

# ABCC10-Mediated Oxaliplatin Resistance in Colorectal Cancer Was Alleviated by Intense Endoplasmic Reticulum Stress (ERS) / IRE1 $\alpha$

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## Research Article

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# Abstract

**Background:** Oxaliplatin resistance is a challenge in treating colorectal cancer (CRC) patients, contributory to the failure in chemotherapy. However, the mechanism of Oxaliplatin resistance has not been completely elucidated. In this study, we explored the key molecule involved in the Oxaliplatin resistance, which could be a candidate therapeutic target to attenuate chemo-resistance in CRC cells.

**Methods:** Microarray screening, western blot and qPCR on clinic CRC samples were conducted to select the target gene ABCC10. The Oncomine Oncology Database and the Cancer Genome Atlas (TCGA) data were analyzed to figure out the correlation between the clinical manifestation and ABCC10. ABCC10 knock-down in CRC cells was conducted to identify its role in the Oxaliplatin resistance. Cell counting kit-8 (CCK-8) assay was conducted to identify the CRC cell viability and Oxaliplatin IC<sub>50</sub>. Flow cytometry was conducted to detect the cell apoptosis exposed to Oxaliplatin. The intracellular Oxaliplatin accumulation was measured by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS).

**Results:** ABCC10 transporter was correlated with CRC relapse and metastasis, indicating a CRC-promoting effect of ABCC10. In ABCC10 knock-down CRC cells the Oxaliplatin sensitivity was significantly elevated due to an increase of intracellular Oxaliplatin accumulation resulted from the diminished drug efflux. We next explored a strategy to inhibit ABCC10 in CRC cells, paying a special interest in the endoplasmic reticulum stress (ERS) / unfolded protein response (UPR) that plays a dual role in tumor development. We found that the CRC cell viability was profoundly decreased and the pro-apoptotic factor CHOP and apoptosis were increased by the intense ERS/UPR instead of the inhibited and mild ERS/UPR. Significantly, the Oxaliplatin sensitivity of CRC cells was enhanced in response to the intense ERS, which was blocked by inhibiting IRE1 $\alpha$  branch of UPR. Finally, we figured out that the intense ERS/UPR down-regulated ABCC10 via regulated IRE1-dependent decay (RIDD) activity.

**Conclusion:** Oxaliplatin was a substrate of ABCC10. The intense ERS/IRE1 $\alpha$  elicited anti-CRC effects through down-regulating ABCC10 so as to increase Oxaliplatin sensitivity, in addition to inducing CHOP. We suggested that introduction of intense ERS/UPR could be a promising strategy to restore chemo-sensitivity when used in combination with Oxaliplatin or other chemotherapeutic drugs pumped out by ABCC10.

## Introduction

According to the latest global cancer burden data released by the International Agency for Research on Cancer in 2020, the number of new cases of colorectal cancer (CRC) reached approximately 1.93 million worldwide, and the death toll 935,173 [1]. Some early or local CRC are amendable to appropriate endoscopic or surgical resection only [2]. For advanced or metastatic CRC, systemic therapy typically including a chemotherapy backbone is strongly proposed either going to be curative or palliative [2]. At present, Fluorouracil in combination with Oxaliplatin confers encouraging overall survival advantage in

metastatic CRC and, as adjuvant therapy, in some stage I and most stage II patients. However, drug resistance reportedly develops in nearly all advanced CRC patients, resulting in a poor 5-year survival rate in advanced CRC patients, especially for stage III CRC patients [3].

The development of chemo-resistance is a complex process achieved through either intrinsic or acquired ways, in which over-expressed ATP-binding cassette transporters (ABC transporters) is considered the leading mechanism [4]. The ABC transporter superfamily includes 49 members mediating the efflux of endogenous and exogenous substances [5]. Over-expressed in several chemo-resistant cancer types, ABC transporters confer resistance to various chemotherapeutics through rapid elimination, thereby decreasing their overall accumulation within the cancer cells [6]-[9]. Inhibitors of ABC transporters were developed with the demand, which, however, initially showed promise but failed clinically, due to high toxicity and other undesired side effects [10]-[12]. Hence, developing safe and effective reagents that can block the activity of ABC transporters in cancer cells has a great clinical significance.

