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Original Article

Expression of SLC22A18 regulates oxaliplatin resistance by modulating the ERK pathway in colorectal cancer

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Abstract: Although oxaliplatin-based chemotherapy is the current standard adjuvant therapy for colorectal cancer (CRC), the molecular mechanisms underlying oxaliplatin resistance remain unclear. Here, we examined the molecular mechanisms underlying SLC22A18-associated oxaliplatin resistance and strategies for overcoming oxaliplatin resistance. We evaluated the association between SLC22A18 and prognosis in 337 patients with CRC and its functional significance and studied the mechanisms through which SLC22A18 affects oxaliplatin resistance development in CRC cells, using CRC cell lines and patient-derived cells (PDCs). SLC22A18 downregulation was positively correlated with worse survival in patients with CRC. Low SLC22A18-expressing cells showed relatively lower sensitivity to oxaliplatin than high SLC22A18-expressing cells. In addition, ERK activation was found to be involved in the mechanisms underlying SLC22A18-related oxaliplatin resistance. To confirm ERK pathway dependence, we used an ERK inhibitor and found that combined treatment with oxaliplatin and the ERK inhibitor overcame oxaliplatin resistance in the low SLC22A18-expressing cells. Ex vivo approaches using PDC confirmed the correlation between SLC22A18 expression and oxaliplatin resistance. Results of the in vivo study showed that SLC22A18 expression regulated oxaliplatin efficacy, and that combined treatment with an ERK inhibitor could be a useful therapeutic strategy when SLC22A18 is downregulated. Together, our findings indicate that SLC22A18 could serve as a biomarker for the prediction of oxaliplatin resistance. In cases of oxaliplatin resistance due to low SLC22A18 expression, resistance can be overcome by combined treatment with an ERK inhibitor.

Keywords: Oxaliplatin-resistance, SLC22A18, colorectal cancer, ERK, combined treatment

Introduction

Colorectal cancer (CRC) is currently the one of the highest ranked cancers in terms of both mortality and incidence rate [1]. Unfortunately, 40% of the CRC patients experience tumor recurrence due to the development of drug resistance [2], with a 5-year survival rate below 10% [3]. Oxaliplatin-based chemotherapy is the current standard adjuvant therapy for advanced CRC and a first-line treatment option in the case of metastasis; it is also one of the most frequently used drugs in combination therapy [4, 5]. Although oxaliplatin induces cell death by inhibiting DNA synthesis, replication, and transcription [6], it has been proven to be less effective against transformed cancerous cells [7], and oxaliplatin resistance is a major cause of failure of CRC chemotherapy. Several molecular mechanisms thought to be responsible for oxaliplatin resistance in CRC have been proposed, including (1) activation of anti-apoptosis pathways [8-10], (2) insufficient drug uptake [11], and (3) intensification of DNA repair processes [12]. Some combined regimens are thought to synergistically overcome drug resistance. Combination treatment strategies such as irinotecan, capecitabine, and bevacizumab

have been evaluated for CRC cancers [13-15]. However, the majority of combination treatments have failed to overcome oxaliplatin resistance in clinical studies, indicating that targeting a single mechanism is not adequate to reverse oxaliplatin resistance. Thus, investigation of individual factors associated with oxaliplatin resistance is paramount to improve current CRC treatment strategies.

Solute carriers (SLC) are transporters that mediate the translocation of inorganic and organic solutes across the transmembrane in various cellular membranes [16]. To date, several SLC family members have been identified as tumor suppressors in various cancers, including SLC5A8 [17], SLC26A3 [18], and SLC39A1 [19]. Additionally, several SLC family members have been studied as predictive biomarkers for platinum anti-cancer drugs [20]. Our previous study demonstrated that SLC22A18 (solute carrier family 22, member 18) is a potential prognostic marker for CRC due to its function as a tumor suppressor. We found that SLC22A18 is significantly low expressed in colon cancer tissues compared to normal adjacent tissues [21]. Recent studies show that SLC22A18 is downregulated via DNA methylation, histone acetylation, or transcription factors such as Sp1 [22-25]. However, the exact function of SLC22A18 in tumor-suppressing mechanisms remains to be elucidated, especially in CRC, and it is still unclear whether the tumor-suppressing role of SLC22A18 is associated with drug resistance.

Therefore, in this study, we evaluated the clinical significance of SLC22A18 expression and investigated the molecular mechanisms underlying SLC22A18-related tumor progression with respect to the development of oxaliplatin resistance and providing a novel therapeutic strategy for CRC chemotherapy.

Materials and methods

Patients and data collection

From 2006 to 2007, paraffin-embedded samples from Stage I (n=76), Stage II (n=87), Stage III (n=91), and Stage IV (n=83) colorectal cancer patients were obtained from the Department of Surgery, Samsung Medical Center (Sungkyunkwan University School of Medicine; Seoul, Korea). All patients underwent a surgical procedure on the colon or rectum and details were prospectively entered into the database. Clinical data were retrospectively analyzed from collected medical records and surgical notes. The experiments conducted on patient samples were approved by the institutional review board of Samsung Medical Center.

Immunohistochemistry and tissue microarray analysis

Tissue microarray analysis (TMA) and immunohistochemistry were employed to analyze the expression status of SLC22A18. Tissue cores (diameter, 2 mm) were carefully transferred to recipient paraffin blocks with 24 holes per block. The filled recipient blocks were embedded in paraffin and 4-μm-thick sections were cut and mounted on slides. The TMA slides were dewaxed by heating at 55°C for 30 minutes followed by three 5-minute washes with xylene, and rehydration using three 5-minute washes with 100%, 95%, and 80% ethanol (dilutions were prepared in pure distilled water). Antigen retrieval was performed by heating the sections at 95°C for 30 minutes in 10 mM sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide for 30 minutes. Background reactivity was eliminated by incubating the sections in a universal blocking serum (Dako Diagnostics, Glostrup, Denmark) for 30 minutes at room temperature. The sections were incubated for 1 hour with antibodies specific to SLC22A18 (LS-C119205; LS Bio, Seattle, WA, USA), then for 30 minutes with a biotin-labeled secondary antibody. Sections were then incubated with streptavidin-peroxidase (Dako Diagnostics) and left to develop. After slight counterstaining with hematoxylin, the sections were dehydrated and mounted under coverslips for microscopy. SLC22A18 expression was evaluated based on the intensity of staining. The intensity of the stained epithelial cells was assessed by a board-certificated pathologist and scored as follows: 0 (no staining), +1 (weak), +2 (moderate), and +3 (strong). The patients were divided into the following two groups based on the assessed score: a low expression group (scores 0 and +1) and a high expression group (scores +2 and +3).

Cell cultures and reagents

SW480, HT29, HCT116, and SW48 colorectal cancer cells were obtained from the American

Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured with RPMI 1640 or DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco) in a 5% CO_2 incubator at 37°C. Oxaliplatin (S1224), ERK inhibitor (S8534), and cetuximab (A2000) were purchased from Selleckchem (Houston, TX, USA). Cell lines were conducted cell line authentication containing short tandem repeat (STR) profiling and mycoplasma test.

Cell proliferation assay

Cell proliferation was measured in triplicate using a colorimetric assay that determines cellular viability by evaluating the metabolic conversion of a water-soluble tetrazolium salt, WST-1 (Roche, Indianapolis, IN, USA). Viability of the colon cancer cells was assessed at various times, and assays were performed by adding WST-1 directly to the culture wells and incubating for 60-120 minutes at 37°C. Absorbance was measured at a wavelength of 450 nm. Three different experiments were performed for each experimental condition.

Transfection of siRNAs and overexpression vectors

Specific siRNAs for *SLC22A18* and the scrambled control siRNA were purchased from Bioneer (Seoul, South Korea). The following two *SLC22A18* siRNA target sequences were used in RNA interference: 5'-GACUGGCAAUAAACUCCUA-3' and 5'-CAGAACUUACCUGCCUCUU-3'. The *SLC22A18* expression vector and control pcDNA3.1 vector were provided by Dr. Jaesang Kim (Department of Life Science, Ewha Womans University, South Korea) [21]. For transfection experiments with using Lipofectamine 2000 or Lipofectamine RNAiMAX (Invitrogen, Waltham, MA USA), cells were seeded into 6-well plates at a density of 5×10^5 cells per well to achieve 60-70% confluence after overnight growth. Lipofectamine-plasmid complexes were prepared according to the manufacturer's instructions. Transfection efficiency and cell survival rate were analyzed 24-72 hours later.

Cell lysates and western blot analysis

To yield whole cell extracts, cells were lysed using Pro-prep buffer (Intron Biotechnology, Seoul, Korea) with protease inhibitors. Protein extract (10-60 μg) was resolved by SDS-PAGE and transferred to PVDF (Polyvinylidene fluoride) membranes. The membranes were probed with primary antibodies against SLC22A18 (LS-C119205, LS Bio), phosphoERK (#612358, BD Biosciences, San Jose, CA, USA), ERK (#9102, Cell Signaling, Danvers, MA, USA), phosphoAKT (#4060, Cell Signaling), AKT (#4691, Cell Signaling), and β-actin (#3700, Cell Signaling), followed by incubation with secondary antibodies conjugated to horseradish peroxidase (Santa-Cruz). β-actin was used as a loading control in western blot analysis.

Apoptosis assay

Colorectal cancer cells (1.5×10^5 cells/ml) were seeded into 100 mm cell culture dishes and cultured overnight. The cells were treated with oxaliplatin (100 μM), ERK inhibitor (10 μM), or a combination of both. After 30 hours, the cells were washed with PBS and detached by trypsinization using trypsin-EDTA solution. The cell suspensions were washed again and then resuspended in Annexin V binding buffer at a density of 1×10^6 cells/ml. 100 μl of the each cell suspension was transferred to 5 ml round-bottom tube and 3 μl of FITC Annexin V (BD Biosciences) was added. The cell mixtures were incubated for 15 minutes at room temperature in the dark. After adding 400 μl of Annexin V binding buffer to each tube, the death of cells was analyzed by flow cytometry (FACSVerse, BD Biosciences) and all the flow cytometric data were analyzed using FlowJo software (TreeStar).

