# Human Drug Efflux Transporter ABCC5 Confers Acquired Resistance to Pemetrexed in Breast Cancer

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**Primary research**

**Keywords:** ABC transporter, Breast cancer, Pemetrexed, Drugs Resistance, Sensitivity

**Posted Date:** January 27th, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-105352/v2



**Version of Record:** A version of this preprint was published on February 25th, 2021. See the published version at https://doi.org/10.1186/s12935-021-01842-x.

# Abstract

## Aim

Pemetrexed, a new generation antifolate drug, is approved for the treatment for locally advanced or metastatic breast cancer, but factors affecting the efficacy and resistance of it have yet to be fully explicit. ATP-binding cassette (ABC) transporters have been reported as prognostic and adverse effects predictors of many xenobiotics. This study was designed to explore whether ABC transporters affect pemetrexed resistance and may contribute to treatment regimen optimization for breast cancer.

## Methods

Firstly, the expression of ABC transporters family members was measured in cell lines, thereafter examined the potential role of ABC transporter in conferring resistance to pemetrexed in primary cancer cell lines isolated from 34 breast cancer patients, and then the role of ABCC5 in mediating transport of pemetrexed and apoptosis pathway in MCF-7 cell line was assessed. Finally, the functions of ABCC5 on therapeutic effect of pemetrexed was evaluated in breast cancer bearing mice.

## Results

The expressions of ABCC2, ABCC4, ABCC5 and ABCG2 were significantly increased in pan-resistance cell line, and the ABCC5, the most obvious one, was 5.21 times higher than that of the control group. The expression of ABCC5 was inversely correlated with sensitivity ($IC_{50}$) of pemetrexed ($r = 0.741$; $p<0.001$) in breast cancer cells from 34 patients. Furthermore, we found that the expression of ABCC5 influenced the efflux and cytotoxicity of pemetrexed in MCF-7 cell line, and the $IC_{50}$ were 0.06 μg/ml and 0.20 μg/ml in ABCC5 knock-down and over-expression cells, respectively. In *in vivo* study, we found ABCC5 affected the sensitivity of pemetrexed in breast cancer bearing mice, and the tumor volume was much larger in ABCC5 over-expression group than that in control group (2.7 folds vs 1.3 folds).

## Conclusions

Our results indicated ABCC5 expression was associated with pemetrexed resistance in vitro and in vivo, and may be a biomarker for regimen optimization of pemetrexed in breast cancer treatment.

# Introduction

Pemetrexed (MTA) is a novel multi-targeted antifolate for the treatment of non-small cell lung cancer and mesothelioma[1-4] through inhibiting thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyltransferase, which are folate-dependent enzymes involved in the *de novo* biosynthesis of thymidine and purine nucleotides[5, 6]. Shaughnessy et al. proved that MTA had a good effect (overall response rate:8%, stable disease:36%, median survival:8 months) on metastatic breast cancer (BC) and well-tolerated in 80% patients in second-line treatment [7], and another study presented

an approximately 30% response rate in advanced BC patients in first-line, and 21% in second-line treatment, too [8].

The mechanism of sensitivity and/or acquired resistance of MTA is complex, mainly including decreased intracellular concentration and alteration of metabolism, etc[9].The cytotoxic activity of MTA is largely attributed to its concentration and retention time in the cells[10]. Thus, transmembrane transport of MTA is a critical determinant for its activity. In this context, the decreased expression of influx transporters and increased expression of efflux transporters could induce the cancer cells more resistant through reducing the intracellular drug exposure[11, 12]. The ATP binding cassette transporter (ABC transporter), also named as multidrug resistance protein (MRP)[13], is capable of conferring resistance to nucleotide analogues such as 5-FU[14, 15], 6-thioguanine (6-TG)[16], 6-mercaptopurine (6-MP)[17], and 9-(2-phosphonylmethoxynyl)-adenine (PMEA)[18, 19]. The expression of ABC transporters is regulated by PXR (pregnane X receptor), CAR (constitutive androstane receptor) and other transcriptional regulators, and the primary functions of them lie in efflux many endogenous and exogenous substrates[20]. ABC transporters comprise approximately of 50 members and are subdivided into seven groups (ABCA to ABCG)[21]. In particularly, the C branch of the ABC transporters (ABCC) superfamily dominated in multidrug resistance[22, 23].The first evidence about the hydrophilic antifolate transport by certain members of the ABCC family was put forward in 1997 by Masuda and his colleagues[24], who demonstrated that MTA was transported into bile only in the wild type rats but not in rats that has hereditary deficiency in ABCC2 function. After this study, many researchers have found more evidences of ABCC affecting the antifolate transport. Vlaming, et al. found that the absence of ABCC2 and/or ABCG2 in mice increased the oral availability of MTA [25,26]. Wielinga, et al. identified an important exporter, ABCC5, which is involved in the transport of MTA and folic acid in HEK 293 cells[28].

