 were significantly decreased in

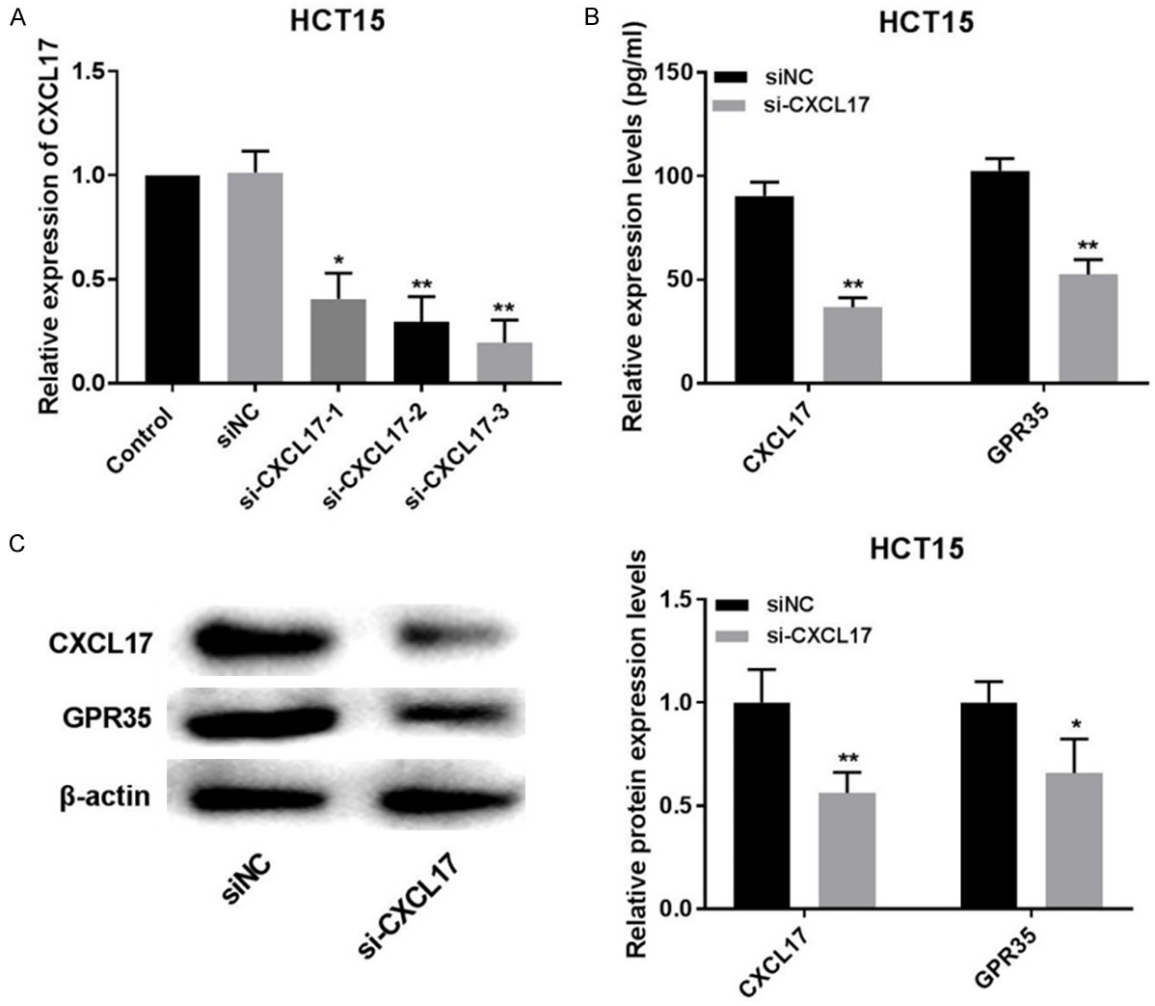


Figure 2. Verifying the efficiency of siRNA transfection and expression levels of CXXL17 and GPR35. A. The transfection efficiency of CXCL17 was verified by RT-qPCR; B. The expression of CXCL17 and GPR35 was detected by ELISA; C. The protein levels of CXCL17 and GPR35 was detected by Western-blot. Compared with siNC group, $^{*}P < 0.05$; $^{**}P < 0.01$.

CXCL17-deleted HCT15 cells (**Figure 2C**). These data show that CXCL17 was successfully knocked down, and expression of its receptor, GPR35, was reduced upon CXCL17 silencing.

Knockdown of CXCL17 inhibits drug resistance and tumorigenesis of CRC

To determine the function of CXCL17 in HCT15 cells, CCK-8 assay was used to measure cell viability, which showed that the viability of cells with CXCL17 knockdown was significantly reduced from day 2 relative to that of cells transfected with siNC (**Figure 3A**, $P < 0.01$). Then, to assess the correlation between CXCL17 and drug resistance, we treated the HCT15 cells with different concentrations of taxol (0, 50, 100, and 200 μM) for 12 hours and then measured the cell viability. The results show that the chemoresistance to taxol of HCT15 cells transfected with si-CXCL17 was lower than that in HCT15 cells transfected with siNC (**Figure 3B**, $P < 0.01$). The migration and invasion of HCT15 cells were also significantly downregulated upon CXCL17 knockdown (**Figure 3C**, **3D**, $P < 0.01$). In addition, CXCL17 knockdown blocked HCT15 cells in the G2/M phase of the cell cycle ($P < 0.01$) and increased their apoptotic rate ($P < 0.05$) (**Figure 3E**, **3F**). These data demonstrate that CXCL17 promotes the proliferation and invasion of HCT15 cells and enhances their resistance to taxol.