The endoplasmic reticulum (ER) is responsible for protein folding, modification and assembly, which are rigorously controlled and highly sensitive to perturbation of ER homeostasis. Under cellular stresses, protein folding is disrupted and leads to an accumulation of unfolded/misfolded proteins, often referred to as ER stress (ERS). To relieve ERS, the unfolded protein response (UPR) is triggered principally through the activation of 3 ERS sensors: inositol requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ), protein kinase RNA-activated-like ER kinase (PERK) and activating transcription factor 6 (ATF6). The ERS sensors are bound to the glucose-related protein 78 (GRP78) and maintained inactivated under homeostatic condition. During ERS, GRP78 dissociates from each of the sensors allowing for the activation the UPR. The UPR attempts to re-establish ER homeostasis by reducing incoming protein load, facilitating protein folding and eliminating unfolded proteins. However, if the ERS persists and the UPR is insufficient to deal with the increasing unfolded protein load, the cells would switch from an adaptive response to an apoptotic response, triggering cell death by increasing the pro-apoptotic factor CCAAT/enhancer-binding protein homologous protein (CHOP) [13][14]. So that it is not surprising that ERS/UPR plays a dual role in deciding the fate of the cancer cells, either facilitating the tumor development or preventing it [15][16]. Emerging evidence also suggested an implication of ERS/UPR in the chemo-sensitization. Chemo-resistant cancer cells re-gained sensitivity to chemotherapies when either the 3 arms of UPR was knocked down [13]. But paradoxical outcomes were also reported that the activated IRE1 $\alpha$ -XBP1s axis and PERK restored sensitivity in myeloma and CRC cells, respectively [17][18]. Due to the contradictory results, it needs to be further investigated to improve our current knowledge in order to develop ERS/UPR-targeted approaches for treating chemo-resistant CRC.

In this study, we explored the effect of the ERS/UPR on Oxaliplatin resistance in CRC cells. We demonstrated that the intense ERS/UPR was able to restore Oxaliplatin sensitivity in CRC cells through down-regulating ABCC10 via IRE1 $\alpha$  pathway.

## Materials And Methods

## **Clinical samples and microarray**

A total of 30 (18 males and 12 females, aged 34~81 years) pairs of CRC specimens and adjacent paratumoral normal tissues were collected from patients who underwent surgical resection at the Beijing Friendship Hospital, Capital Medical University from January, 2005 to December, 2012. All cases were reviewed by a pathologist and diagnosed as CRC. Microarray analysis was performed on randomly selected 5 pairs of specimens using Affymetrix Clariom D Human with the support from Beijing Cnkingbio Biotechnology Corporation (China).

## **Bioinformatics**

Gene expression and clinic data in CRC patients was analyzed based on the Oncomine Oncology Database, the Cancer Genome Atlas (TCGA) data from cBioPortal and GEPIA2.

## **Cell culture**

CRC cell lines including HT-29 (ATCC HTB-38, RRID: CVCL\_0320), HCT-116 (ATCC CCL-247, RRID:CVCL\_0291), RKO (ATCC CRL-2577, RRID:CVCL\_0504), LS174T (ATCC CL-188, RRID:CVCL\_1384) and Caco-2 (ATCC HTB-37, RRID:CVCL\_0025) were purchased from the American Type Culture Collection (ATCC). Normal colonic epithelial cell line NCM460 (ZKC1143-1, RRID:CVCL\_0460) was purchased from the Beijing Zoman Biotechnology Co., Ltd. CRC cells and NCM460 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) and Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco), respectively, supplemented with 10% of fetal bovine serum (FBS, Biological Industries, Israel), 100 U/mL of penicillin-streptomycin (Gibco) and 1% of mycoplasma removal agent Myco-3 (Applichem, German). Cells were grown at 37 °C in the presence of 5% CO<sub>2</sub>.

## **Reagents**

4-phenylbutyric acid (4-PBA), a specific ERS antagonist, was purchased from Sigma-Aldrich (P21005, USA). Tunicamycin (Tm), a ERS inducer, was purchased from Cell Signaling Technology (CST, USA, #12819). STF-083010, an IRE1 $\alpha$ -specific inhibitor, was purchased from MedChemExpress (MCE, China, HY-15845). 4-PBA, Tm and STF-083010 were dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich).

## **Cell counting kit-8 assay**

Cell viability was assessed using a cell counting kit-8 (CCK-8, Dojindo Laboratories, Japan, CK04) assay according to the manufactory's instruction. The optical density (OD) values were measured at 450 nm with a microplate reader (Fluoroskan Ascent, Thermo Fisher Scientific, USA). The cell viability was expressed as percentage of the vehicle controls and the IC<sub>50</sub> values (half maximal inhibitory concentration) were calculated. All experiments were performed in 6 repeats.

## **Apoptosis analysis by flow cytometry**

CRC cells were stained with Alexa® Fluor 488 Annexin V/PI kit (Invitrogen, USA, V13241) according to the manufacturer's instruction. Apoptosis was analyzed using a BD FACSCyte flow cytometer (Beckman Coulter, USA). All experiments were performed in triplicate.

