Supplementary Materials and methods

Cell viability assay. After adhering to a 96-well plate for 24 h, the cells (6,000 cells per well) were treated with or without various concentrations of shikonin (DMSO as a control) for 10 h, and cell viability was determined using a Cell Counting Kit-8 (cat. no. HY-K0301; MedChemExpress) according to the manufacturer's instructions.

Cell culture, treatment and antibodies. NCM460 cells (cat. no. C1227) were obtained from WarnerBiotech, and authenticated via STR profiling and mycoplasmas were tested monthly. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nanjing KeyGen Biotech Co., Ltd.) supplemented with high glucose and fetal bovine serum (10X; Gibco; Thermo Fisher Scientific, Inc.) and penicillin/streptomycin (100X; Sigma-Aldrich; Merck KGaA). Prior to treatment, the cells were cultured for 12 h in an incubator (37˚C, 5% CO_2). IL-6 (25 ng/ml, 10 h of treatment) was used to detect the expression of p-EGFR, while various concentrations of shikonin (10, 15 and 20 μM, 10 h of treatment) were to determine the potential effect of shikonin on β-catenin levels. The antibodies (1:1,000 dilution) used were as follows: anti-p-EGFR (cat. no. 3777; Cell Signaling Technology, Inc.), anti-β-catenin (cat. no. 8480; Cell Signaling Technology, Inc.) and anti-EGFR (cat. no. ET1603-37; HuaBio). Western blot analysis was performed as described in Materials and methods section in the manuscript.

Annexin V/PI assay. Cells (5,000 cells per well) were seeded in 96-well plates for 12 h and treated with or without shikonin (10 or 20 μM, DMSO as a control) for 10 h. The following experiments were performed with Annexin V-FITC/PI cell apoptosis detection assay (cat. no. KGA108; Nanjing KeyGen Biotech Co., Ltd.) according to the manufacturer's instructions. The stained cells were imaged under a fluorescence microscope (Guangzhou Micro-shot Technology Co., Ltd.).

Figure S1. Shikonin promotes apoptosis as evidenced by the enhanced number of positively stained spots in the Annexin V/PI immunofluorescence staining assay. (A) Spots of Annexin V (green) and PI (red) imaged in HCT116 cells. (B) Spots of Annexin V (green) and PI (red) imaged in SW480 colon cancer cells. Scale bars, 50 μm.