Transcriptome sequencing for DEG analysis

To obtain a global view of the role of CXCL17 in the regulation of CRC cells, we performed transcriptome analysis of HCT15 cells transfected

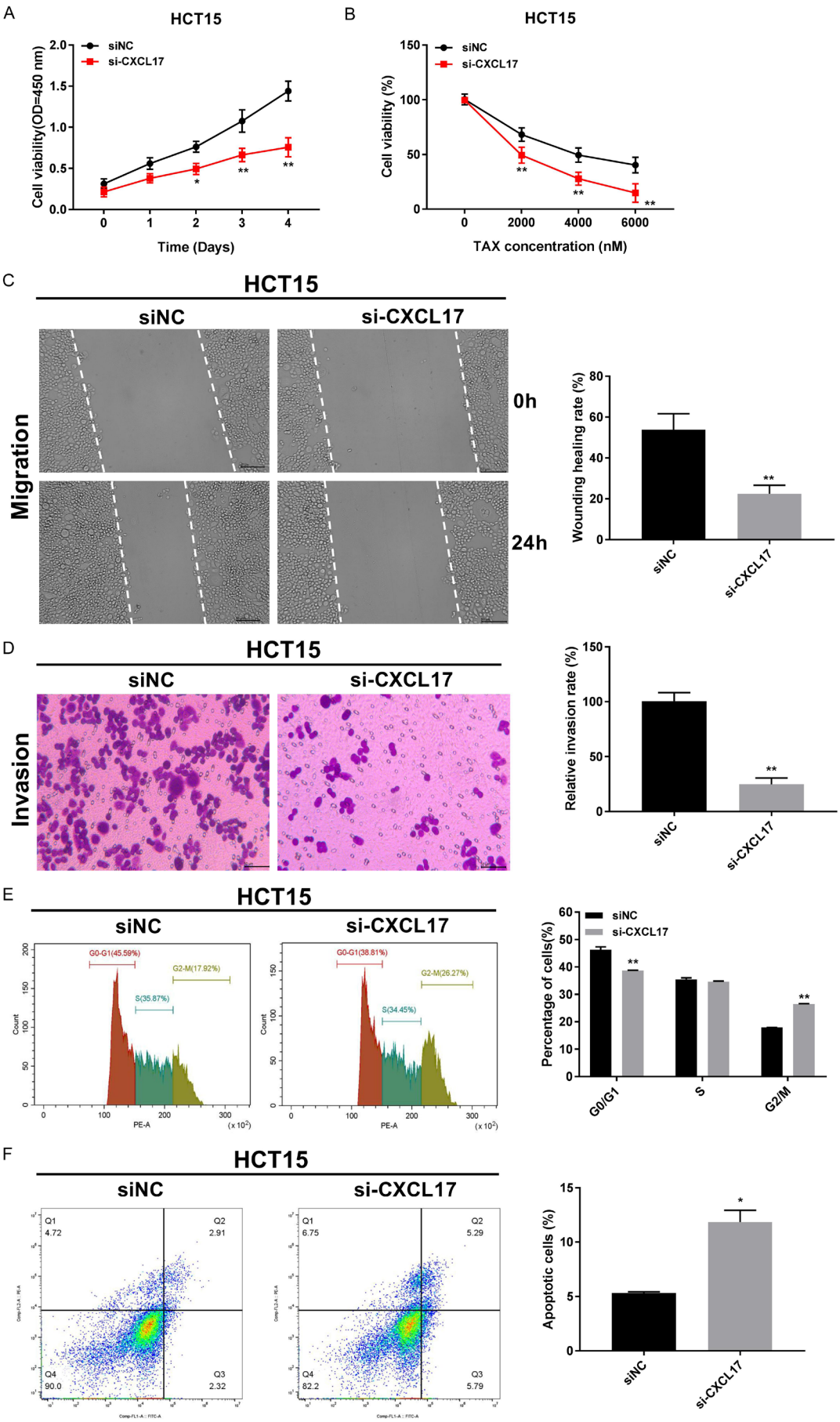

A
HCT15
siNC
si-CXCL17
Cell viability(OD=450 nm)
Time (Days)
B
HCT15
siNC
si-CXCL17
Cell viability (%)
TAX concentration (nM)
C
HCT15
siNC
si-CXCL17
Migration
0h
24h
Wounding healing rate (%)
D
HCT15
siNC
si-CXCL17
Invasion
Relative invasion rate (%)
E
HCT15
siNC
si-CXCL17
G0-G1(45.59%)
G2-M(17.92%)
S(35.87%)
G0-G1(38.81%)
G2-M(26.27%)
S(34.45%)
PE-A
Percentage of cells(%)
G0/G1
S
G2/M
F
HCT15
siNC
si-CXCL17
Q1 4.72
Q2 2.91
Q4 90.0
Q3 2.32
Q1 6.75
Q2 5.29
Q4 82.2
Q3 5.79
Apoptotic cells (%)

Figure 3. CXCL17 plays a critical role in proliferation and drug-resistance of HCT15 cells. A, B. CCK-8 assay was performed to detect cell viability after the treatment with CXCL17 silence or different concentrations of taxol; C. Wound-healing assay for cell migration ability of HCT15 cells upon siNC or si-CXCL17 transfection; D. Transwell assay was applied to analyze cell invasion ability; E. Cell cycle was detected by flow cytometry; F. Cell apoptosis was detected by flow cytometry. Scale bar was 50 μm. Compared with siNC group, $^{*}P < 0.05$; $^{**}P < 0.01$.