Patient-derived cell isolation and dissociation

Surgically resected colon cancer tissues were obtained from patients from Samsung Medical Center, Seoul, Korea. Tissues were washed with 70% ethanol three times followed by cold PBS (phosphate buffered saline, pH7.4) washes until the supernatant was clear. Next, tissues were chopped into approximately 5-mm pieces and further washed with cold PBS. These pieces were then incubated with digestion buffer (Dulbecco's modified Eagle medium with 2.5% fetal bovine serum, 1% penicillin/streptomycin [Invitrogen], 75 U/mL collagenase type IV [Gibco], and 125 μg/mL dispase type II [Gibco]) at 37°C for 30-60 minutes. Digested cells were then centrifuged at 200×*g* for 3 minutes to separate adenoma from single cells. Dissociated cells were passed through a 40-μm cell strainer and washed several times with

PBS. Isolated colon cancer cells were counted and embedded in Matrigel on ice and seeded into 24-well cell culture plates.

Subcutaneous mouse model

The efficacy of anti-cancer drugs was measured using an in vivo assay to evaluate the effect of SLC22A18 expression on cancer growth. Briefly, approximately 1×10^6 cells with overexpressed or downregulated SLC22A18 were suspended in 50 μl HBSS, supplemented with 50% Matrigel, and injected subcutaneously into the flanks of 6-week-old female BALB/c nude mice (Orient Bio Group, Seoul, Korea). Tumor size was measured using a caliper, inoculation or as soon as a reduction in vitality was observed. For the drug efficacy test, oxaliplatin (10 mg/kg) and/or an ERK inhibitor (5 mg/kg) were administered intraperitoneally twice a week 13 days after cell inoculation. And tumor volume was calculated using the following formula: (short length × long length × width)/2. Mice were sacrificed 6-8 weeks after inoculation or as soon as a reduction in vitality was observed. For the drug efficacy test, oxaliplatin (10 mg/kg) and/or an ERK inhibitor (5 mg/kg) were administered intraperitoneally twice a week 13 days after cell inoculation.

Statistical analysis

Data from proliferation were analyzed using GraphPad Prism 5.0 software (CA, USA) by applying one-way analysis of variance (ANOVA) with post hoc analysis using the Bonferroni post hoc test. All experiments were performed at least three times. For clinical data analysis, statistical processing was conducted using SPSS version 19.0 software (SPSS Inc., Chicago, IL, USA). Survival rates were estimated using the Kaplan-Meier method and compared using the log-rank test. Differences between groups were considered significant when $P<0.05$.

Results

Association of SLC22A18 with clinicopathological features

To evaluate SLC22A18 expression status in relation to clinicopathological parameters in CRC, TMA from 337 patients (Stage I, n=76; Stage II, n=87; Stage III, n=91; and Stage IV, n=83) was performed (**Figure 1A**). Patients were divided into the following two groups: low-expression (scores 0 and +1) and high expression (scores +2 and +3) according to the criteria described in Materials and Methods. Kaplan-Meier survival analysis showed a clear association with better PFS (progression-free survival) and OS (overall survival) in the high SLC22A18-expressing group (P=0.034, P=0.008, respectively; **Figure 1B**) compared to that of the low SLC22A18-expressing group. **Table 1** shows the clinicopathological parameters according to SLC22A18 expression status. There were no significant differences between the expression levels of SLC22A18 and clinical characteristics of patients based on age, primary tumor location, lymphatic invasion, and perineural invasion. In contrast, lack of SLC22A18 expression was significantly correlated with sex (P=0.008), preoperative carcinoembryonic antigen (CEA) level (P=0.015), tumor stage (P<0.001), cell type (P=0.001), and vascular invasion (P=0.021) (**Table 1**, Supplementary Figure 1).

Oxaliplatin-induced cell death is dependent on the expression of SLC22A18

SLC22A18 protein expression was evaluated in four colon cancer cell lines. For our study, we divided these into the two following groups: low expression cell lines (SW480 and HT29) and high expression cell lines (HCT116 and SW48) (**Figure 2A**). In order to identify the relationship between SLC22A18 expression level and oxaliplatin resistance, we performed cell viability assays using WST-1. Although cell viability was negatively correlated with increasing concentrations of oxaliplatin in all four colon cancer cell lines, reactivity was divided into two categories, a low sensitivity category (SW480 and HT29) and a high sensitivity category (HCT116 and SW48) (**Figure 2B**).

From these data, we hypothesized that SLC22A18 expression level may be a predictor of oxaliplatin sensitivity in CRC. To confirm the effects of SLC22A18 on the cellular response to oxaliplatin, we transiently transfected SW48 with *SLC22A18* siRNAs and transfected HT29 with an *SLC22A18* expression vector (**Figure 2C**). SLC22A18-knockdown SW48 cells showed increased viability compared with control

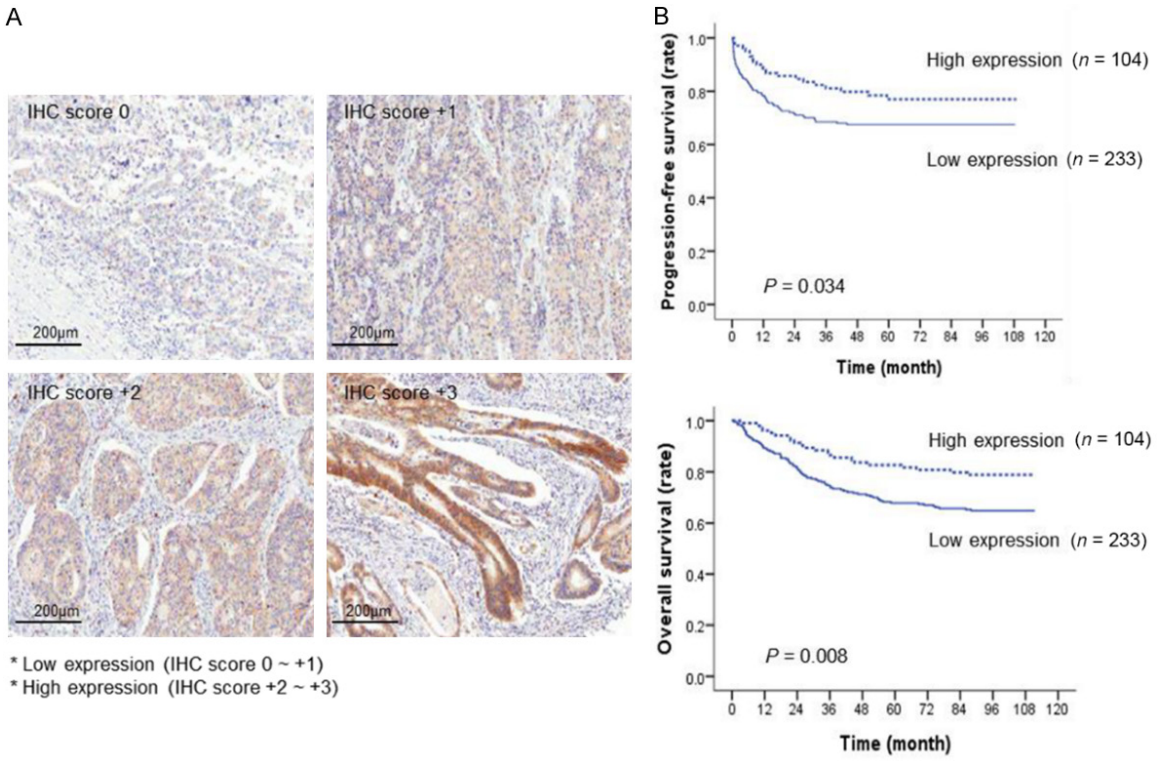

Figure 1. Indicators of poor survival in CRC patients with low levels of SLC22A18. A. Representative immunohistochemistry for SLC22A18 in CRC. SLC22A18 scores, i.e., 0, no; +1, low; +2, moderate; and +3, strong expression of SLC22A18 protein. Scale bar =200 μm. B. Kaplan-Meier analysis of both progression-free survival and overall survival of all patients (upper panel) and patients with disease recurrence (lower panel) based on SLC22A18 expression. Patients with scores of 0 or +1 were considered to have low expression; patients with scores of +2 or +3 were considered to have high expression. The log-rank test was used for statistical analyses.

cells cultured in the presence of oxaliplatin. The cell-death inhibition rates of SLC22A18-knockdown SW48 cells were significantly higher than those of the corresponding negative siRNA control (**Figure 2D**). To corroborate the results from SLC22A18 knockdown experiments, we transiently transfected HT29 cells with the SLC22A18-overexpression vector for 24 hours and then treated them with increasing concentrations of oxaliplatin (0-400 μM) for an additional 24 hours. Contrary to the knockdown results, SLC22A18-overexpressing HT29 cells showed decreased viability compared with control cells cultured in the presence of oxaliplatin. The cell-death promotion rates of SLC22A18-overexpressing HT29 cells were 2.56 times higher at 400 μM oxaliplatin than those of the corresponding vector control (**Figure 2E**). Taken together, our data indicate that SLC22A18 can affect the sensitivity of colon cancer cells to oxaliplatin.

Effects of combined treatment with oxaliplatin and ERK inhibitor on colon cancer progression

To explore the signaling pathways involved in mediating SLC22A18-elicited resistance to oxaliplatin in colon cancer cells, we investigated ERK pathways, which include well-known molecules targeted by cancer treatment strategies [26, 27], in SLC22A18-knockdown SW48 cells or overexpressing HT29 cells. SLC22A18-knockdown SW48 cells exhibited increased phosphorylation levels of ERK (**Figure 3A**, left panel). In contrast, SLC22A18 overexpression diminished phosphorylated ERK levels in HT29 cells (**Figure 3A**, right panel). Based on these data, we speculate that ERK is involved in mediating the SLC22A18-elicited resistance to oxaliplatin in colon cancer cells.

