Appendix 1

Methods

Gene chip analysis and HCS of cell function

The Affymetrix Clariom™ S gene chip was used to determine the gene expression spectrum of MKN-45/X cells before and after Ubenimex treatment and assess changes in gene expression. In this study, the fold change of RI values of MKN-45/X cells was evaluated by the HCS, from which genes with the most significant differential fold changes were screened out. The effect of silencing CD13 expression on the mRNA level of the above genes was observed by HCS experiment, and the key genes downstream of CD13 were selected.

Plasmid construction and gene amplification

EGFP-labeled ANPEP (CD13; GenBank number NM_001150), EMP3 (GenBank number NM_001425) overexpression plasmids and negative control plasmids (empty vector control plasmid; CMV-MCS-3FLAG-IRES-EGFP-SV40-Neomycin) were used in this study. The following primer pairs were designed based on the ANPEP and NAB1 mRNA sequences: ANPEP-p1, 5'-TACCGG ACTCAGATCTCGAGCGCCACCATGGCCAAGGGCTTCTATATTTCCAAG-3'; ANPEP-p2, 5'-TCCTTGTAGTCCATGGATCCTTTGCTGTTTTCTGTGAACCACTGGAGCAC-3'; EMP3-p1, 5'-GAGGATCCCCGGGTACCGGTCGCCACCATGGCGGAGCCGAGCGGC-3'; EMP3-p1, 5'-TCACCATGGTG GCGACCGGGCTGACACTCAACTGAGCA-3'. The PCR mixture contained 2 μL of cDNA template, 1 μL of primer (10 μmol/L), 10 μL of PCR mix and dH_2O to a total volume of 20 μL. The PCR program was as follows: 94 ℃ for 5 min; 40 cycles of 94 ℃ for 30 s, 58 ℃ for 30 s and 72 ℃ for 5 min; and a final extension at 72 ℃ for 5 min. The final PCR products were separated by electrophoresis using 1% polyacrylamide gels, and the target fragment was purified and recovered using an agarose gel extraction kit (Watson Biomedical Inc., Shanghai, China).

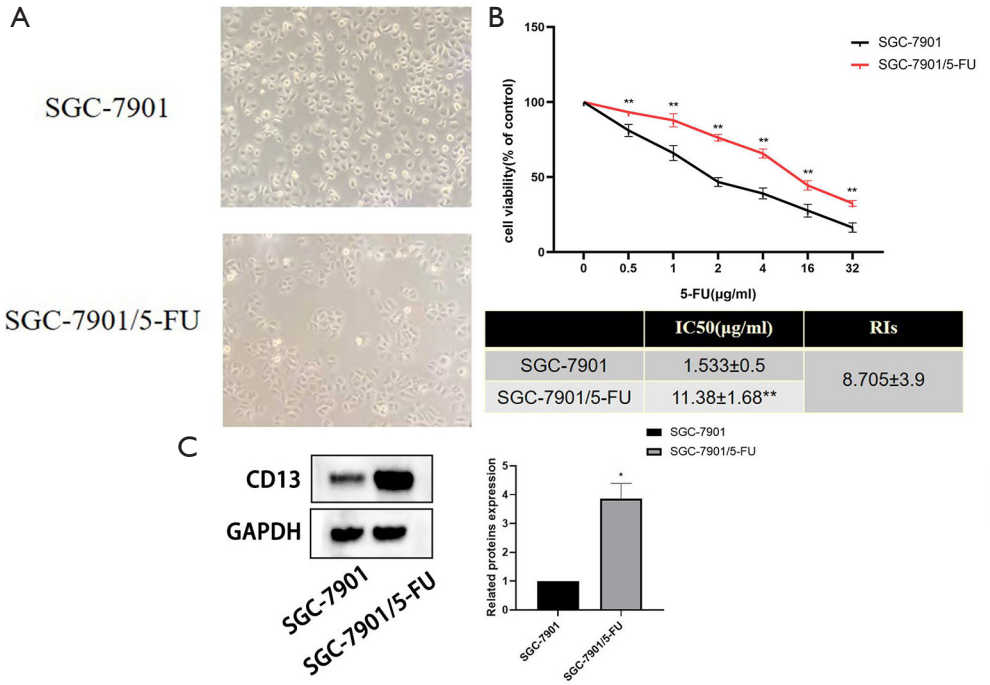
Proliferative activity and cell sensitivity assays