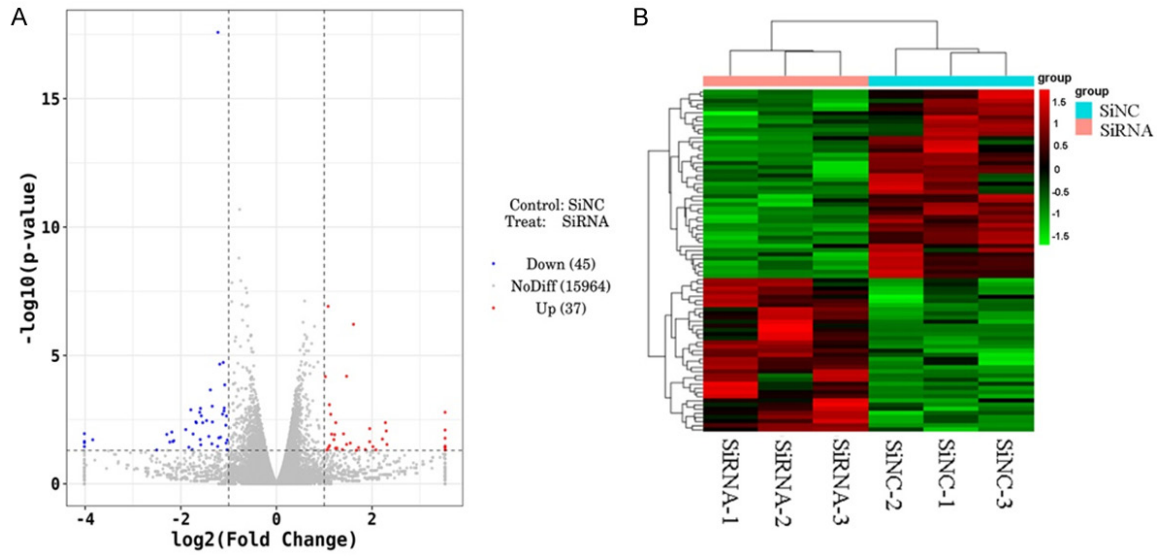


Figure 4. Global view of RNA sequencing results in control and CXCL17 knockdown colorectal cancer cells. A. Volcano mapping of DEGs by the R language ggplots2 package; B. Bivariate clustering analysis of concatenated sets and samples of differential genes for all comparison groups by the R language Pheatmap package.

with siNC or si-CXCL17. The analysis revealed that there were 37 significantly up-regulated DEGs and 45 that were downregulated upon CXCL17 knockdown (**Figure 4A**). Among the top 10 altered genes, *OSGIN1* and *AKR1C1* expression increased; conversely, *CHAC1* and *ULBP1* expression decreased in CXCL17-knockdown HCT15 cells (Supplementary Table 3). In addition, the heatmap showed significant differences in gene expression between the siNC and si-CXCL17 groups (**Figure 4B**).

Gene enrichment analysis to screen key pathways

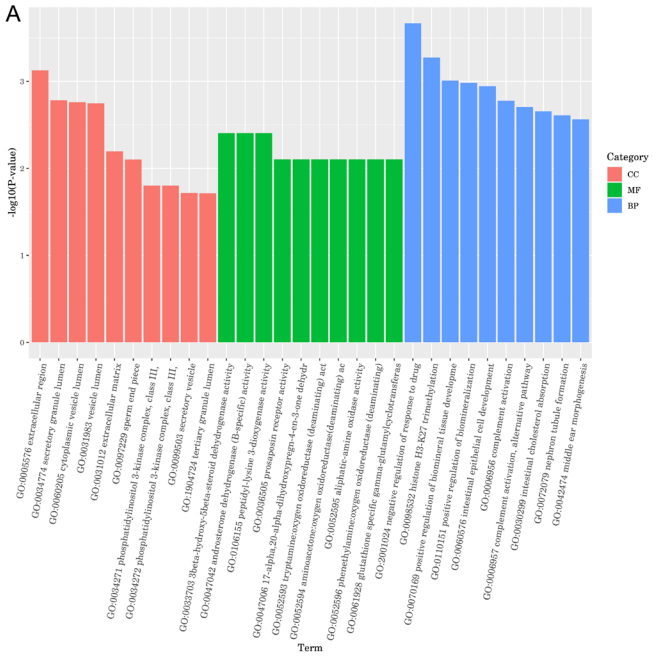
The results of the GO enrichment analysis of the DEGs were classified into cellular components (CC), molecular functions (MF), and biological processes (BP). The results show that the function of CXCL17 was mainly enriched in the extracellular region of the CC (GO:0005576), negative regulation of the drug response by BP (GO:2001024), and histone H3-K27 trimethylation (GO:0098532) (**Figure 5A**). KEGG enrichment analysis showed that certain processes, including SLE, immune disease development, the IL-17 signalling pathway, and phenylalanine metabolism, were significantly enriched in CRC cells upon CXCL17 knockdown (**Figure 5B**).

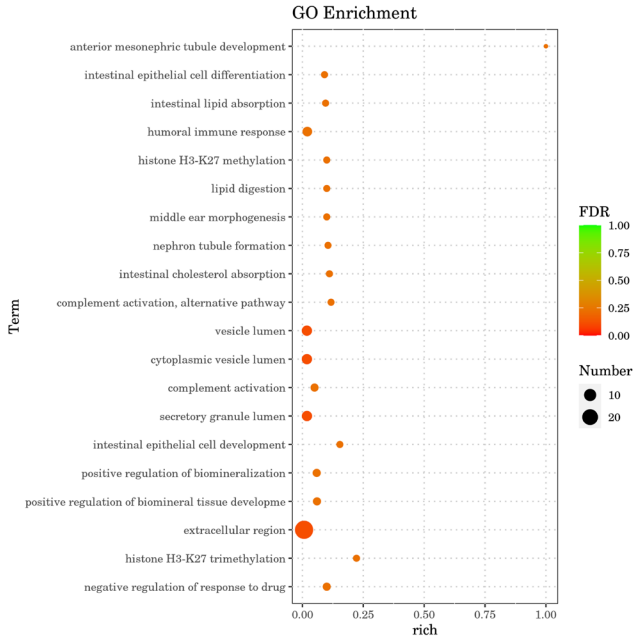
Knockdown of CXCL17 inhibits the IL-17 signalling pathway