To identify ERK pathway dependence, we used an ERK inhibitor in SLC22A18 low-expression cell lines. Cell viability was reduced upon treat-

Table 1. Patient characteristics

	Patients (n=337)	SLC22A18 expression		
		Low (n=233)	High (n=104)	*p*-value
Age, n (%)				0.966
<65 years	142 (42.1%)	98 (42.1%)	44 (42.3%)	
≥65 years	195 (57.9%)	135 (57.9%)	60 (57.7%)	
Gender, n (%)				0.008
Male	207 (61.4%)	154 (66.1%)	53 (51.0%)	
Female	130 (38.6%)	79 (33.9%)	51 (49.0%)	
Preoperative CEA level, n (%)				0.015
<5 ng/ml	206 (61.1%)	137 (58.8%)	69 (66.3%)	
≥5 ng/ml	108 (32.0%)	86 (36.9%)	22 (21.2%)	
unknown	23 (6.8%)	10 (4.3%)	13 (12.5%)	
Primary tumor location, n (%)				0.390
Right colon	72 (21.4%)	46 (19.7%)	26 (25.0%)	
Left colon	143 (42.4%)	101 (43.3%)	42 (40.4%)	
Rectum	122 (36.2%)	86 (36.9%)	36 (34.6%)	
Tumor stage, n (%)				<0.001
I	76 (22.6%)	37 (15.9%)	39 (37.5%)	
II	87 (25.8%)	60 (25.8%)	27 (26.0%)	
III	91 (27.0%)	64 (27.5%)	27 (26.0%)	
IV	83 (24.6%)	72 (30.9%)	11 (10.6%)	
Cell type, n (%)				0.001
WD/MD	305 (90.5%)	203 (87.1%)	102 (98.1%)	
PD/MUC/SRC	32 (9.5%)	30 (12.9%)	2 (1.9%)	
Vascular invasion, n (%)				0.021
Positive	44 (13.1%)	37 (15.9%)	7 (6.7%)	
Negative	293 (86.9%)	196 (84.1%)	97 (93.3%)	
Lymphatic invasion, n (%)				0.091
Positive	85 (25.2%)	65 (27.9%)	20 (19.2%)	
Negative	252 (74.8%)	168 (72.1%)	84 (80.8%)	
Perineural invasion, n (%)				0.666
Positive	17 (7.3%)	17 (7.3%)	9 (8.7%)	
Negative	311 (92.3%)	216 (92.7%)	95 (91.3%)	

ment of HT29 and SW480 cells with oxaliplatin (100 μM) and an ERK inhibitor (10 μM), by approximately 20% and 50%, respectively. Interestingly, we found that combined treatment with oxaliplatin and ERK inhibitor significantly induced cell death in HT29 and SW480 cells, compared to treatment with oxaliplatin or ERK inhibitor alone (**Figure 3B**, **3D**, Supplementary Figure S2A and S2B). To determine if this synergistic inhibitory effect involved ERK activation, we evaluated the protein levels by western blotting. Phosphorylated ERK levels decreased in HT29 and in SW480 cells treated with ERK inhibitor or combined treatment, respectively (**Figure 3C**, **3E**). As ERK inhibitors are not used clinically, we tested cetuximab, an anti-EGFR monoclonal antibody widely used in the clinical treatment of CRC [28]. Viability of the HT29 cell line (KRAS wild type) was reduced upon treatment with oxaliplatin (100 μM) and cetuximab (200 μg/mL), by approximately 20% and 45%, respectively. The result showed that combined treatment with oxaliplatin and cetuximab significantly induced cell death in HT29 cells, compared to treatment with oxaliplatin or cetuximab alone (Supplementary Figure 3A). Western blotting revealed that phosphorylated ERK levels were decreased upon cetuximab or combined treatment with oxaliplatin and cetuximab, respectively (Supplementary Figure 3B).

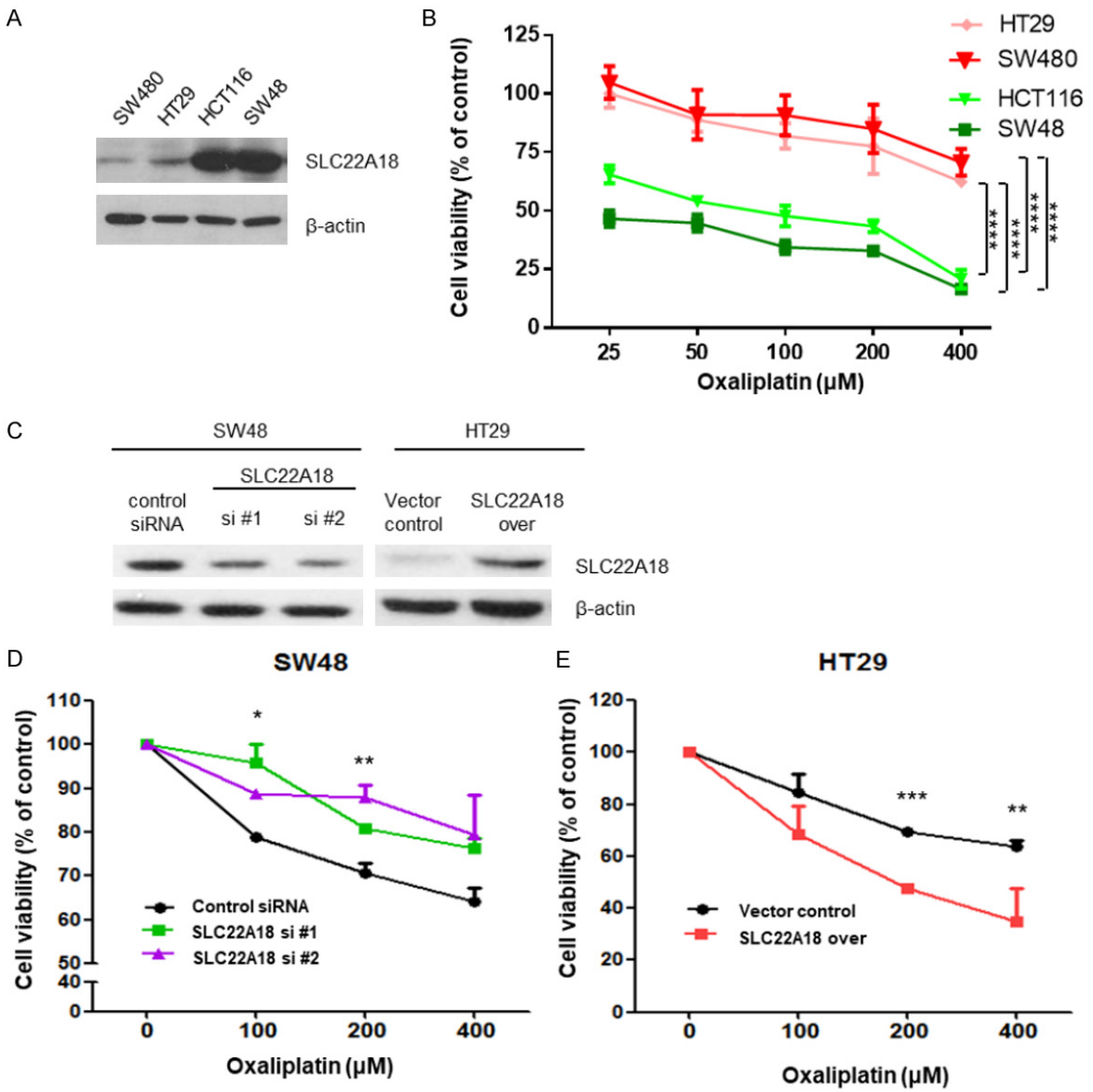

Figure 2. Expression level of SLC22A18 affected oxaliplatin response. (A) Western blot (upper panel) and densitometry (lower panel) of SLC22A18 expression in four colon cancer cell lines. (B) Four colon cancer cell lines were treated with increasing concentrations of oxaliplatin for 24 hours and cell viability was determined by WST-1 assay. Each bar represents the mean ± SD. (C) SW48 cells were transfected with control siRNA or SLC22A18-specific siRNAs (left panel) and HT29 cells were transfected with an SLC22A18 expression vector or empty vector (right panel). Total cell lysates were subjected to western blotting for confirmation of SLC22A18 downregulation or overexpression. Actin was used as a loading control. After 24 hours of transfection, transfected SW48 cells (D) and HT29 cells (E) were treated with increasing concentrations of oxaliplatin for 24 hours and cell viability was determined by WST-1 assay. Experiments were conducted in triplicate. Results shown are mean value ± SE. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$.