### **Western blot**

Total protein was extracted with RIPA lysis (Applygen, China) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE). PVDF membranes (Millipore, USA) were incubated with appropriate primary antibody overnight at 4 °C. The primary antibodies included rabbit polyclonal anti-GRP78 (1: 1000, Abcam, USA, ab21685, RRID:AB\_2119834), rabbit monoclonal anti-IRE1α (1:1000, CST, #3294, RRID:AB\_823545), mouse monoclonal anti-DDIT3/CHOP (1:1000, Abcam, ab11419, RRID:AB\_298023) and rabbit polyclonal anti-ABCC10 (1:1000, Abcam, ab107053, RRID:AB\_10864747). Mouse monoclonal anti-β-actin (Santa Cruz, USA, sc-8432, RRID:AB\_626630) was used as the internal control. The membranes were then incubated with corresponding secondary antibody at room temperature for 2 h. The immunoblotting bands were detected using an enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific) on a Fusion FX Vilber Lourmat (France). Image J software (NIH, USA) was employed for densitometric analysis of the immunoblotting bands.

### **Quantitative reverse transcription PCR (qRT-PCR)**

Total RNA was extracted using TRIzol reagent (Sigma-Aldrich). Reverse transcription of extracted mRNA was performed using a 5×All-In-One RT MasterMix (ABM, USA). qPCR was performed using a Powerup SYBR Master Mix (Thermo Fisher Scientific) and detected on an ABI7500 qPCR instrument (Applied Biosystems, USA). The reactions were incubated at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were performed in triplicate. The relative mRNA expression was calculated using the  $2^{-\Delta\Delta Ct}$  method. The oligonucleotide sequences of the qPCR primers are listed in Supplementary information, Table S1.

### **siRNA transfection**

siRNA specific to ABCC10 (siABCC10) as well as negative control siRNA (siControl) was ordered from RiboBio Co., Ltd. (China). Cells were transfected with siRNA using Opti-MEM I reduced serum medium (Gibco) and Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturers' instructions.

### **Lentiviral shRNA infection**

Lentiviral shRNA to ABCC10 (shABCC10) as well as control shRNA (shControl) was constructed by the support from Sangon Biotech (China). Three days post lentiviral shRNA infection, the infection efficiency was evaluated by observing the GFP fluorescence with a fluorescence microscope (Leica DMI3000B, Leica, German).

### **UPLC-MS/MS**

Intracellular drug concentration was detected by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). A Waters Acquity UPLC H-Class system (USA) was used for liquid chromatography. The chromatographic separation was performed on a ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm) and the temperature was maintained at 30 °C. The mobile phase consisted of acetonitrile:water (7:93, for Oxaliplatin detection) or methyl alcohol:water (55:45, for Paclitaxel detection) at a flow rate of 0.3 mL/min. The injection volume was 10 μL. MS/MS analysis was performed on a Waters Xevo TQ-XS triple quadrupole mass spectrometer.

## Statistical analysis

Results were presented as the mean±SEM. For relative quantification, the value of the control group was considered 1 or 100%. Student *t*-test, ANOVA followed by Tukey's post hoc test and *Chi*-square test were conducted using GraphPad prism 7 (GraphPad Software, USA) and SPSS20.0 (IBM, USA). *P*-value of 0.05 or less was considered statistically significant. GraphPad prism 7 and Photoshop CC 2017 (Adobe, USA) were used to create the artwork.

## Results

### ABCC10 was associated with Oxaliplatin sensitivity and CRC malignancy.

The chemo-resistance of cancer cells primarily attributed to the over-expression of the ABC transporter superfamily. Among these ABC transporters, we paid a special interest in ABCC10 because of the following reasons: ☐ According to the analysis on the clinic CRC specimens and the TCGA database, we found that although there was no significant difference in the ABCC10 expression between the tumors and adjacent normal tissues (Fig. 1a and b), the CRC patients with higher ABCC10 were apt to have metastasis, recurrence and shorter survival time (Fig. 1c~f). ☐ The IC<sub>50</sub> of Oxaliplatin in 5 CRC cells including Caco-2, RKO, HCT-116, LS174T and HT-29 was 56.6 ng/mL, 65.8 ng/mL, 23.9 ng/mL, 60.8 ng/mL and 69.8 ng/mL, respectively, which was positively correlated with the expression level of ABCC10 (Fig. 1g~j), indicating that the CRC cells with lower ABCC10 were more Oxaliplatin sensitive.

### Knock down of ABCC10 sensitized CRC cells to Oxaliplatin.

In siABCC10 (100 nM) treated Caco-2, LS174T and RKO cells, Oxaliplatin IC<sub>50</sub> was declined from 56.2 ng/mL to 15.1 ng/mL, 72.6 ng/mL to 33.0 ng/mL, and 40.6 ng/mL to 8.9 ng/mL, respectively (Fig. 2a). Caco-2 cells treated with siABCC10 or siControl were exposed to 80 ng/mL of Oxaliplatin for 24 h and the intracellular accumulation of Oxaliplatin was measured by UPLC-MS/MS. Cells treated with 100 nM and 200 nM of siABCC10 had significantly increased Oxaliplatin accumulation (179.4±8.8 pg/mL and 152.1±5.1 pg/mL, respectively) compared with siControls (77.0±6.5 pg/mL) and 50 nM of siABCC10 treated cells (60.6±3.9 pg/mL) (Fig. 2b).