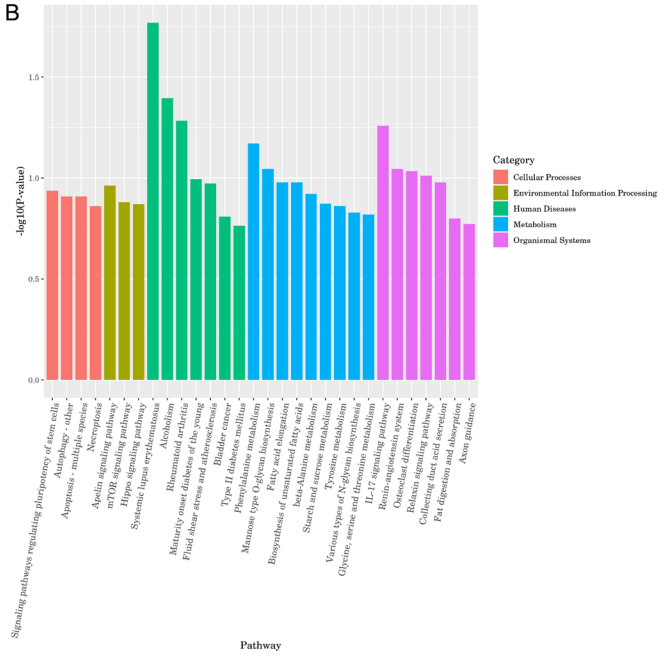
The upregulated genes *FOSB* and *MMP1* were enriched in the IL-17 signalling pathway, which was further validated by RT-qPCR (**Figure 6A**). We then determined the protein levels of the key effectors of the IL-17 signalling pathway. Western blot analysis revealed that knockdown of CXCL17 significantly reduced the expression levels of IL-17A and IL-17F, indicating that CXCL17 silencing inhibited IL-17 signalling (**Figure 6B**, $P < 0.01$).

Knockdown of CXCL17 inhibits the IL-17 signalling pathway to affect drug resistance and tumourigenic properties of CRC cells

To further investigate the relationship between the IL-17 signalling pathway and drug resist-


A
-log10(P-value)
Term
Category
CC
MF
BP
GO:0005576 extracellular region
GO:0034774 secretory granule lumen
GO:0060205 cytoplasmic vesicle lumen
GO:0031983 vesicle lumen
GO:0031012 extracellular matrix
GO:0097229 sperm end piece
GO:0034271 phosphatidylinositol 3-kinase complex, class III,
GO:0034272 phosphatidylinositol 3-kinase complex, class III,
GO:0090568 secretory vesicle
GO:1904724 tertiary granule lumen
GO:0033703 3beta-hydroxy-5beta-steroid dehydrogenase activity
GO:0047042 androsterone dehydrogenase (B-specific) activity
GO:0106155 peptidyl-lysine 3-dioxygenase activity
GO:0030508 prosaposin receptor activity
GO:0047006 17-alpha,20-alpha-dihydroxypregn-4-en-3-one dehydr
GO:0052593 cryptanimine:oxygen oxidoreductase (deaminating) ac
GO:0052594 aminoacetone:oxygen oxidoreductase (deaminating) act
GO:0052595 aliphatic-amine oxidase activity
GO:0052596 phenethylamine:oxygen oxidoreductase (deaminating)
GO:0061928 glutathione specific gamma-glutamylcyclotransferas
GO:2001024 negative regulation of response to drug
GO:0098532 histone H3-K27 trimethylation
GO:0070189 positive regulation of biomineral tissue developme
GO:0110151 positive regulation of biomineralization
GO:0060576 intestinal epithelial cell development
GO:0006956 complement activation, alternative pathway
GO:0030449 regulation of complement activation, alternative pathway
GO:0030299 intestinal cholesterol absorption
GO:0072079 nephron tubule formation
GO:0042474 middle ear morphogenesis


GO Enrichment
Term
rich
FDR
Number
anterior mesonephric tubule development
intestinal epithelial cell differentiation
intestinal lipid absorption
humoral immune response
histone H3-K27 methylation
lipid digestion
middle ear morphogenesis
nephron tubule formation
intestinal cholesterol absorption
complement activation, alternative pathway
vesicle lumen
cytoplasmic vesicle lumen
complement activation
secretory granule lumen
intestinal epithelial cell development
positive regulation of biomineralization
positive regulation of biomineral tissue developme
extracellular region
histone H3-K27 trimethylation
negative regulation of response to drug


B
-log10(P-value)
Pathway
Category
Cellular Processes
Environmental Information Processing
Human Diseases
Metabolism
Organismal Systems
Signaling pathways regulating pluripotency of stem cells
Autophagy - other
Apoptosis - multiple species
Necroptosis
Apelin signaling pathway
mTOR signaling pathway
Hippo signaling pathway
Systemic lupus erythematosus
Alcoholism
Rheumatoid arthritis
Maturity onset diabetes of the young
Fluid shear stress and atherosclerosis
Bladder cancer
Type II diabetes mellitus
Phenylalanine metabolism
Mannose type O-glycan biosynthesis
Biosynthesis of unsaturated fatty acids
Fatty acid elongation
beta-Alanine metabolism
Starch and sucrose metabolism
Tyrosine metabolism
Various types of N-glycan biosynthesis
Glycine, serine and threonine metabolism
IL-17 signaling pathway
Renin-angiotensin system
Osteoclast differentiation
Relaxin signaling pathway
Collecting duct acid secretion
Fat digestion and absorption
Axon guidance