Confirmation of the efficacy of combined treatment in SLC22A18-downregulated cell lines

To confirm if this synergistic inhibitory effect involved ERK activation, we tested the efficacy of drug treatment in SLC22A18-downregulated cell lines. SLC22A18-knockdown SW48 cells showed increased viability compared with control cells cultured in the presence of oxaliplatin. Additionally, SLC22A18-knockdown SW48 cells showed the same effect as that observed upon ERK inhibitor treatment as compared to controls. However, the cell-death inhibition rates of the combined treatment were significantly high-

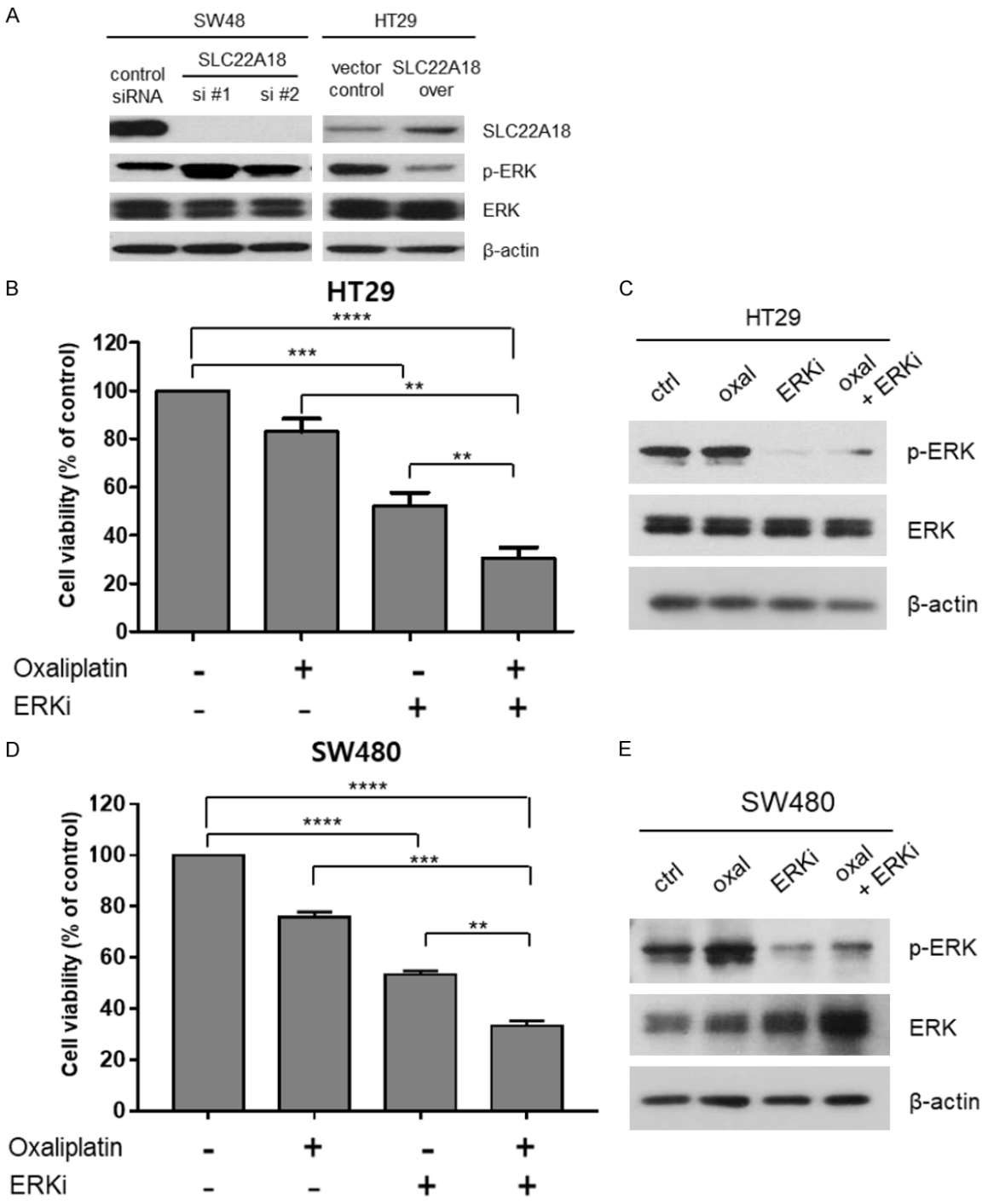

Figure 3. Combined treatment with oxaliplatin and ERK inhibitor in low SLC22A18-expressing CRC cell lines. A. Expression of phosphorylated and total ERK in controls or SLC22A18-specific siRNA-transfected SW48 cells (left panel) and in HT29 cells transiently transfected with an SLC22A18 expression vector (right panel). B. HT29 cells were treated with oxaliplatin (100 μM), ERK inhibitor (10 μM), or a combination of both. After 24 hours of treatment, cell viability was determined by WST-1 assay. C. The levels of phosphorylated and total ERK in HT29 cells were evaluated using western blotting after treatment. Actin was used as a loading control. D. SW480 cells were treated with oxaliplatin (100 μM), ERK inhibitor (10 μM), or a combination of both. After 24 hours of treatment, cell viability was determined by WST-1 assay. E. The levels of phosphorylated and total ERK in SW480 cells were evaluated using western blotting after treatment. Actin was used as a loading control. Results shown are mean value ± SE. $^{*}P<0.05$; $^{***}P<0.001$; $^{****}P<0.0001$.

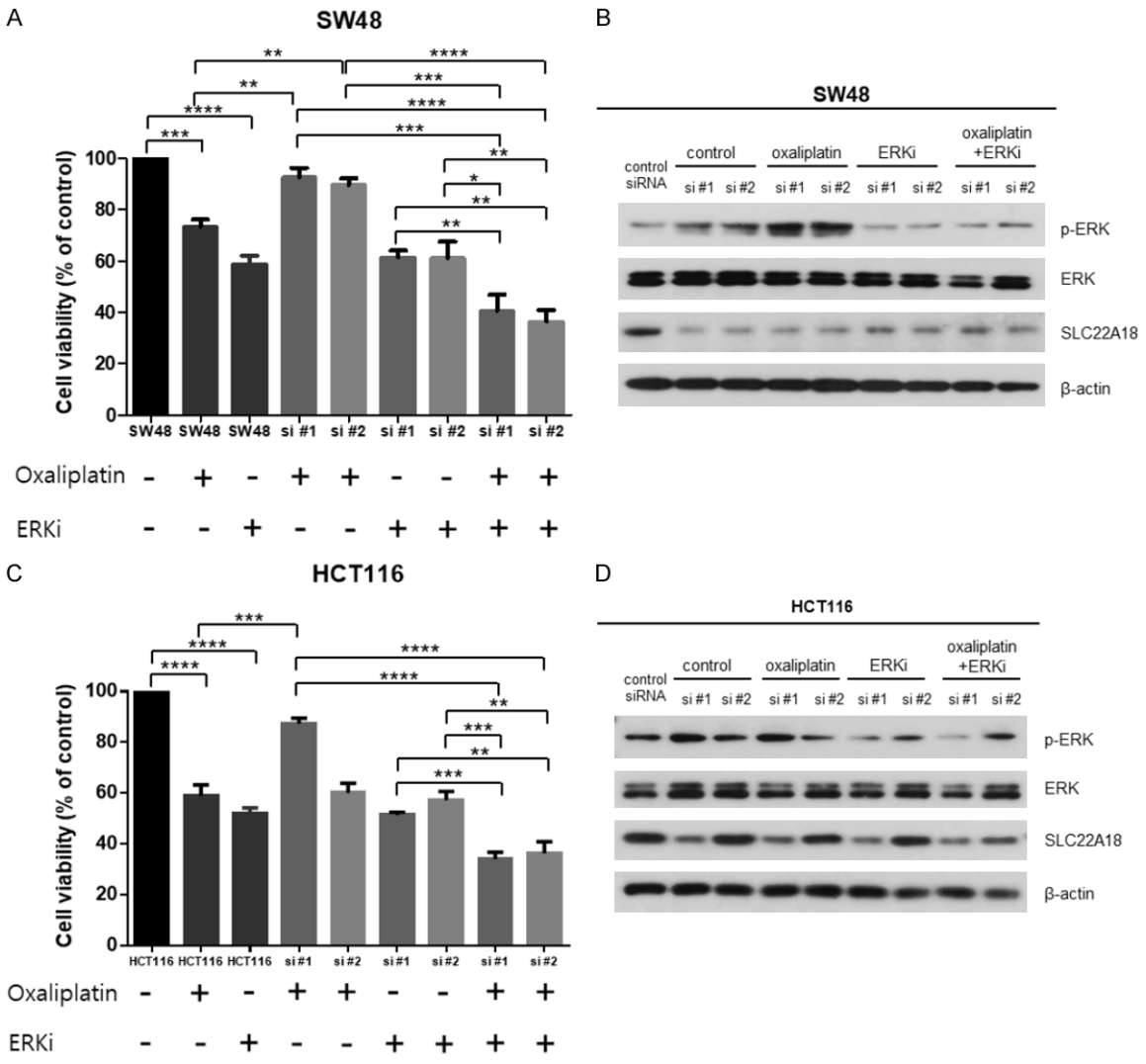

Figure 4. Combined treatment in the background of SLC22A18 downregulation. A. SW48 cells were transfected with control siRNA or SLC22A18-specific siRNAs. After 24 hours, transfected cells were treated with oxaliplatin (100 μM), ERK inhibitor (10 μM), or a combination of both for 24 hours. B. The levels of phosphorylated and total ERK, and SLC22A18 were evaluated using western blotting after treatment in siRNA-transfected SW48 cells. Actin was used as a loading control. C. SW48 cells were transfected with control siRNA or SLC22A18-specific siRNAs. After 24 hours, transfected cells were treated with oxaliplatin (100 μM), ERK inhibitor (10 μM), or a combination of both for 24 hours. D. The levels of phosphorylated and total ERK, and SLC22A18 in siRNA-transfected SW48 cells were evaluated using western blotting after treatment. Actin was used as a loading control.

er than those of oxaliplatin or ERK inhibitor treatment alone in SLC22A18-knockdown SW48 cells (**Figure 4A**). To confirm that this synergistic inhibitory effect involved ERK activation, we evaluated protein levels by western blotting. Phosphorylated ERK levels increased in SLC22A18 downregulated SW48 cells. Treatment of SLC22A18-downregulated SW48 cells with 10 μM ERK inhibitor inhibited ERK phosphorylation by half, while 100 μM oxaliplatin treatment did not exert any effects (mild inhibitory effects) on ERK phosphorylation. As speculated, we found that compared to treatment with oxaliplatin, combined treatment with oxaliplatin and ERK inhibitor significantly decreased ERK phosphorylation levels in SLC22A18-downregulated SW48 cells (**Figure 4B**). SLC22A18 was downregulated in HCT116 cells upon transfection with si-SLC22A18 #1. However, transfection with si-SLC22A18 #2 did not show substantial knockdown efficiency in HCT116 cells. SLC22A18 knockdown in

HCT116 cells upon transfection with SLC22A18 #1 resulted in increased viability compared with controls cultured in the presence of oxaliplatin, whereas HCT116 cells which were transfected using si-SLC22A18 #2 showed no increase in viability compared with controls cultured in the presence of oxaliplatin. Additionally, SLC22A18-knockdown in HCT116 cells upon transfection with si-SLC22A18 #1 resulted in the same efficacy as that observed upon treatment with ERK inhibitor compared with controls. However, the cell death inhibition rates of combined treatment were significantly higher than those of oxaliplatin or ERK inhibitor treatment alone in SLC22A18-knockdown HCT116 cells (**Figure 4C**). To confirm that this synergistic inhibitory effect involved ERK activation, we evaluated protein levels by western blotting. Phosphorylated ERK levels increased in SLC22A18 downregulated HCT116 cells transfected with si-SLC22A18 #1. In contrast, SLC22A18 downregulated HCT116 cells transfected with si-SLC22A18 #2 (no downregulation) showed no increase in phosphorylated ERK levels. Treatment of SLC22A18 downregulated HCT116 cells with ERK inhibitor (10 μM) inhibited ERK phosphorylation by half, while oxaliplatin (100 μM) exerted no or mild inhibitory effects on ERK phosphorylation. As speculated, we found that combined treatment with oxaliplatin and ERK inhibitor significantly decreased ERK phosphorylation in SLC22A18 downregulated HCT116 cells, compared to treatment with oxaliplatin (**Figure 4D**).