The increase of intracellular Oxaliplatin could be due to a decrease in the efflux of Oxaliplatin and/or an increase in the uptake of Oxaliplatin. To investigate the exact reason, ABCC10 knock-down Caco-2 cells

and controls were incubated with 80 ng/mL of Oxaliplatin for 24 h, followed by further incubation in medium without Oxaliplatin for 0, 30, 60 and 120 min. The remained intracellular Oxaliplatin in shABCC10 group was  $5814.3 \pm 590.4$  pg/mL,  $3947.1 \pm 508.8$  pg/mL,  $3240.0 \pm 200.0$  pg/mL and  $1305.0 \pm 330.0$  pg/mL at 0, 30, 60 and 120 min, respectively, which was significantly increased compared with that in shControl group ( $1985.7 \pm 461.5$  pg/mL,  $307.5 \pm 13.6$  pg/mL,  $315.0 \pm 33.3$  pg/mL and  $440.0 \pm 40.8$  pg/mL at 0, 30, 60 and 120 min, respectively) (Fig. 2c), demonstrating that the increased accumulation of intracellular Oxaliplatin by ABCC10 down-regulation was fundamentally because of the reduced efflux of Oxaliplatin.

These results indicated that Oxaliplatin was a substrate of ABCC10 transporter and the highly expressed ABCC10 accelerated Oxaliplatin efflux. Down-regulation of ABCC10 could impede Oxaliplatin efflux, which increased the intracellular Oxaliplatin accumulation and conferred the chemo-sensitivity of CRC cells.

### **ERS/UPR was activated in CRC.**

The ERS/UPR plays a dual role in tumor development, but its role in the Oxaliplatin resistance has not been fully elucidated. Here, the microarray and GO Enrichment analysis on CRC specimens revealed that the ERS/UPR, especially the IRE1 $\alpha$  pathway, was activated in the CRC tissues compared with the adjacent normal tissues (Fig. 3a). GRP78, the biomarker of ERS, and its downstream IRE1 $\alpha$  were hyper-expressed in tumors (Fig. 3b). The same results were obtained from the TCGA analysis showing that multiple genes in the IRE1 $\alpha$  pathway were highly expressed in the CRC tissues (Fig. 3c).

### **Inactivation of ERS was unable to significantly reduce CRC cell viability.**

In tumor cells, the adaptive ERS/UPR was conducive to the proliferation, invasion, angiogenesis and other malignant biological activities [19], prompting that inactivating ERS/UPR seems to be an anti-tumor approach. Therefore, in this study, the CRC cells were treated with an ERS inhibitor, 4-PBA (5 mM), for 12, 24 or 48 h. Unexpectedly, inactivation of ERS was incompetent to reduce CRC cell viability. Conversely, CRC cell viability was invigorated in some cases (Fig. 4a).

### **Mild ERS/UPR neither inhibited CRC cell viability.**

Given that the ERS/UPR could also exhibit anti-CRC effect, the CRC cells were exposed to different doses of Tm, which can provoke ERS by blocking the N-glycosylation in the post translational modification. As shown in Fig. 4, despite that low dose of Tm (0.5, 1 and 2  $\mu$ g/mL) induced the ERS and the activation of IRE1 $\alpha$  pathway in the CRC cells (Fig. 4b), the cell viability was not influenced significantly or just slightly reduced (Fig. 4c).

### **Intense ERS/UPR elicited significant anti-CRC effect.**

On the contrary, high dose of Tm (10  $\mu$ g/mL) evidently abated the CRC cell viability upon the activation of the ERS/IRE1 $\alpha$  pathway (Fig. 5a and b). It is worth noting that the viability of the NCM460 (normal colonic epithelial cell line) was not significantly decreased by high dose of Tm (Fig. 5c). Whereas,

inhibition of the ERS by 4-PBA (5 mM) weakened its viability, which was not reversed by the additional Tm (Fig. 5c). Moreover, the intense ERS profoundly up-regulated the pro-apoptotic factor CHOP and promoted the CRC cell apoptosis, while the mild ERS did not (Fig. 5d and e). These results suggested the intense ERS/UPR instead of the mild ERS/UPR could elicit significant anti-CRC effect without obvious side effects on normal colonic epithelial cells.