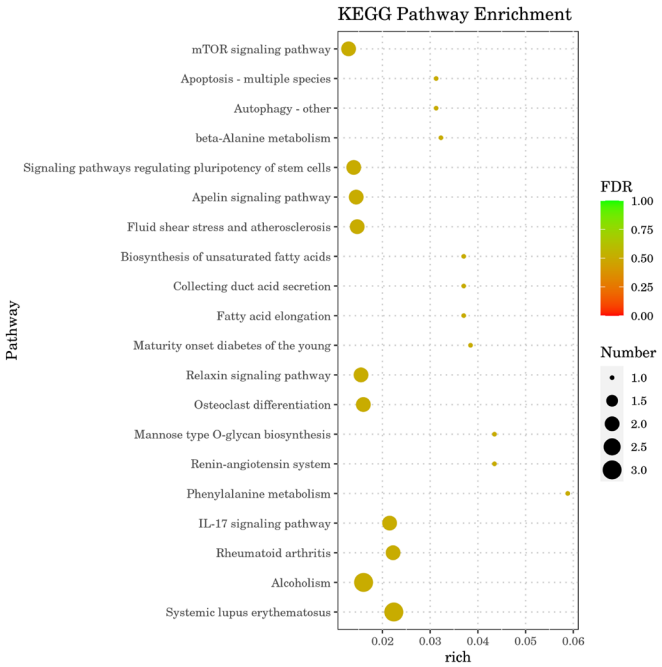

KEGG Pathway Enrichment
Pathway
rich
FDR
Number
mTOR signaling pathway
Apoptosis - multiple species
Autophagy - other
beta-Alanine metabolism
Signaling pathways regulating pluripotency of stem cells
Apelin signaling pathway
Fluid shear stress and atherosclerosis
Biosynthesis of unsaturated fatty acids
Collecting duct acid secretion
Fatty acid elongation
Maturity onset diabetes of the young
Relaxin signaling pathway
Osteoclast differentiation
Mannose type O-glycan biosynthesis
Renin-angiotensin system
Phenylalanine metabolism
IL-17 signaling pathway
Rheumatoid arthritis
Alcoholism
Systemic lupus erythematosus

Figure 5. Enrichment analysis of DEGs upon CXCL17 deletion in CRC cells. A. Bar and bubble chart of GO enrichment analysis. B. Bar and bubble plots of KEGG enrichment analysis.

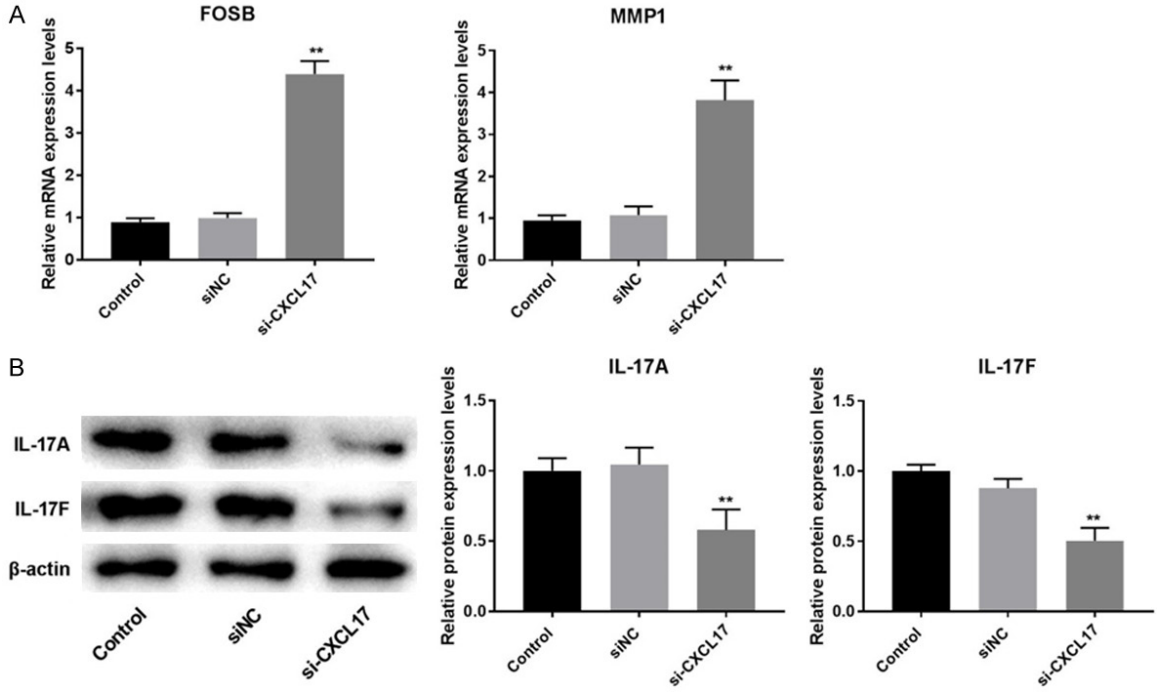


Figure 6. CXCL17 deletion suppresses the IL-17 signaling pathway. A. RT-qPCR was used to detect the mRNA levels of FOSB and MMP1, which were enriched in the IL-17 signaling pathway; B. representative result of western blotting analysis for the protein levels of key effectors in IL-17 signaling pathway. Column diagrams were performed to account the relative protein levels in the right bottom panel. Compared with siNC group, $^{**}P < 0.01$.

ance, we treated HCT15 cells with IL-17A protein after CXCL17 silencing to examine cell activity, migration, and invasion. CXCL17 knockdown followed by IL-17A supplementation significantly reduced the viability of CRC cells (**Figure 7A**, $P < 0.01$). Cells were treated with different concentrations of taxol to detect cellular drug resistance. The results show that drug resistance was reduced after knockdown of CXCL17 and was enhanced following the addition of IL-17A (**Figure 7B**, $P < 0.01$). The migration and invasion abilities of HCT15 cells were both significantly enhanced after treatment with IL-17A relative to those of CXCL17-silenced cells (**Figure 7C**, **7D**, $P < 0.01$). In addition, IL-17A supplementation reversed the G2/M phase block ($P < 0.01$) and reduced the apoptosis ($P < 0.05$) induced by CXCL17 knockdown in HCT15 cells (**Figure 7E**, **7F**). Thus, the combined findings demonstrate that IL-17 may be a downstream signalling pathway of CXCL17, which plays a key role in tumourigenesis and drug resistance.