Previous studies have proven that the expression of ABC transporter in human cancer could regulate the efficacy of chemotherapy, but the contribution of ABC transporters to drug-resistance, especially to novel antifolate drug has yet to been fully elucidated. This study was designed to explore whether ABC transporters influence the MTA sensitivity and resistance in BC.

# Materials And Methods

### Cell lines

MCF-7 and MCF-7-ADR (MCF-7-adriamycin-resistant breast cancer cells) cell lines were obtained from the Culture Collection Company (ATCC-LGC Promochem, Teddington, UK). Cells were routinely grown in DMEM (Invitrogen) medium supplemented with 10% FBS (Invitrogen) and 100 units of penicillin/streptomycin per mL (Invitrogen) at 37 ℃ and 5% $CO_2$ under humidifying conditions. The medium was refreshed every two or three days regularly until the cells reached 80-90% confluence, and the cells were transferred to next experiment or make stock solutions. The mycoplasma contamination was tested every month.

## Reagents

MTA (reference substance solution), which was used in quantitative analysis in a reverse phase-high performance liquid chromatography (RP-HPLC) experiment, was purchased from Sigma-Aldrich (St. Louis, MO). MTA used for cell incubation, was purchased from Eli Lilly company. ABCC5 human shRNA was bought from OriGENE company (Locus ID 10057, Product ID TL315024). Rabbit antihuman Anti-MRP5 antibody [M5II-54](ab137070, Abcam), rabbit antihuman Bax mAb (5023, CST), Rabbit antihuman caspase-3 mAb (14220, Cell Signaling Technology), cleaved caspase-3 mAb (9664, Cell Signaling Technology) and β-Actin (13E5) rabbit mAb (4970, Cell Signaling Technology) were used as the primary antibodies, and the goat anti-rabbit IgG (final dilution 1:2000; LI-COR Biosciences, Lincoln, NE, USA) was used as the secondary antibody.

## Generation of ABCC5 adenovirus

For recombinant adenovirus construction, the ABCC5-gene cDNA (AdvABCC5) and the green fluorescence protein gene cDNA (AdvCtrl, control) were cloned by PCR and inserted into pHBAD-EF1-MCS-3flag-CMV-EGFP vector (supplemental Fig.1). The pDC315-ABCC5 and pBHGloxE1,3Cre were co-transfected into HEK293 cells using Lipo-FiterTM transfection reagent (QIAGEN) to generate the recombinant adenoviruses. The recombinant AdvABCC5 and AdvCtrl adenoviruses produced in the HEK293 cells were purified and the virus titer was measured by plaque assay. The stock solutions of AdvABCC5 and AdvCtrl were $1\times10^{11}$ plaque formation unit (PFU)/ml, respectively.

## Collection of tumor tissue specimens from patients and primary breast cancer cell isolation

A total of 34 patients with confirmed primary BC (2 cm or larger), who consecutively underwent neoadjuvant chemotherapy containing anthracyclines at the Breast Tumor Department, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, were enrolled into this study from January 2014 to December 2015. Tumor specimens were obtained by surgical excision before MTA chemotherapy. Informed consents were signed and retrieved from all patients, following a protocol approved by the Ethics Committee of Xinhua Hospital, Shanghai Jiaotong University School of Medicine.

To isolate the BC cells, at least two consecutive frozen sections were prepared for each paraffin embedded tumor tissue sample, and one of the sections was then subjected to the hematoxylin-eosin staining to confirm the presence of cancer cell, and the adjacent one was transferred to cancer cell isolation as described in the previous publication [29]. Briefly, the blood, fat and fibro connective tissue were removed from the tumor tissue pieces, and then the residual was cut into 1-2 mm pieces for enzymatic disaggregation. The small tissue pieces were incubated with 2.5% crude trypsin for 30 minutes at 37°C and with collagenase (0.15%) overnight. Cells released after enzymatic treatments were further tested for cell viability and were cultured to perform subsequent experiments.