### **Intense ERS/UPR sensitized CRC cells to Oxaliplatin.**

Next, we wondered if the intense ERS was capable of raising the chemo-sensitivity to Oxaliplatin. The CRC cells were treated with increasing doses of Oxaliplatin in the presence or absence of Tm (10  $\mu\text{g}/\text{mL}$ ). When treated with Oxaliplatin alone, the  $\text{IC}_{50}$  was 52.2 ng/mL in Caco-2 cells and 67.0 ng/mL in RKO cells (Fig. 5f). The cell viability was further attenuated in response to additional Tm treatment, indicated by the reduced  $\text{IC}_{50}$  (20.4 ng/mL in Caco-2 cells and 15.6 ng/mL in RKO cells) (Fig. 5f). A rescue assay demonstrated that when the IRE1 $\alpha$  pathway was blocked by STF-083010 (200  $\mu\text{M}$ ), the cell viability was evidently recovered ( $\text{IC}_{50}$ =80.8 ng/mL in Caco-2 cells and  $\text{IC}_{50}$ =71.4 ng/mL in RKO cells) (Fig. 5f). These results suggested that the intense ERS/UPR was competent to enhance Oxaliplatin sensitivity of CRC cells via IRE1 $\alpha$  pathway.

### **Intense ERS/UPR increased intracellular Oxaliplatin accumulation by down-regulating ABCC10 via IRE1 $\alpha$ pathway.**

Since ABCC10 was responsible for Oxaliplatin efflux and sensitivity, we then asked whether ABCC10 was a target of the intense ERS/UPR. As shown in Fig. 6, high dose of Tm (10  $\mu\text{g}/\text{mL}$ ) significantly down-regulated ABCC10 protein in several CRC cells (Fig. 6a). However, when the IRE1 $\alpha$  pathway was blocked by STF-083010 (200  $\mu\text{M}$ ), the expression of ABCC10 was restored (Fig. 6b). The intracellular accumulation of Oxaliplatin in Tm-treated CRC cells reached  $6066.7 \pm 759.8$  pg/mL, which was significantly increased compared with controls ( $1333.3 \pm 47.1$  pg/mL). While, the blockage of IRE1 $\alpha$  pathway by STF-083010 decreased Oxaliplatin accumulation to  $8333.3 \pm 23.6$  pg/mL (Fig. 6c). To confirm the disruptive role of Tm in ABCC10, a determined substrate of ABCC10, Paclitaxel [20], was used as a positive control in this study. Caco-2 cells were exposed to Paclitaxel (80 ng/mL) or Paclitaxel (80 ng/mL)+Tm (10  $\mu\text{g}/\text{mL}$ ) for 24 h. The intracellular accumulation of Paclitaxel was clearly increased in response to the intense ERS/UPR, but the increase was reversed by pre-treatment of STF-083010 (200  $\mu\text{M}$ ) (Fig. 6d).

Finally, we investigated the mechanism by which the intense ERS/UPR decreased ABCC10. Owing to the endoribonuclease domain, IRE1 $\alpha$  mediates the cleavage of multiple RNAs in a process known as regulated IRE1-dependent decay (RIDD). The target mRNAs of RIDD contain a consensus sequence, CUGCAA, which could form a hairpin secondary structure in order to be cleaved. By the use of the RNAfold Web server, we found that ABCC10 mRNA (NM\_001198934.2) contains the CUGCAA consensus sequence at the site of 1445~1450, suggesting that ABCC10 mRNA was a putative target of RIDD (Fig. 6e). Thus, we traced ABCC10 mRNA levels in Caco-2 cells exposed to Tm (10  $\mu\text{g}/\text{mL}$ ) at 2, 4 and 8 h, in

the presence or the absence of the IRE1 $\alpha$  RNase activity inhibitor STF-083010 (200  $\mu$ M). ABCC10 mRNA was remarkably decreased when treated with Tm, while STF-083010 increased it up to 8 h (Fig. 6f).

## Discussion

In this study, we for the first time provided evidence that Oxaliplatin is the substrate of ABCC10 transporter. Highly expressed ABCC10 in CRC cells conferred resistance to Oxaliplatin through accelerating drug efflux, contributory to the metastasis and recurrence of CRC. The intense ERS/UPR is capable of down-regulating ABCC10 through IRE1 $\alpha$  pathway, which sensitized CRC cells to Oxaliplatin by increasing intracellular drug accumulation.

Oxaliplatin has been commonly used in combination with other chemotherapeutics, especially for those failed in 5-Fluorouracil remedy [21]. However, like most chemotherapeutics, Oxaliplatin resistance usually emerged in surprisingly 90% of metastatic patients. Drug resistance could be attributed to the decreased drug uptake, impaired DNA adduct formation, alteration in DNA repair genes, defects in cell apoptosis and, the most frequently, over-expression of ABC transporters in tumor cells [22].