Knockdown of CXCL17 affects tumour growth in mice by suppression of the IL-17 signalling pathway

To evaluate the effects of CXCL17 on tumour growth, we performed subcutaneous xenograft experiments using HCT15 cells transfected with siNC or si-CXCL17, with or without IL-17A supplementation. Knockdown of CXCL17 inhibited tumour growth in mice, resulting in a significant decrease in tumour volume and weight, and supplementation with IL-17A reversed the reduction in tumour growth upon CXCL17 silencing (**Figure 8A-C**, $P < 0.01$). In addition, H&E staining showed that CXCL17 silencing resulted in loose tumour tissues compared to the control. Meanwhile, addition of the cytokine IL-17A tightened the tumour tissues compared to their CXCL17-silenced counterpart (**Figure 8D**). Immunohistochemistry revealed that the number of positively stained cells was significantly reduced by CXCL17 knockdown. Following the addition of the cytokine IL-17A, the

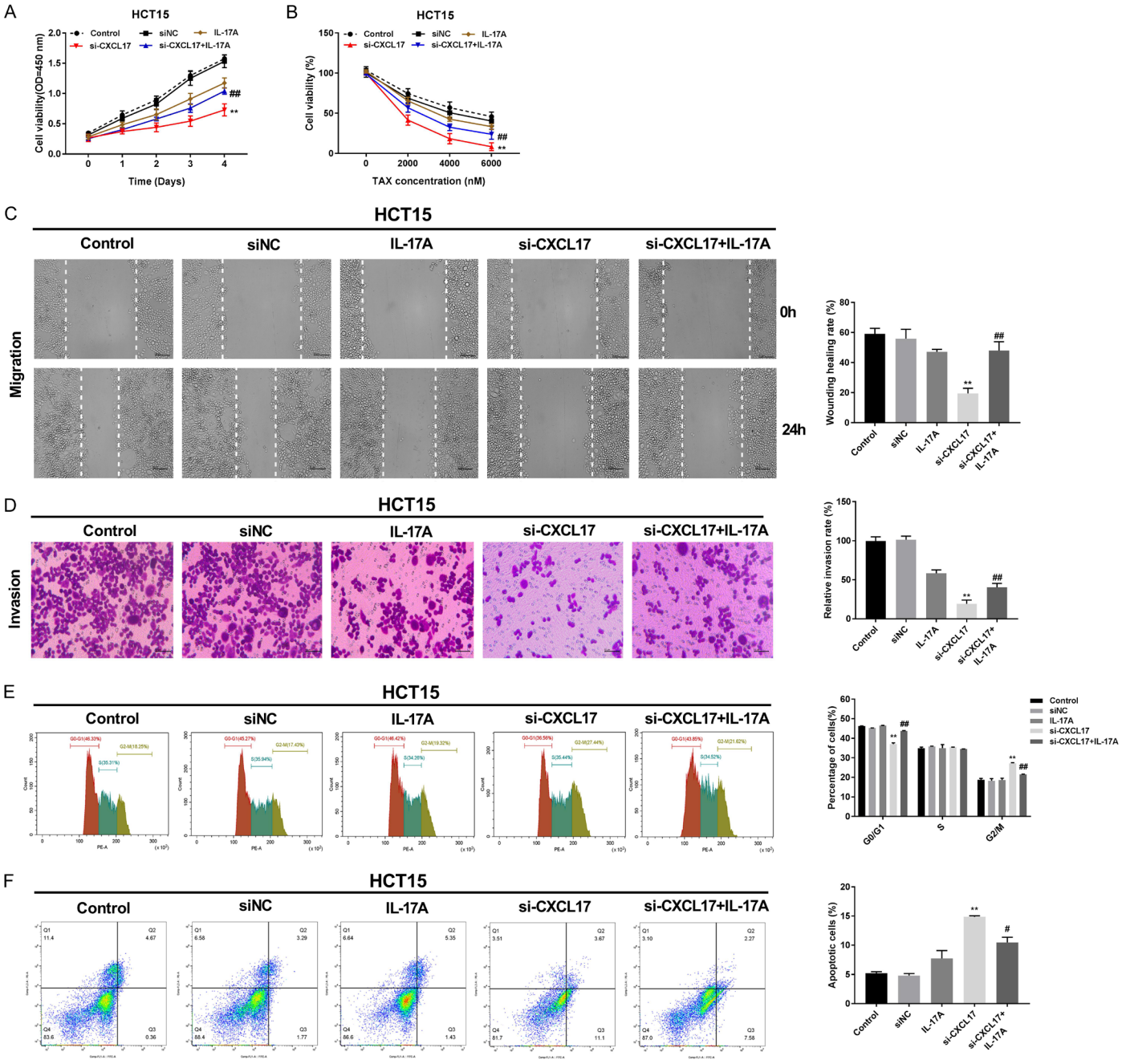

A
HCT15
Control
siNC
IL-17A
si-CXCL17
si-CXCL17+IL-17A
Cell viability(OD=450 nm)
Time (Days)
B
HCT15
Cell viability (%)
TAX concentration (nM)
C
HCT15
Migration
0h
24h
Wounding healing rate (%)
D
HCT15
Invasion
Relative invasion rate (%)
E
HCT15
Percentage of cells(%)
G0/G1
S
G2/M
F
HCT15
Apoptotic cells (%)

Figure 7. IL-17 signaling pathway is involved in tumorigenesis and drug resistance of HCT15 cells. A. CCK-8 assay was performed to detect cell viability of HCT15 cells with or without CXCL17 silence upon IL-17A supplementation; B. CCK-8 assay for cell resistance was applied to detect the cell viability of HCT15 cells with or without CXCL17 silence which treated with different concentrations of taxol; C. Wound-healing assay was used to evaluate cell migration ability of HCT15 cells treated with IL-17A followed by siNC or si-CXCL17 transfection; D. Transwell assay was applied to analyze cell invasion ability of HCT15 treated with IL-17A followed by siNC or si-CXCL17 transfection; E. Flow cytometry was used to detect the cell cycle of HCT15 cells treated with IL-17A followed by siNC or si-CXCL17 transfection; F. Flow cytometry was used to detect the cell apoptosis of HCT15 cells treated with IL-17A followed by siNC or si-CXCL17 transfection. Scale bar was 50 μm. Compared with the siNC group, $^{**}P < 0.01$; compared with the si-CXCL17 group, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