In addition, we tested combined treatment with cetuximab, which is used in the clinical treatment of CRC. SLC22A18-knockdown SW48 cells showed increased viability compared with controls cultured in the presence of oxaliplatin, and the same efficacy as cetuximab (200 μg/mL) compared with controls. However, the cell-death inhibition rates of combined treatment were significantly higher than those of oxaliplatin or cetuximab treatment alone in SLC22A18-knockdown SW48 cells (Supplementary Figure 4A). To confirm that this synergistic inhibitory effect involved ERK activation, we evaluated protein levels by western blotting. Treatment of SLC22A18 down-regulated SW48 cells with cetuximab inhibited ERK phosphorylation by half, while oxaliplatin (100 μM) exerted no or mild inhibitory effects on ERK phosphorylation. As speculated, we found that combined treatment with oxaliplatin and cetuximab significantly decreased ERK phosphorylation in SLC22A18 downregulated SW48 cells compared to treatment with oxaliplatin (Supplementary Figure 4B).

Ex vivo analyses confirmed the correlation between SLC22A18 expression and oxaliplatin resistance

To further confirm our findings, *ex vivo* analyses were performed using patient-derived cells (PDCs). First, we evaluated the expression pattern of SLC22A18 in each PDC and selected two representative PDCs (Supplementary Table 1) that showed relatively higher and lower expression of SLC22A18 (**Figure 4A**, upper panel). Subsequently, to determine whether oxaliplatin resistance was associated with SLC22A18 expression, cell survival assays were performed using WST-1 reagent (**Figure 4A**, lower panel). As expected, oxaliplatin resistance was observed in PDCs with relatively low SLC22A18 expression (PDC #2), whereas those with high SLC22A18 expression (PDC #1) showed a comparatively sensitive response to oxaliplatin. To confirm the previously identified signaling pathways involved in SLC22A18-related resistance to oxaliplatin using CRC cell lines, we tested combined treatment with ERK inhibitor in PDC #2. Cell viability was reduced upon treatment of PDC #2 with oxaliplatin (100 μM) and ERK inhibitor (5 μM) by approximately 20% and 50%, respectively. Combinatorial treatment with oxaliplatin and ERK inhibitor significantly induced cell death, compared to treatment with oxaliplatin or ERK inhibitor alone (**Figure 5B**). To determine if this synergistic inhibitory effect involved ERK activation, we evaluated protein levels by western blotting. Phosphorylated ERK levels decreased upon treatment of PDC #2 with ERK inhibitor or combined treatment with oxaliplatin and ERK inhibitor (**Figure 5C**). Additionally, the viability of PDC #2 was reduced upon treatment with oxaliplatin (100 μM) or cetuximab (200 μg/mL) by approximately 30% and 60%, respectively. The result showed that combined treatment with oxaliplatin and cetuximab significantly induced cell death in PDC #2, compared to treatment with oxaliplatin or cetuximab alone (Supplementary Figure 5A). Western blotting revealed that phosphorylated ERK levels were decreased by cetuximab or combined treatment (Supplementary Figure 5B).

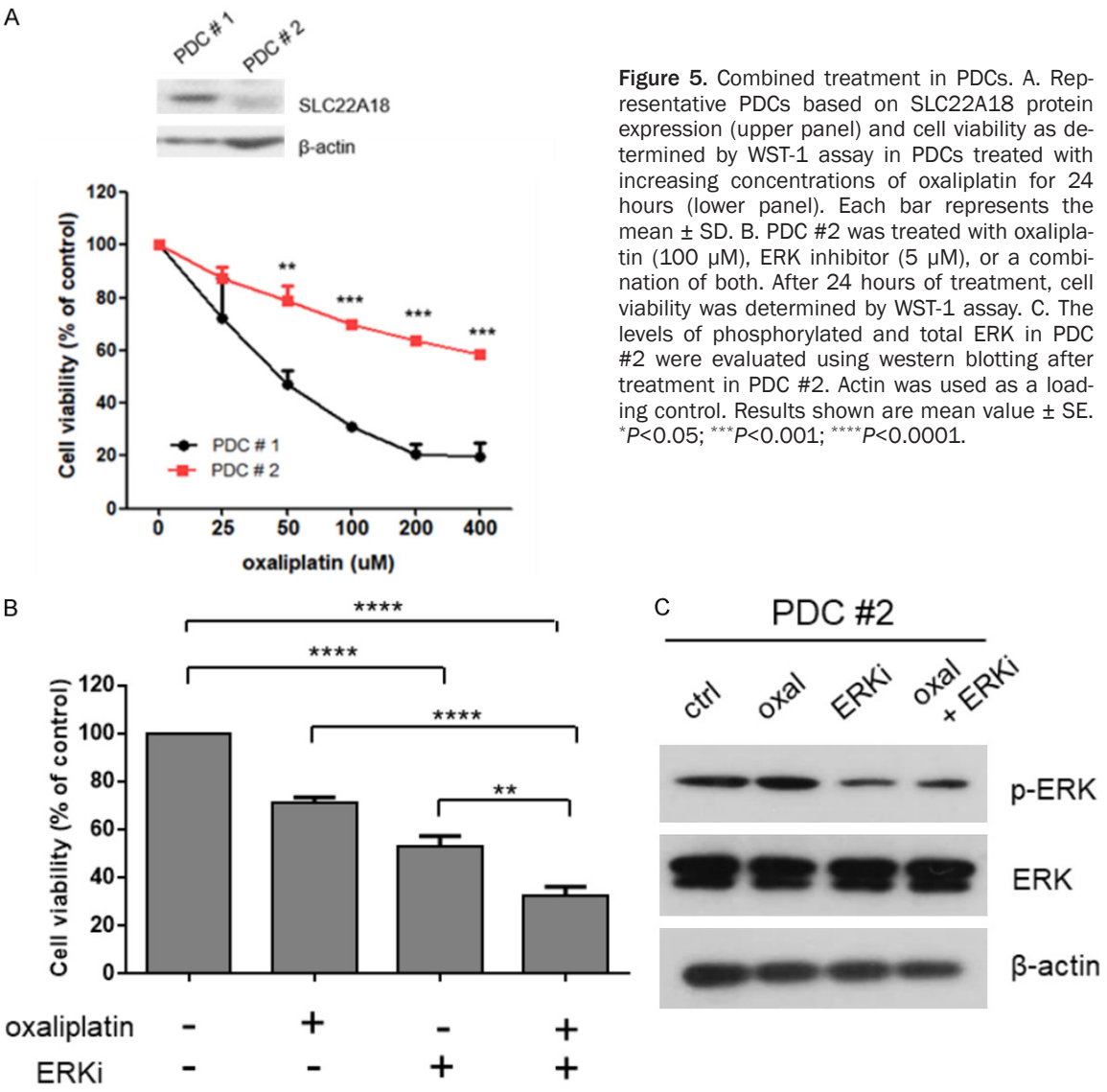

Figure 5. Combined treatment in PDCs. A. Representative PDCs based on SLC22A18 protein expression (upper panel) and cell viability as determined by WST-1 assay in PDCs treated with increasing concentrations of oxaliplatin for 24 hours (lower panel). Each bar represents the mean ± SD. B. PDC #2 was treated with oxaliplatin (100 μM), ERK inhibitor (5 μM), or a combination of both. After 24 hours of treatment, cell viability was determined by WST-1 assay. C. The levels of phosphorylated and total ERK in PDC #2 were evaluated using western blotting after treatment in PDC #2. Actin was used as a loading control. Results shown are mean value ± SE. $^{*}P<0.05$; $^{***}P<0.001$; $^{****}P<0.0001$.