CRC cells permanently exposed to a high dose of Oxaliplatin had an up-regulation of ABCB1 and a poor response to Oxaliplatin [23]. Silencing ABCC2 increased Oxaliplatin accumulation and cytotoxicity in Caco-2 CRC cell line [24]. Evidence provided by Ma *et al.* supported the hypothesis that the Oxaliplatin resistance in CRC correlated with the ABCG2 over-expression in a subset of cancer stem cells [25]. In this study, we picked out ABCC10, also known as multidrug resistance-associated protein 7 (MRP7), as a novel putative candidate responsible for Oxaliplatin efflux. *In vitro* experiments uncovered that ABCC10 conferred tumor cells with multidrug resistance [26]-[31]. While ABCC10 knock-out mice exhibited susceptibility to Paclitaxel treatment [32]. However, there has been no research demonstrating the role of ABCC10 in Oxaliplatin efflux in CRC cells. Only one analysis based on an investigation of 1444 single nucleotide polymorphisms (SNPs) from a cohort of 623 stage II-IV CRC patients pointed out that 6 transporter genes, ABCC10 included, could be predictive to identify the patients who were more likely to benefit from Oxaliplatin treatment [33]. Here, we explored that the CRC cells with higher ABCC10 expression were more resistant to Oxaliplatin. With the ABCC10 knock down, the intracellular accumulation of Oxaliplatin was increased and the Oxaliplatin sensitivity was significantly restored consequently.

To date, 3 generations of ABC transporter modulators have been developed to restore chemo-sensitivity or eliminate chemo-resistance. They could either block or inactivate ABC transporters to increase the intracellular concentration of chemotherapeutic drugs within tumor cells. These ABC transporter modulators indeed showed efficacy in pre-clinical trials but always not effective in clinic trials. Some of them produced cardiotoxicity and inhibited hepatic and intestinal cytochrome P450 enzymes thereby causing systemic toxicity [34]. Moreover, nearly all of the ABC transporter modulators are targeting ABCG2, ABCB1, ABCC1 and ABCC2, while ABCC10 modulator has been unavailable yet.

In light of the role of ERS/UPR in the tumor development, we tried to investigate the possibility that induced ERS/UPR in regulating Oxaliplatin resistance. We found that the intense ERS/UPR instead of the mild ERS/UPR inhibited CRC cell viability and promoted apoptosis with little or no side effect on human normal colonic epithelial cell NCM460. Actually, some anti-cancer drugs and natural compounds worked through, in part, inducing ERS/UPR suggesting induction of intense ERS/UPR as a prospective anti-CRC strategy. Nelfinavir, an HIV protease inhibitor, inhibited small-cell lung cancer cell proliferation and induced cell death *in vitro* and *in vivo*, which was caused by induction of the UPR [35]. Vitamin E succinate-induced apoptosis was coupled to the ERS/UPR in human gastric cancer cells [36]. Our results revealed a novel anti-CRC mechanism of ERS/UPR by augmenting Oxaliplatin sensitivity when applied in combination.

We next investigated how the ERS/UPR multiplied the anti-CRC effect of Oxaliplatin, focusing on its efflux pump, ABCC10. Protein homeostasis, referred to as proteostasis, is under a control of an exquisite network of mechanism in cells. Among them, the ER exerts a substantial effect for it's the organelle where the protein synthesis, folding, trafficking and degradation are carefully orchestrated. Disrupted protein homeostasis resulted from permanent or intense ERS destroys protein integrity and functionality. But in another way, tumor cell bioactivities could be inhibited as a result of the intense ERS/UPR-induced protein homeostasis perturbation. The ERS decreases protein products from 3 arms of UPR, PERK, ATF6 and IRE1 $\alpha$ . IRE1 $\alpha$  is a transmembrane protein containing a kinase and an endoribonuclease domain, the latter mediates the RIDD. Degradation of the specific RIDD targeted RNAs may impact particular signaling pathways in a cell-specific manner [37]. Here, we identified a novel regulatory mechanism of ABCC10 in CRC cells via the RIDD way. Furthermore, ABCC10 was not the exclusive target of the ERS/UPR. We detected significant down-regulation of other ABC transporters that were as well responsible for Oxaliplatin efflux, including ABCB1, ABCC2, ABCG2, by the intense ERS/UPR (Supplementary information, Fig. S1).

## Conclusion

Our results demonstrated that the intense ERS/UPR showed potent anti-CRC effect probably by sensitizing CRC cells to Oxaliplatin via down-regulating ABCC10 through IRE1 $\alpha$  RIDD activity, in addition to increasing pro-apoptotic factor CHOP. Application of Oxaliplatin in combination with ERS/UPR inducer would exert better curative effect on CRC patients, especially those refractory to Oxaliplatin.