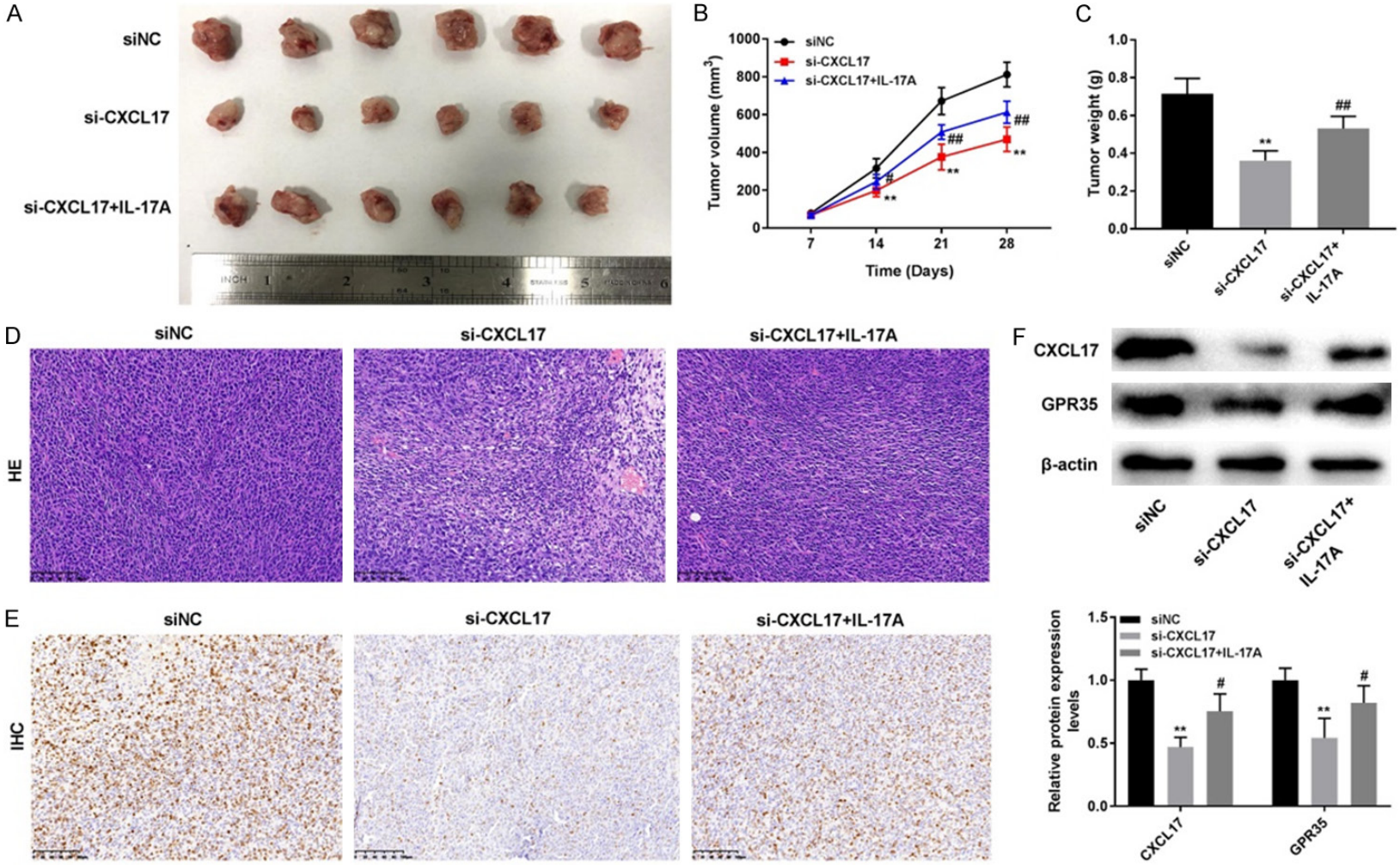


Figure 8. CXCL17 elimination suppresses tumorigenesis via regulating IL-17 signaling pathway. A-C. Measurement of tumor volume and weight from subcutaneously injected xenograft model with or without CXCL17 silence upon IL-17 addition; D. HE staining was used to observe tumor pathological changes (Scale bars, 50 μm) with or without CXCL17 silence upon IL-17 addition; E. Immunohistochemistry was performed to assess CXCL17 expression (Scale bars, 50 μm) in tumor; F. Western-blot assay was applied to detect CXCL17 and receptor GPR35 expression in tissues. Compared with the siNC group, $^{**}P < 0.01$; compared with the si-CXCL17 group, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

number of positively stained cells was significantly increased compared to in CXCL17-deleted HCT15 cells (**Figure 8E**). Additionally, the protein levels of CXCL17 and its receptor GPR35 were reduced in mouse tumour tissues upon CXCL17 knockdown, and IL-17A addition significantly restored the downregulation of CXCL17 and GPR35 expression (**Figure 8F**, $P < 0.01$). Taken together, these results demonstrate that the CXCL17-GPR35 pathway may play an important role in tumour growth in vivo through the IL-17 signalling pathway.

Discussion

Because CRC is one of the most malignant diseases worldwide, its therapy is challenging. OXA is a common and efficient antitumour drug for CRC chemotherapy. However, OXA resistance is one of the most challenging issues encountered during CRC treatment [5, 6]. Acquisition of resistance is a complex event involving multiple regulatory networks [31]. Therefore, elucidating the underlying mechanisms is particularly important for the treatment of drug-resistant CRC. The present study shows that the CXCL17-GPR35 axis is involved in the regulation of cell proliferation, migration, and invasion of HCT15 cells, which are processes related to activation of the IL-17 signalling pathway. Elevated expression of CXCL17 and its receptor-GPR35 was found in drug-resistant CRC cells. CXCL17 knockdown inhibited tumourigenesis and drug resistance, which were restored by IL-17A supplementation.