## Animal studies

Twenty-four female Balc/b nude mice (5 weeks, 18 g) were purchased from the Shanghai Super B&K Laboratory Animal Corp. Ltd. (Shanghai, China), and all the mice were raised in specific pathogen-free environment with free access to food and water. All animal studies were approved by the Research Ethics Committee at Xinhua hospital, affiliated to Shanghai Jiaotong University, school of Medicine.

On day 0, every mouse was injected with $1\times10^7$ MCF-7 cells subcutaneously into the right armpit. When tumors were approximately 100 $mm^3$ in size on day 30, adenoviruses containing ABCC5(AdvABCC5) ($5\times10^{11}$ PFU) were injected into the tumors of 12 mice to over-express ABCC5 in the tumor cells, and the vehicle (AdvCtrl) was applied to the other 12 control mice. The expression of ABCC5 was checked by diffused green fluorescence. When the volumes of tumors were approximately 150 $mm^3$on day 35, 6 of the ABCC5 over-expression mice and 6 mice in the control group were treated with intravenous injections (via the tail vein) of MTA (20 mg/kg, saline) once a day, and the same dose of vehicle was administrated to the others from day 35 to 46. The tumor volumes (V) were measured using a caliper once a day ($V = width^2 \times length/2$). Mice were killed at the end of 7-week, and tumor volume and weight were measured. The animal experiments' design has been shown in Fig.1

## Measuring the MTA in MCF-7 cell using RP-HPLC

MTA concentrations in the MCF-7 cell were determinated based on a developed HPLC method. The chromatographic separation and quantification were performed on an RP-column (ZORBAX Eclipse XDB-C8, 250 mm×4.6 mm, 5 μm; Agilent) with the column temperature maintained at 25 °C, and the MTA was detected with a ultraviolet detector at a wavelength of 240 nm. The mobile phase was composed of water plus 0.02 M phosphate buffer (pH 4.0)/acetonitrile (86:14, V:V) and delivered at a flow rate of 1 mL/min. The sample pretreatment was completed using an ultrafiltration method (0.22 μm). All experiments were completed on an Agilent 1260 HPLC system. A calibration curve was constructed at a range of 80-625 ng/mL for the MTA measurement. The injection volume was 20 μL, and all analysis was performed in triplicate.

## Cell preparation for cellular uptake analysis

MCF-7 cells were seeded at $2\times10^5$/well into six-well flat-bottom tissue-culture plates in triplicate. After 24 hours, the cells were infected with ABCC5, Ctrl and siABCC5-RNA expressing adenovirus, separately. The cells were incubated with adenoviral particles for another 24 hours and then refreshed with medium containing 50 μM MTA(21.37 μg/mL). At the particular time points (0, 0.5, 1, 2, 4, 24 hours), the cells were washed three times with cold PBS (0.1 M, pH 7.4) and then resuspended in 0.2 mL of RPMI-1640 medium and homogenized. After centrifugation at 13000 ×g for 10 min, the supernatant was harvested and stored at -80°C for the detection of MTA by the RP-HPLC method as mentioned above.

## Cell viability assay

Cell viability assay was performed using CCK-8 kit (Dojindo Laboratories) according to the manufacturer's instructions. Briefly, cells were plated in 96-well plates at a density of 2000 cells/well, and

then treated with different concentrations of MTA for 72 h. Then CCK-8 solution diluted with DMEM/F12 with 10% FBS at 1/10 ratio was added to each well and incubated for 2 hours at 37°C. Finally, the absorbance at 450 nm was measured using a microplate reader. The (%) cell viability was calculated using the formulae; (OD treatment group-ODBlank)/(OD control group-OD Blank) × 100. The $IC_{50}$ value was determined using GraphPad Prism software. All experiments were performed in triplicate, and the presented data represent the mean of three biological repeats.

### Western blot

Protein extracts were separated through 5% to 12% SDSPAGE, and then transferred to nitrocellulose membranes, probing with mouse monoclonal antibodies against ABCC5 or β-actin, and then followed by incubation with either IRDye 700 or 800 secondary antibodies and visualized using the Odyssey Infrared Imaging System software (Li-Cor, Lincoln, NE).