## Abbreviations

4-PBA: 4-phenylbutyric acid; ABC transporter: ATP-binding cassette transporter; ATF6: activate transcription factor 6; CCK-8: cell counting kit-8; CHOP: CCAAT/enhancer-binding protein homologous protein; CRC: colorectal cancer; ERS: endoplasmic reticulum stress; GRP78: 78-kDa glucose-regulated protein; IC<sub>50</sub>: half maximal inhibitory concentration; IRE1: inositol-requiring enzyme 1; MRP: multidrug resistance-associated protein; OD: optical density; PERK: protein kinase RNA-activated-like ER kinase; RIDD: regulated IRE1-dependent decay; Tm: Tunicamycin; UPLC-MS/MS: ultra-high performance liquid

chromatography coupled to tandem mass spectrometry; UPR: unfolded protein response; XBP1: X-box binding protein 1.

## **Declarations**

### **Ethics approval and consent to participate**

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of the Capital Medical University Ethical Committee. Informed consent was obtained from all subjects involved in the study.

### **Consent for publication**

All the listed authors have participated in the study, and have seen and approved the submitted manuscript.

### **Availability of data and materials**

The data presented in this study are available on request from the corresponding author on reasonable request.

### **Competing interests**

The authors declare no conflicts of interest.

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### **Authors' contributions**

Conceptualization, S.Y. and D.Z.; Methodology, X.L., H.C., H.S., X.G., T.S., B.W., F.J. and S.Y.; Validation, S.Y. and D.Z.; Formal Analysis, X.L. and S.Y.; Writing-Original Draft Preparation, X.L. and H.C.; Writing-Review & Editing, S.Y. and D.Z.; Supervision, S.Y. and D.Z.; Project Administration, S.Y. and D.Z.; Funding Acquisition, S.Y., T.S., B.W. and D.Z. All authors read and approved the final manuscript.

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## Figures

### Figure 1

ABCC10 is negatively correlated with Oxaliplatin sensitivity and contributes to CRC malignancy. (a) ABCC10 protein is undifferentiated between CRC tissues and adjacent normal mucosal tissues. n=30. (b) There is no obvious difference in ABCC10 mRNA level between CRC tissues and normal mucosa according to TCGA data analysis. (c) CRC patients with higher ABCC10 are prone to metastasis. M0: no metastasis, M: metastasis. \* P<0.05 (d) CRC patients with higher ABCC10 are prone to recurrence post initial therapy. \* P<0.05 (e) ABCC10 level in CRC patients with tumor is significantly higher when compared with those without tumor. \* P<0.05 (f) CRC patients with lower ABCC10 survive those with higher ABCC10. (g) The basic ABCC10 protein expression in 5 CRC cell lines. n=3. (h) The basic ABCC10 mRNA expression in 5 CRC cells lines. n=3. (i) The CRC cells are treated with a gradient of Oxaliplatin (10, 20, 40, 80 and 160 ng/mL) for 24 h. The IC50 of Oxaliplatin in 5 CRC cell lines is calculated according to the cellular viability. n=6. (j) ABCC10 mRNA level has a significant positive correlation with IC50 of Oxaliplatin in 5 CRC cell lines. n=6.

## Figure 2

Oxaliplatin is a substrate of ABCC10 and knock down of ABCC10 sensitizes CRC cells to Oxaliplatin. (a) CRC cells treated with siABCC10 (100 nM) or siControl (100 nM) are incubated in a gradient of Oxaliplatin (10, 20, 40, 80 and 160 ng/mL) for 24 h. Oxaliplatin IC<sub>50</sub> in siABCC10 groups is evidently decreased compared with siControl groups. n=6. \*\* P<0.01 (b) Oxaliplatin accumulation in Caco-2 cells exposed to Oxaliplatin (80 ng/mL) for 24 h is measured by UPLC-MS/MS. The remained intracellular Oxaliplatin is significantly increased when ABCC10 is knocked down by 100 nM and 200 nM of siABCC10 compared with 50 nM of siABCC10 and siControls. n=3. \*\* P<0.01 (c) Caco-2 cells are incubated in 80 ng/mL of Oxaliplatin for 24 h. Then Oxaliplatin is withdrawn and the cells are incubated for another 0, 30, 60 or 120 min. In ABCC10 knock down cells, the intracellular Oxaliplatin residual is much more than that in controls. n=3. \*\*\* P<0.001

## Figure 3

ERS/UPR is activated in CRC tissues. (a) Microarray and GO analysis show that the ERS/UPR, especially the IRE1 $\alpha$  pathway, is activated in CRC compared with normal mucosa. (b) The ERS biomarker GRP78 is hyper-expressed and its down-stream IRE1 $\alpha$  is activated in CRC. n=30. \*\*\* P<0.001 (c) The Oncomine Oncology Database analysis suggests that a series of genes involved in the IRE1 $\alpha$  pathway are up-regulated in CRC. \*\* P<0.01, \*\*\* P<0.001