Chemokines are low-molecular-weight proteins involved in the chemotaxis of leukocytes to inflammatory sites, which are important components of the tumour microenvironment (TME). They can directly or indirectly participate in the antitumour immune response by targeting non-immune cells in the TME, including tumour cells and vascular endothelial cells, thereby regulating tumour cell proliferation, tumour stem cell-like cell characteristics, and tumour invasion and metastasis, thereby affecting cancer progression, patient treatment, and prognosis [32-34]. The previous studies have demonstrated that some chemokines and its receptor are involved in cell invasion, migration, and proliferation in colon cancer, such as the CXCR4-CXCL12 [35], CXCR6-CXCL16 [36], and CXCR5-CXCL13 axes [37]. CXCL17 is another inflammatory factor that plays an important role in a variety of tumours, such as gastric cancer [17] and breast cancer [38], by binding to its receptor GPR35. Yao et al. have reported that CXCL17 and its receptor GPR35 are expressed at higher levels in colon cancer samples than in tumour-adjacent tissues [18]. In our study, we found that drug-resistant tumour cells had high expression levels of CXCL17 and GPR35 and that GPR35 expression was significantly reduced after knockdown of CXCL17. In addition, inhibition of CXCL17 expression significantly reduced the viability, migration, and invasion of drug-resistant CRC cells with or without taxol supplementation. Inhibition of CXCL17 expression arrested cells in the G2/M phase and promoted apoptosis. Consistent with previous results, our findings suggested that CXCL17 and GPR35 play pathogenic roles in CRC and drug-resistant CRC. CXCL17 also plays an important role in angiogenesis, which is essential for tumour growth [12]. Antiangiogenic drugs can be delivered directly to the target via the blood and are less likely to develop resistance. Therefore, CXCL17 can be used as a target for antiangiogenic drugs to complement CRC treatment. In summary, these findings provide a new perspective of drug resistance and for increasing the efficiency of chemotherapy.

Interleukin (IL)-17 is a proinflammatory cytokine secreted by Th17 cells that plays a significant role in the immune system and cancer development, including initiation, metastasis, and proliferation [29, 42]. In addition, IL-17 can promote resistance to DPP [43] which is widely used in CRC treatment [44]. However, the exact function of IL-17A remains unclear. On the one hand, IL-17A is stimulates cancer cell proliferation and migration [45, 46]; On the other hand, it has an inhibitory effect on tumorigenesis

[47]. In our study, activation of the IL-17 signalling pathway by the CXCL17-GPR35 axis enhanced cell viability, migration, and invasion, further promoting drug resistance and CRC tumourigenesis and thus confirming that IL-17 may be a promising therapeutic target for drug resistance in CRC. In addition, IL-17 inhibits the production of CXCL9/10 in CRC cells, thereby increasing $CD8^+$ cytotoxicity and Treg infiltration into CRC cells to further promote the development of CRC [49].

In this study, we found that CXCL17 and its receptor GPR35 were abundant in drug-resistant CRC cells and that deletion of CXCL17 reduced cell migration, invasion, drug resistance, and the development of CRC. Moreover, CXCL17 knockdown affected the CRC cell cycle and promoted apoptosis. CXCL17 knockdown decreased the expression of IL-17 pathway-related proteins. The addition of IL-17A partly reversed the effects of CXCL17 silencing on CRC drug resistance by increasing cell viability, migration, and invasion. In summary, our data suggest that the CXCL17-GPR35 biological axis is a critical factor for mediating drug resistance in CRC and that CXCL17 inhibition may be an efficient target for treating CRC by regulating the IL-17 signalling pathway.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. The list of siRNA sequences

Gene name	Sense	Antisense
siNC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
si-CXCL17-1	CAGGGAACCUUCCAUUAUATT	UAUAAUGGAAGGUUCCCUGTT
si-CXCL17-2	GCCCACUCUUCCAAUUAAATT	UUUAAUUGGAAGAGUGGGCTT
si-CXCL17-3	CAGACACGGUCUUAUUAAATT	UUUAAUAAGACCGUGUCUGTT

Supplementary Table 2. The list of primer sequences

Primer name	Sequences (5'-3')
GAPDH-F	CCGGGAAACTGTGGCGTGATGG
GAPDH-R	AGGTGGAGGAGTGGGTGTCGCTGTT
CXCL17-F	GTTGCTGCCACTAATGCTGA
CXCL17-R	GGTGCCTTTGGTGTCTTGTT
GPR35-F	GTGTTCGTGGTCTGCTTCCT
GPR35-R	GAGAGTCCTGGCTTTTGTGG
FOSB-F	AGGAAGAGGAGAAGCGAAGG
FOSB-R	CTTCGTAGGGGATCTTGCAG
MMP1-F	GGTCTCTGAGGGTCAAGCAG
MMP1-R	AGTTCATGAGCTGCAACACG

Supplementary Table 3. Top 10 up-regulated and down-regulated results of DEGs

Genes	Description	log2Fold Change	Pval	Up/Down
TULP2	TUB like protein 2	2.312	0.029	Up
C2	Complement C2	2.297	8.79E-03	Up
NPC1L1	NPC1 like intracellular cholesterol transporter 1FT	2.279	4.16E-03	Up
SLC8A2	Solute carrier family 8 member A2	2.216	0.018	Up
BOLA2B	Bola family member 2B	2.081	0.048	Up
MMP10	Matrix metallopeptidase 10	2.017	0.036	Up
H2AC13	H2A clustered histone 13	1.951	7.14E-03	Up
OSR1	Odd-skipped related transcription factor 1FT	1.949	0.023	Up
SPRN	Shadow of prion protein	1.860	0.046	Up
GPR37L1	G protein-coupled receptor 37 like 1	1.718	0.039	Up
POM121L2	POM121 transmembrane nucleoporin like 2	-4.016	0.022	Down
MB	Myoglobin	-3.841	0.019	Down
SYT11	Synaptotagmin 11	-2.503	0.048	Down
NKX3-2	NK3 homeobox 2	-2.293	0.012	Down
CFAP74	Cilia and flagella associated protein 74FT	-2.231	0.024	Down
ENPEP	Glutamyl aminopeptidase	-2.188	9.71E-03	Down
SUSD4	Sushi domain containing 4	-2.167	0.023	Down
VSIG2	V-set and immunoglobulin domain containing 2FT	-2.146	0.021	Down
SVOP	SV2 related protein	-1.895	7.73E-03	Down
GPR132	G protein-coupled receptor 132	-1.831	0.038	Down