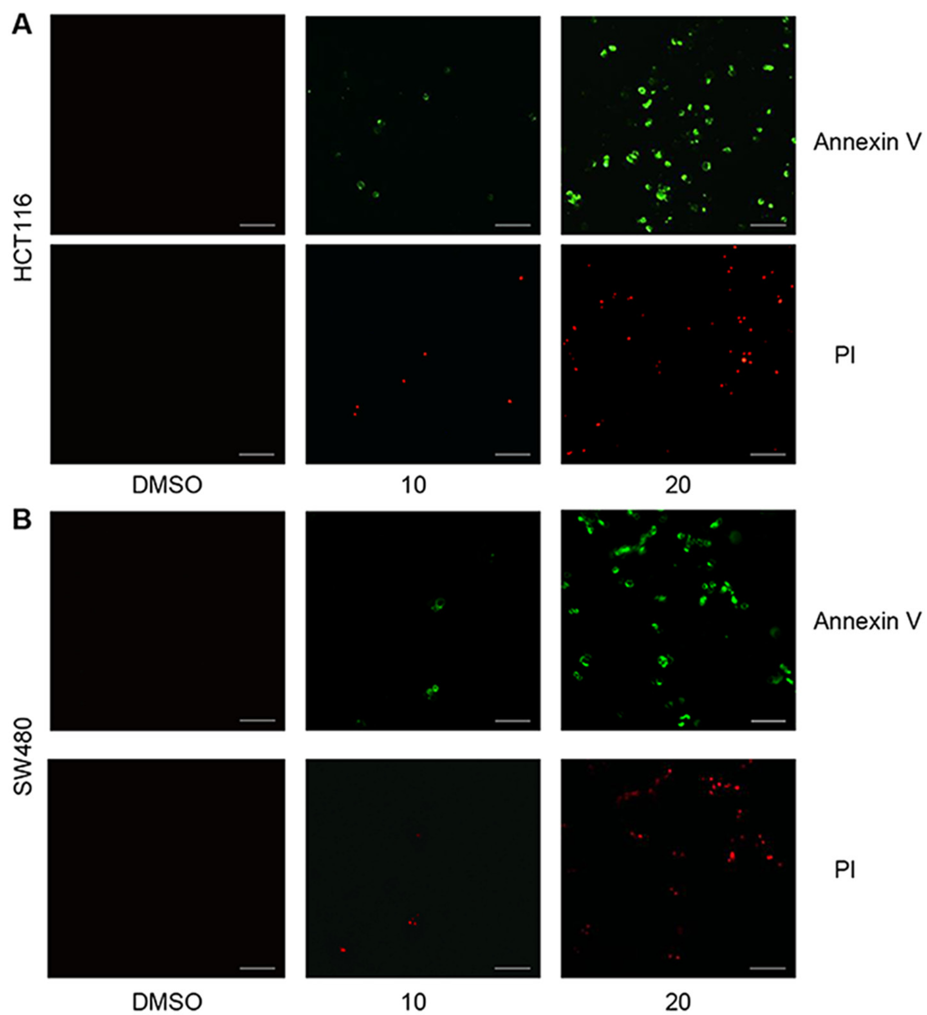

Figure S2. Treatment with 25 ng/ml IL-6 does not induce the phosphorylation of EGFR in HCT116 and SW480 cells. Quantitative data of p-EGFR were normalized to GAPDH or t-EGFR. The quantitative data of the control group were arbitrarily set as 1. All data represent the mean ± SEM. ns, no significance. IL, interleukin; EGFR, epidermal growth factor receptor.

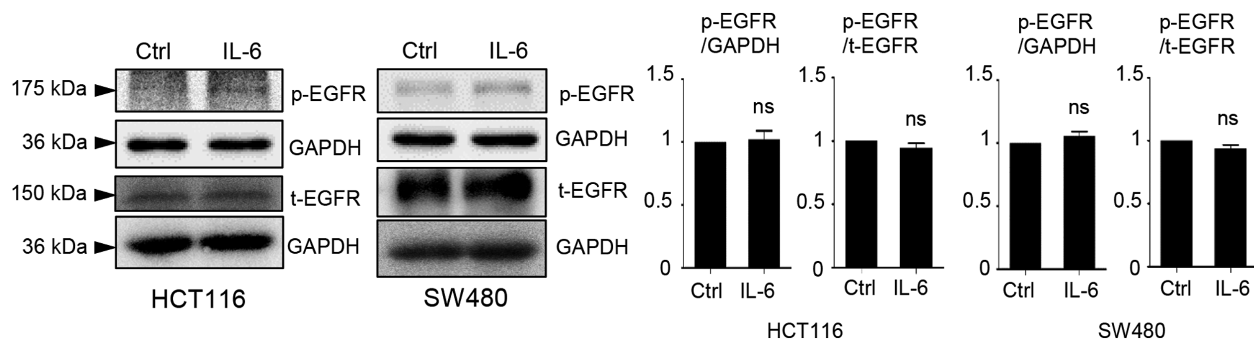

Figure S3. Shikonin does not affect the expression of β-catenin in the both cancer cell lines. Shikonin (10, 15 and 20 μM) was added to the medium for 10 h. Quantitative data of β-catenin were normalized to GAPDH. The quantitative data of the DMSO group (0 μM SKN) were arbitrarily set as 1. All data represent the mean ± SEM. ns, no significance.

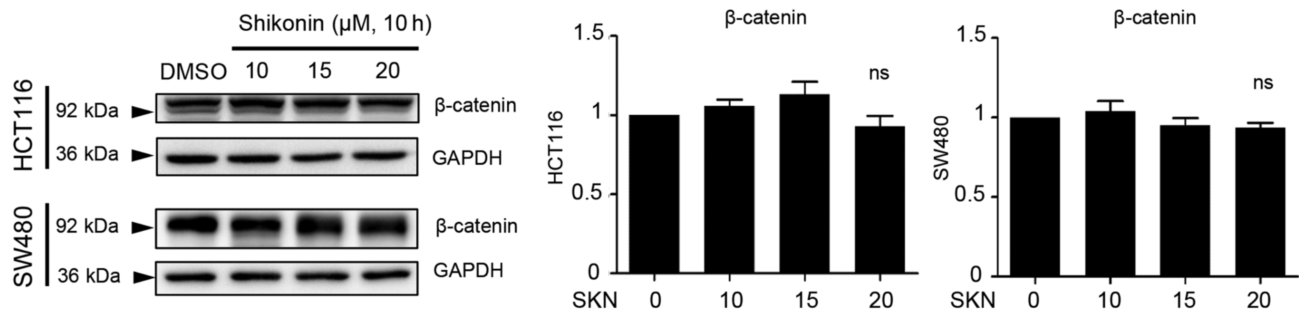

Figure S4. Effect of SKN on NCM460 cells. Cell viability of the DMSO group (0 μM SKN) was arbitrarily set as 1. *P<0.05 vs. DMSO group. SKN, shikonin.