In vivo analyses confirmed effects of combined treatment with oxaliplatin and ERK inhibitor

Based on these results, it was clear that SLC22A18 expression was associated with oxaliplatin resistance in vitro. To establish whether SLC22A18 expression is related to oxaliplatin resistance in vivo, we developed a subcutaneous mouse model by injecting SW48, which downregulated SLC22A18 expression via siRNA silencing. The results presented in **Figure 6A** showed that tumor volume was in accordance with SLC22A18 knockdown, with or without drug treatment. Tumor volume measurements revealed that oxaliplatin suppressed the growth of xenograft tumors in the control siRNA group (**Figure 6A**). However, the SLC22A18-knockdown group did not show oxaliplatin activity. Additionally, the ERK inhibitor showed greater sensitivity than oxaliplatin in the SLC22A18-knockdown group. However, the growth inhibition rates of the combined treatment were significantly higher than those of oxaliplatin or ERK inhibitor treatment alone in the SLC22A18-knockdown group (**Figure 6A**). The tumor weight at the end of the experiment showed the same results (**Figure 6B**). Additionally, the tumors subjected to the combined treatment were significantly smaller than those in the others group (**Figure 6C**). To confirm whether SLC22A18 expression is related to oxaliplatin resistance in vivo, we developed a subcutaneous mouse model by injecting SLC22A18-overexpressing HT29 cells. Oxaliplatin

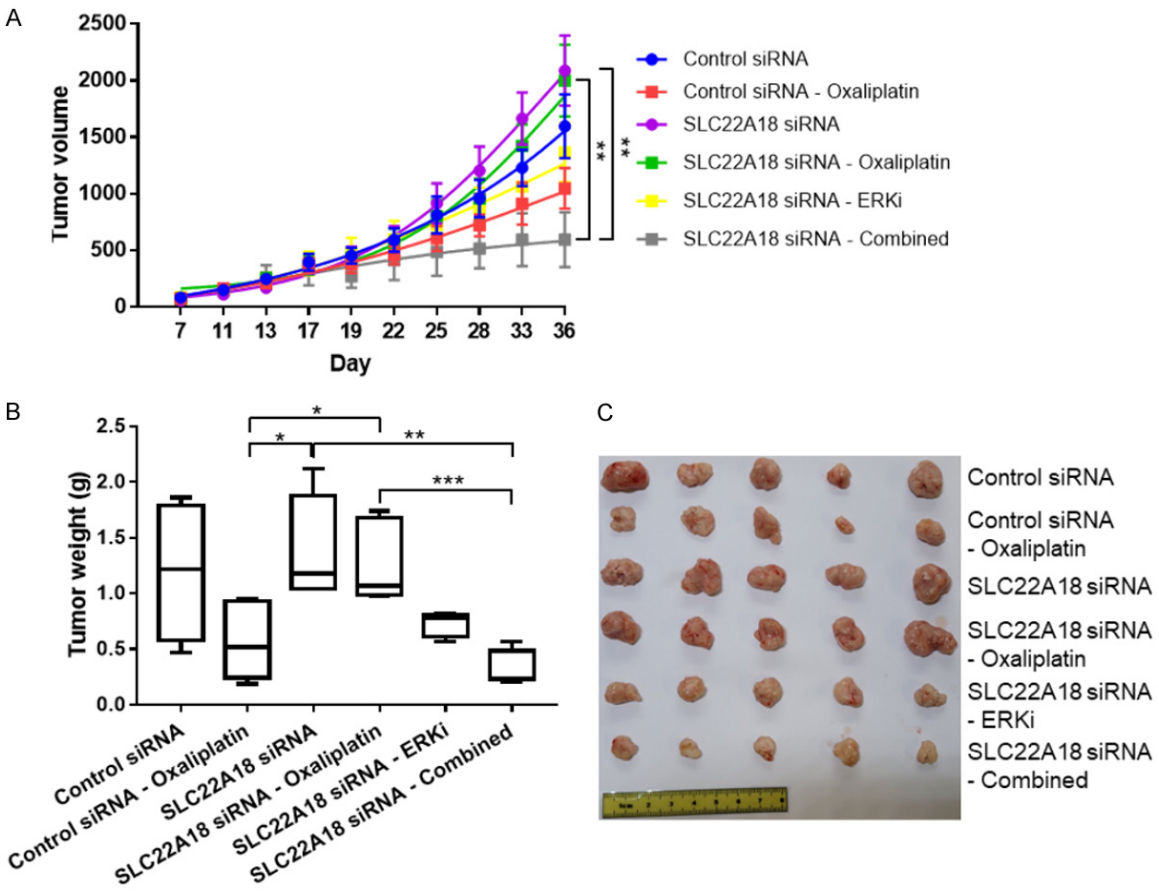

Figure 6. Combined treatment with oxaliplatin and ERK inhibitor after injection of SLC22A18-downregulating SW48 in vivo. A. The graph shows tumor volume and drug efficacy during 5 weeks after injection of control siRNA-transfected SW48 and SLC22A18 siRNA-transfected SW48. B. After concluding the experiment, the tumor weight of each mouse was measured. C. Photographs show the excised tumors at the end of the study.

inhibited tumor growth in the SLC22A18-overexpressed group compared to that in the control group (Supplementary Figure 6A). However, no statistical significance was observed. The tumor weights of oxaliplatin-treated mice in the SLC22A18-overexpressed group were significantly lower than those of oxaliplatin-treated mice in the SLC22A18-overexpressed group and control group (Supplementary Figure 6B). The tumors in the oxaliplatin-treated SLC22A-18-overexpressed group were significantly smaller than those in the oxaliplatin-treated control group (Supplementary Figure 6C).

Discussion

Plasma membrane transporters have received much attention for their potential as therapeutic targets in colon cancer. In this study, we found that SLC22A18 expression was correlated with the prognosis of patients and response to oxaliplatin in CRC cell lines. In addition, we also found that SLC22A18 overexpression resulted in significantly decreased phosphorylated ERK levels, whereas knockdown of SLC22A18 resulted in increased phosphorylated ERK levels in CRC cell lines. To inhibit the ERK pathway in low SLC22A18-expressing cells, we used an ERK inhibitor and found that combined treatment with oxaliplatin and ERK inhibitor overcame oxaliplatin resistance. Furthermore, in KRAS wild type cells, we found that combined treatment with oxaliplatin and cetuximab overcame oxaliplatin resistance in low SLC22A18-expressing cells.

By analyzing gene expression data obtained from TCGA, Jung Y et al. [21] demonstrated that a low level of SLC22A18 correlates with poor prognosis in CRC patients. In agreement, we found that a lack of SLC22A18 protein expression was significantly associated with PFS and

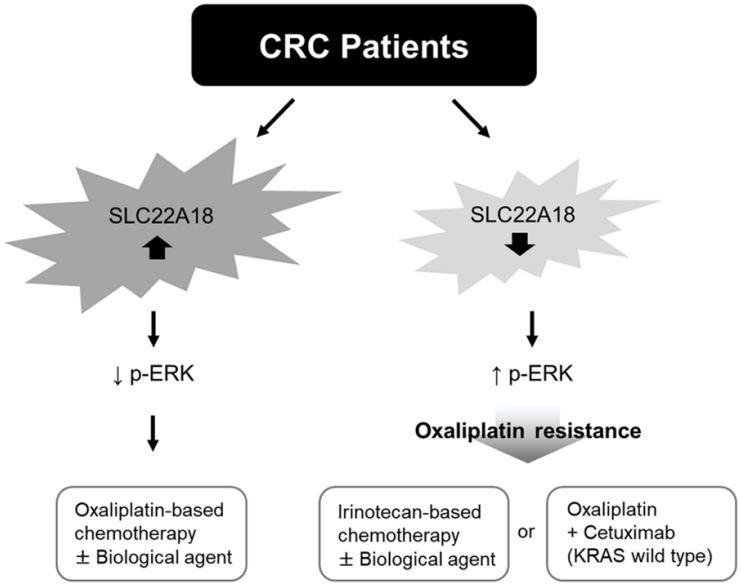

Figure 7. Treatment model and strategy according to the expression level of SLC22A18.

OS in 337 CRC patients (**Figure 1B**). Furthermore, we discovered that a lack of SLC22A18 expression was significantly correlated with sex, preoperative CEA level, tumor stage, cell type, and vascular invasion (**Table 1**). However, a limitation is that the clinical outcomes related to all stages of CRC only allowed the examination of the relationship between SLC22A18 and survival rates. Among 337 CRC patients, we analyzed 72 CRC patients who were at Stage III and were treated with oxaliplatin-based chemotherapy. Though there were no significant results, there was a tendency toward a relationship between SLC22A18 and oxaliplatin resistance in patients treated with oxaliplatin (Supplementary Figure 7). These observations are consistent with our hypothesis that low expression of SLC22A18 correlates with oxaliplatin resistance, thereby decreasing survival rates. Though discrepancies exist, likely due to the small number of included patients (n=72), our findings support the requirement for further testing of greater numbers of oxaliplatin-treated CRC patients. These results showed that SLC22A18 is a prognostic factor and could be predict the response to oxaliplatin in CRC patients.

Through *in vitro* experiments, we identified an inverse relationship between SLC22A18 expression level and oxaliplatin resistance (**Figure 2**). The SLC family affects cancer progression through ERK activation in gastric cancer and colon cancer [29, 30]. Previous our study showed that suppression of KRAS promoted SLC22A18 expression, and expression of SLC22A18 in turn inhibit KRASG12D-mediated anchorage independent growth indicating a mutual negative interaction [21]. KRAS mutation regulate ERK activation through MEK1/2 signaling pathway [31] Furthermore, ERK activation regulate progression of colon cancer [32] and is a well-known cause of oxaliplatin resistance [33-35]. Hence, we investigated whether ERK activation was involved in SLC22A18-mediated oxaliplatin resistance. We found that ERK signaling pathways are involved in SLC22A18-mediated oxaliplatin resistance (**Figure 3**). Our data showed that combined treatment with oxaliplatin and ERK inhibitor synergistically inhibited CRC cell viability through the regulation of ERK activity. However, ERK inhibitors are not used in clinical chemotherapy. Cetuximab inhibits ERK, which is a downstream effector of EGFR, and it may be that even though ERK is activated in low SLC22A18-expressing cells, they undergo cell death through the ERK pathway under conditions of cetuximab treatment. Our data indicated that oxaliplatin and cetuximab synergistically inhibited CRC cell viability through the regulation of ERK activity. Next, we employed *ex vivo* approaches using patient-derived cells (PDCs) to confirm our *in vitro* data. PDCs offer a model that can recapitulate features of primary patient tumors, thereby helping to overcome the potential shortcomings of *in vitro* models. Our PDC data showed the same results as from the cell lines. In vivo study, we observed that expression of SLC22A18 regulated oxaliplatin efficacy. Hence, our study went one step further than the existing studies with respect to exploring the role of SLC22A18 in oxaliplatin resistance in CRC.