## Figure 4

Neither inactivation of ERS nor induction of mild ERS/UPR significantly reduce CRC cell viability. (a) Inhibition of the ERS/UPR by 4-PBA (5 mM) for 12 h, 24 h or 48 h dose not suppress the cellular viability significantly in most of the CRC cells. Even, some CRC cells displays increased viability. n=6. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001 (b) Low dose of Tm (0.5, 1 and 2  $\mu$ g/mL) increases GRP78 and activates IRE1 $\alpha$  pathway of the UPR. n=3. \*\*\* P<0.001 (c) Except for the treatment of 2  $\mu$ g/mL of Tm on RKO cells for 24 h, the cellular viability is generally not inhibited a lot when treated with low dose of Tm (0.5 and 2  $\mu$ g/mL). n=6. \* P<0.05 Tm denotes Tunicamycin.

## Figure 5

Intense ERS/UPR significantly reduces CRC cell viability, promotes apoptosis and sensitizes CRC cells to Oxaliplatin. (a) High dose of Tm (10  $\mu$ g/mL) treatment for 4 h, 8 h, 12 h and 24 h increases GRP78 and activates IRE1 $\alpha$  pathway. n=3. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001 (b) The cellular viability of multiple CRC cells exposed to Tm (10  $\mu$ g/mL) are remarkably decreased. The additional ERS inhibitor 4-PBA (5 mM) can't effectively restore the viability. n=6. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001 (c) Unlike on the CRC cells, high dose of Tm (10  $\mu$ g/mL) has no obvious inhibitory effect on the normal human colonic epithelial NCM460

cells; while 4-PBA significantly inhibits their viability. Moreover, the viability of NCM460 cells is kept restrained when co-treated with 4-PBA and Tm. n=6. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001 (d) The pro-apoptotic factor CHOP in RKO cells is profoundly increased by high dose of Tm (10 µg/mL) for 4, 8, 12 and 24 h, while kept hypo-expressed upon the treatment of low dose of Tm (0.5, 1 and 2 µg/mL) for 24 h. n=3. \*\* P<0.01, \*\*\* P<0.001 (e) The intense ERS/UPR induced by high dose of Tm (10 µg/mL) significantly accelerates the CRC cell apoptosis; but the mild ERS/UPR induced by low dose of Tm (0.5, 1 and 2 µg/mL) has no such effect. n=3. \* P<0.05 (f) The CRC cells are treated with a gradient of Oxaliplatin (10, 20, 40, 80 and 160 ng/mL) for 24 h. Compared with the Oxaliplatin-treated cells, additional Tm (10 µg/mL) significantly reduces the cell viability. However, upon the pre-treatment of STF-083010 (200 µM) for 1 h to block the IRE1α pathway, the reduced cell viability is distinctively recovered. n=6. \*\* P<0.01 compared with Oxa., ## P<0.01 and ### P<0.001 compared with Oxa.+Tm. Oxa. denotes Oxaliplatin; Tm denotes Tunicamycin, STF denotes STF-083010.

## Figure 6

Intense ERS/UPR increases intracellular Oxaliplatin accumulation by down-regulating ABCC10 via IRE1α pathway. (a) High dose of Tm (10 µg/mL) treatment for 24 h clearly decreases ABCC10 in all 5 CRC cell lines. n=3. \*\* P<0.01, \*\*\* P<0.001 (b) High dose of Tm (10 µg/mL) treatment for 24 h activates the IRE1α pathway of UPR and down-regulates ABCC10. While pre-treatment of STF-083010 (200 µM) for 1 h partly recovers ABCC10 expression. n=3. \*\* P<0.01, \*\*\* P<0.001 (c) Oxaliplatin accumulation is significantly increased in response to Tm (10 µg/mL) in Caco-2 cells. While pre-treatment of STF-083010 (200 µM) decreased intracellular Oxaliplatin accumulation. n=3. \*\* P<0.01 (d) Tm (10 µg/mL) increases intracellular accumulation of Paclitaxel, a determined substrate of ABCC10, which is reversed by pre-treatment of STF-083010 (200 µM). n=3. \*\* P<0.01 (e) ABCC10 mRNA contains the CUGCAA consensus sequence which forms a hairpin structure predicted by RNAfold Web server. (f) ABCC10 mRNA level is significantly decreased when exposed to Tm (10 µg/mL). Additional STF treatment restores ABCC10 mRNA level up to 8 h. n=3. \*\*\* P<0.001. Tm denotes Tunicamycin, STF denotes STF-083010.

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