Human GC parental cell line and 5-FU resistant cell were dispensed into 96-well culture plates (5×10^3 cells/well) and incubated at 37 ℃ for 0–48 h to evaluate proliferative activity. In addition to evaluate the resistant cell sensitivity to 5-FU, SGC-7901/5-FU cells were incubated with various concentrations of 5-FU (0.5, 1, 2, 4, 16, 32 μg/mL) for 48 h, in the presence or absence of ubenimex (0.12 mg/mL); or were incubated with 5-FU (12 μg/mL) for 0–72 hours in the presence or absence of ubenimex (0.12 mg/mL). Finally, 10 μL CCK-8 solution was added into each well. the cells were then incubated for another 4 h, and the absorbance at 450 nm was read by a microplate reader (EL340; Bio-Tek Instruments, Hopkinton, MA, USA). The resulting absorbance is used to generate the growth curve to assess the proliferative activity or cell sensitivity.

Western blotting

Before lysis, cells were incubated with different treatments to determine the expression level of protein, then, cold PBS washed the cells and proteins were extracted from the cells by resuspension in cold radioimmunoprecipitation assay (RIPA) buffer containing 1% phenylmethylsulfonyl fluoride (PMSF) and 1% phosphatase inhibitor for 15 min. The samples were centrifuged at 12,000 rpm for another 15 min at 4 °C, and the supernatants were recovered for continued analysis. The bicinchoninic acid (BCA) protein assay kit (Solarbio, Beijing, China) was used for the measurement of protein concentration. Subsequently, SDS-PAGE was used to separate proteins and which were transferred to polyvinylidene difluoride (PVDF) membranes. After incubating with different primary antibodies overnight at 4 ℃, the membranes incubated for another 1.5 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. Subsequently, immunoblots were visualized using enhanced chemiluminescence (LAS-4000), the experiments were repeated at least three times.

https://dx.doi.org/10.21037/tcr-22-345



	IC50(μg/ml)	RIs
SGC-7901	1.533±0.5	8.705±3.9
SGC-7901/5-FU	11.38±1.68**	

Figure S1 Establishment of 5-FU resistant GC cells. (A) SGC7901/5-FU cells and their parental cells. (B) SGC7901/5-FU cells and their parental cells were treated with increasing concentrations of 5-FU (0.5, 1, 2, 4, 16, 32 μg/mL) for 48 h. Cell viability was determined by the CCK-8 method. The results are expressed as the means ± SD of three replicates. **P<0.01. (C) Western blotting analysis of CD13 in parental and SGC-7901/5-FU cells. GAPDH is shown as a loading control. Quantification was performed by a densitometry analysis and normalized to GAPDH levels. The bars indicate the means ± SD from the 3 independent experiments (*P<0.05). 5-FU, 5-fluorouracil; RI, resistance index; GC, gastric cancer; CCK-8, Cell Counting Kit-8; SD, standard deviation.

A

Gene Symbol	mRNA Fold Change(After CD13 down-regulation	Regulation
EMP3	8.699537426	Down
PTK2	8.408637398	Down
RELA	7.714647979	Down
BAK1	7.613932323	Down
BECN1	7.032987828	UP
BCL2L1	7.025541418	Down
BCL-2	7.023929478	Down
BCL2L4	6.824932378	Down
DIABLO	6.808841425	UP
CASP3	6.799591928	Down
APAF1	6.762938928	UP
ATG12	6.389432328	Down
ATG5	5.906001318	UP
MAP1LC3	5.785109163	Down
HMGB1	5.619668501	Down

Fold change

B

Gene Symbol	mRNA Fold Change(After EMP3 down-regulation	Regulation
PTK2	8.408637398	Down
RELA	7.714647979	Down
BECN1	7.047199391	UP
BCL2L1	6.666254896	Down
BCL-2	6.658808486	Down
BAK1	6.613932323	Down
BCL2L4	6.500690446	Down
DIABLO	6.301693346	UP
CASP3	6.285602393	Down
APAF1	5.776352896	UP
ATG12	5.739699896	Down
ATG5	5.366193296	UP
MAP1LC3	4.882762286	Down
HMGB1	4.761870131	Down

Fold change

Figure S2 The effect of *CD13* silence and *EMP3* silence on the expression of indicated genes. (A) The *CD13* gene was silenced to observe its effect on the expression of other related genes and ranked. (B) The *EMP3* gene was silenced to observe its effect on the expression of other related genes and ranked. EMP3, epithelial membrane protein 3.

https://dx.doi.org/10.21037/tcr-22-345