Our data indicate that SLC22A18 could be used as a biomarker to predict oxaliplatin resistance. Although detailed randomized studies

and pathway analyses are required, the results provided by this study strongly suggest that targeting ERK activation may be a potential therapeutic strategy for CRC patients with low SLC22A18 expression. Therefore, in Stage IV, patients with high expression of SLC22A18 could exhibit better treatment outcomes in response to oxaliplatin-based chemotherapy with or without biological agent. Whereas patients with low expression of SLC22A18 would be treated by irinotecan-based chemotherapy with or without biological agent, which is not affected by SLC22A18 expression (Supplementary Figure 8), or oxaliplatin with cetuximab (in case of KRAS wild type) (**Figure 7**). If ERK inhibitors could be developed for clinical chemotherapy, they could be useful for patients with low expression of SLC22A18.

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Disclosure of conflict of interest

None.

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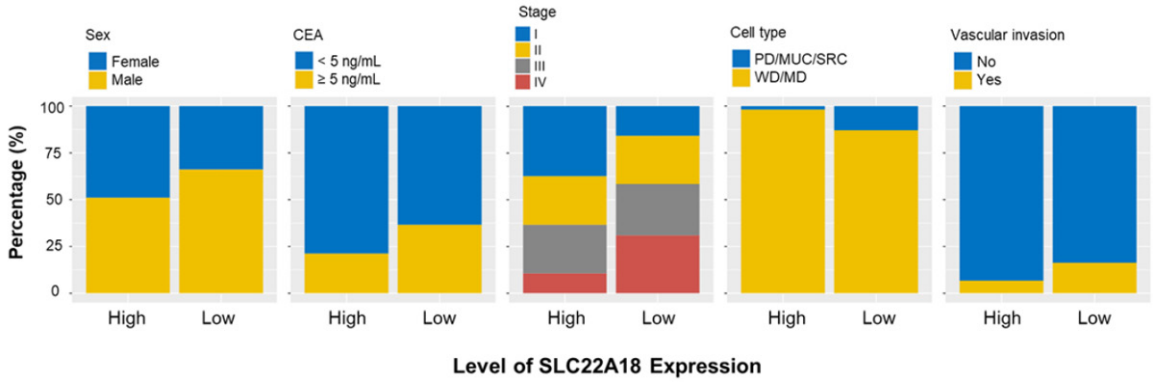
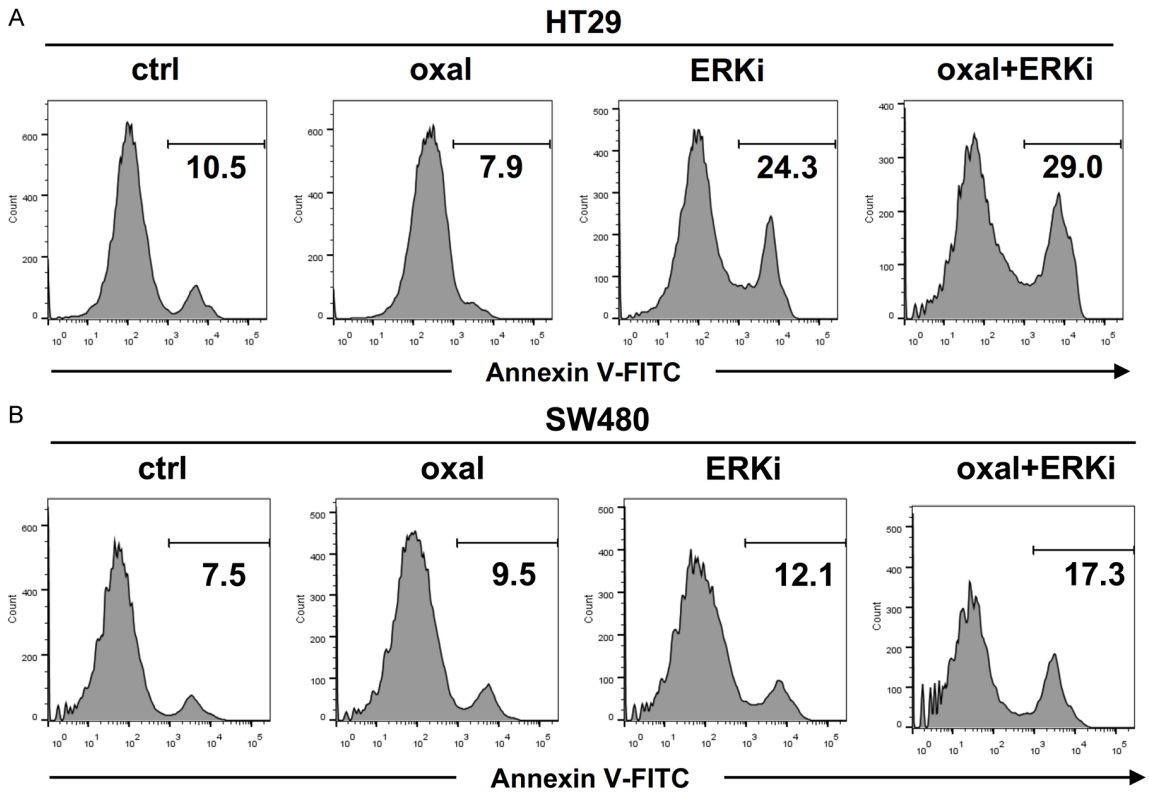
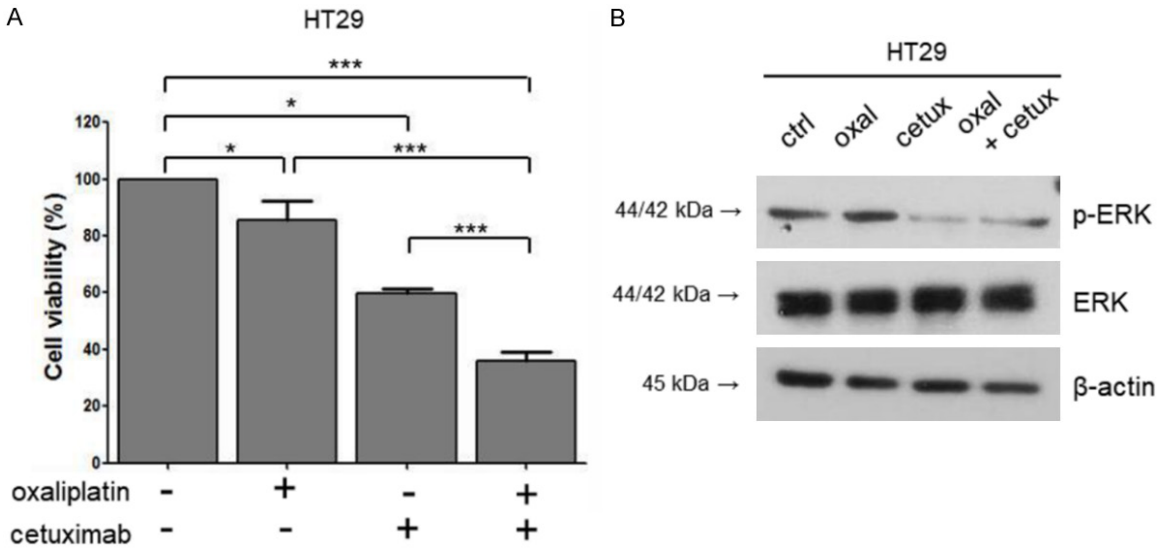


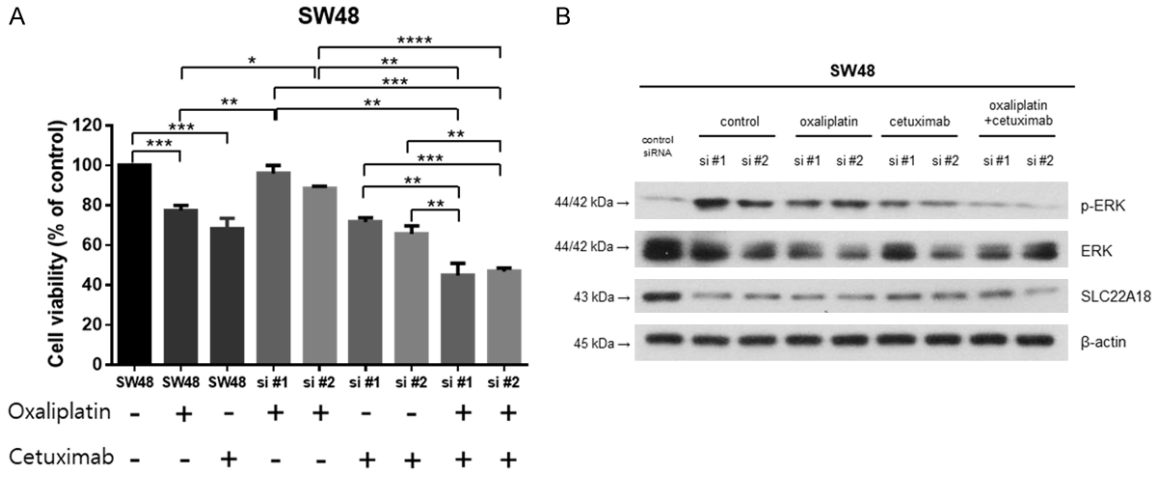
Supplementary Figure 1. Expression of SLC22A18 was correlated with sex, preoperative carcinoembryonic antigen level, tumor stage, cell type, and vascular invasion.



Supplementary Figure 2. A. HT29 cells were treated with oxaliplatin (100 μM), ERK inhibitor (10 μM), or a combination of both. After 30 hours of treatment, the expression of annexin-V was analyzed by flow cytometry. B. SW480 cells were treated with oxaliplatin (100 μM), ERK inhibitor (10 μM), or a combination of both. After 30 hours of treatment, the expression of annexin-V was analyzed by flow cytometry. The numbers on the histograms indicate percentage.



Supplementary Figure 3. A. HT29 cells were treated with oxaliplatin (100 μM), cetuximab (200 μg/mL), or a combination of both. After 24 hours of treatment, cell viability was determined by WST-1 assay. B. The levels of phosphorylated and total ERK in HT29 cells were evaluated using western blotting after treatment. Actin was used as a loading control. Results shown are mean value ± SE. $^{*}P<0.05$; $^{***}P<0.001$; $^{****}P<0.0001$.

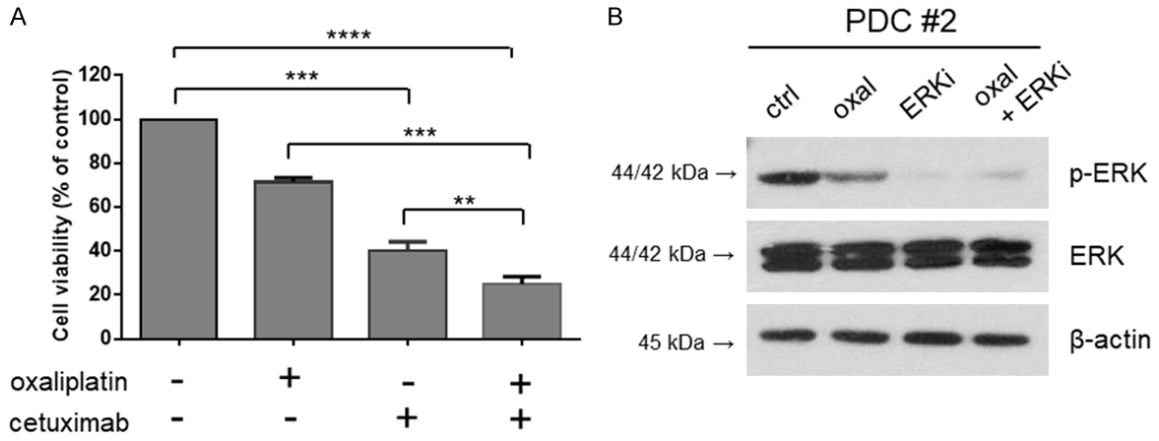


Supplementary Figure 4. A. SW48 cells were transfected with control siRNA or SLC22A18-specific siRNAs. After 24 hours, transfected cells were treated with oxaliplatin (100 μM), cetuximab inhibitor (200 μg/mL), or a combination of both for 24 hours. B. The levels of phosphorylated and total ERK, and SLC22A18 in siRNA-transfected SW48 cells were evaluated using western blotting after treatment. Actin was used as a loading control.

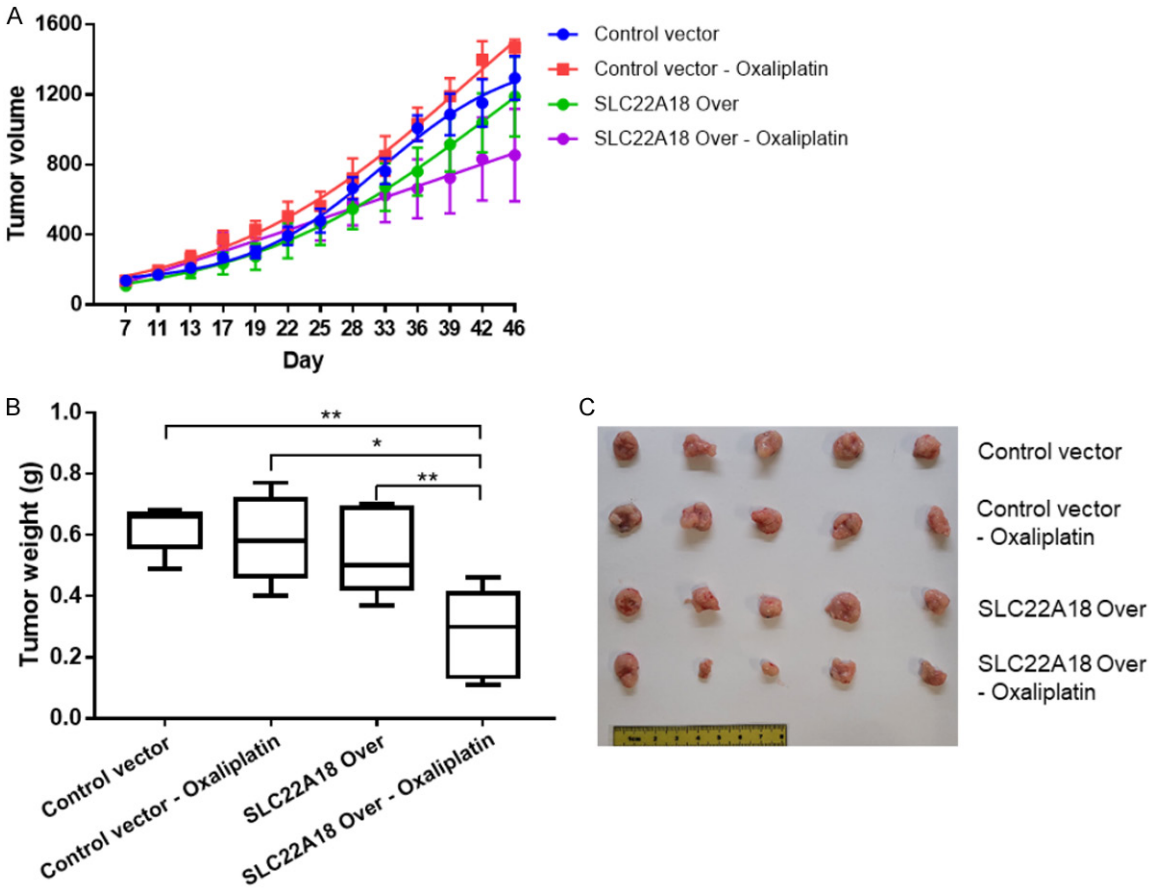
Supplementary Table 1. PDC characterisrics

Sample ID	PDC #1	PDC #2
Age	37	87
Sex	M	M
Cancer	Adenocarcinoma	Adenocarcinoma
Cell Type	Moderately Differentiated	Well Differentiated
TNM Stage	IVA	IIA
Microsatellite instability	MSS*	MSS*
Location of Tumor	Transverse Colon	Sigmoid Colon

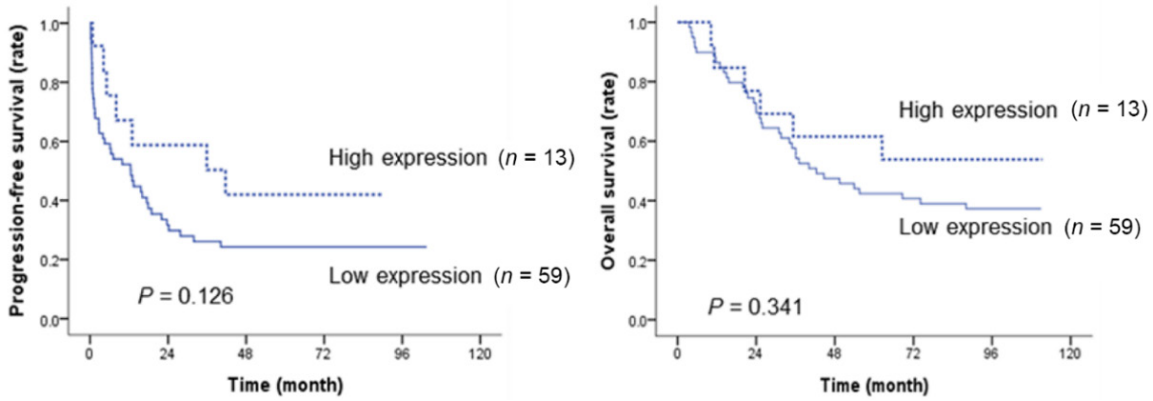
*MSS: Microsatellite stable.



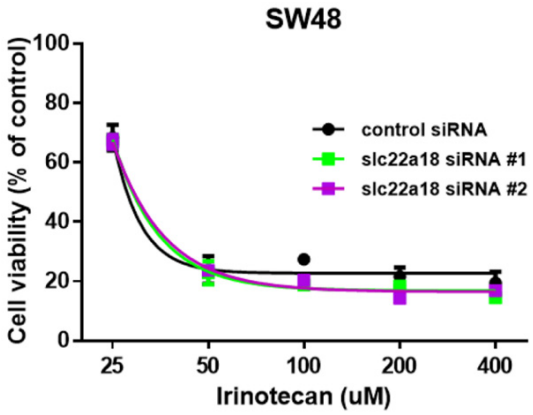
Supplementary Figure 5. A. PDC #2 was treated with oxaliplatin (100 μM), cetuximab inhibitor (200 μg/mL), or a combination of both. After 24 hours of treatment, cell viability was determined by WST-1 assay. B. The levels of phosphorylated and total ERK in PDC #2 were evaluated using western blotting after treatment. Actin was used as a loading control. Results shown are mean value ± SE. $^{*}P<0.05$; $^{***}P<0.001$; $^{****}P<0.0001$.



Supplementary Figure 6. A. The graph shows tumor volume and oxaliplatin efficacy during 7 weeks after injection of control vector-transfected HT29 and SLC22A18-overexpressed HT29. B. After concluding the experiment, the tumor weight of each mouse was measured. C. Photographs show the excised tumors at the end of the study.



Supplementary Figure 7. Kaplan-Meier analysis of progression-free survival and overall survival for patients who received oxaliplatin chemotherapy, as per SLC22A18 expression. Patients with scores of 0 or +1 were considered to have low expression; patients with scores of +2 or +3 were considered to have high expression. The log-rank test was used for statistical analyses.



Supplementary Figure 8. After transfection with SLC22A18 siRNA, SW48 cells were treated with increasing concentrations of irinotecan for 24 hours and cell viability was determined by WST-1 assay. Experiments were conducted in triplicate.