### RNA Isolation and Real-Time RT-PCR

Total RNA was extracted from the cells using RNeasy mini kit (QIAGEN) and qRT-PCR were performed on the cDNAs generated from 250 ng of total RNA by using HotStart-IT® SYBR® Green qPCR Master Mix along with UDG (2X) by a user-friendly TM kit (USB Corporation). ABC transporters subfamily primers were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd. (supplemental Table.1).

### Immunofluorescence microscopy

Cells were initially seeded onto coverslips, and then harvested and washed three times with PBS. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and blocked with 1% (w/v) bovine serum albumin, 0.1% Triton X-100, and 0.05% Tween-20 overnight at 4°C to avoid nonspecific staining. Next, the cells were incubated with goat polyclonal antibody, anti-ABCC5 for overnight. Subsequently, the secondary antibody (TRITC-rabbit-anti-goat, 1:100) was added and the cells were incubated for 1 hour in dark room. DAPI staining was used to visualize the cell nuclei. The images were captured by Leica DMI300B inverted fluorescent microscope.

### Data analysis

The results were presented as mean ± SD. The graphics and calculations were finished using Microsoft excel software (Microsoft Corp) or Prism 5.0 software (GraphPad Software Inc). The $IC_{50}$ values were calculated by nonlinear regression from a sigmoidal dose-response curve (variable slope, bottom value 0) using Prism software. Pearson's correlation test was used to analyze the correlation between the expression of target gene and the $IC_{50}$ values. The $p$ value<0.05 was considered statistically significant using unpaired t test analysis unless stated otherwise.

## Results

### The expression of ABC transporters was increased in MCF-7ADR cell lines

The expression of ABC subfamily was measured by qPCR in resistance cell lines MCF-7-ADR and control cells(MCF-7), and 18S expression was utilized as an internal standard to normalize all the data. Almost all the ABC transporters were up-regulated in MCF-7-ADR cells compared with control cells. The expressions of ABCC1 (up to 1.50 times), ABCC2 (1.46 times), ABCC4 (4.31 times), and ABCC5 (5.21 times) were significantly increased, and among them, the change of ABCC5 was most obvious, which imply a critical role of it in the drug-resistance of MTA(supplementary Fig. 2)

## ABCC5 expression in BC tissues correlates with MTA induced cell toxicity

To investigate the correlation between ABCC expression and cellular sensitivity ($IC_{50}$) to MTA in patients, we enrolled 34 BC patients and tested the $IC_{50}$ of primary BC cells isolated from their tissue samples. In addition, we performed the mRNA expression analysis of 11 ABC transporter family genes in the BC cells from the samples **(**supplemental Fig.3**)**. A *pearson's* correlation analysis was performed to evaluate the association between ABCC expression and BC cell viability. The ABCC2 expresses highest in all ABC transporter family but no association was found with MTA sensitivity (R=0.07, $p$=0.71), and only ABCC5, as shown in Fig.2, presented a significant correlation with the $IC_{50}$ of MTA(R=0.741, $p$<0.001).The ABCC5 may dominate the ABC transporter-mediated MTA drug resistance in BC.

## Effect of ABCC5 on efflux of MTA

To validate the functional significance of ABCC5 in drug efflux, we transducted MCF-7 cells with AdvABCC5 or AdvCtrl. As shown in Fig. 3A the transduction of MCF-7 cells with AdvABCC5 resulted in approximately 10-fold increase of ABCC5 protein expression. The immunofluorescence microscopy showed that ABCC5 was expressed on the cell membrane and an obvious increase was presented of ABCC5 levels in the AdvABCC5 group compared to the control (AdvCtrol, Fig 3B). The ABCC5 siRNA was used to knockdown ABCC5 expression, and the result showed ABCC5 mRNA was totally suppressed (Fig.3C). Next, we interrogated if alterations in ABCC5 expression in MCF-7 could influence the efflux of MTA and thereby alter its intracellular concentration. The MCF-7, ABCC5-overexpressing MCF-7 or ABCC5-knockdown MCF-7 cells were treated with 21.37 μg/mL MTA for 0, 0.5, 1, 2, 3, 4 or 24 hours at 37°C, and then the cells were collected and processed to extract the intracellular MTA, and the supernatant was analyzed for the intracellular MTA concentrations. The intracellular MTA concentration was much lower in the ABCC5-overexpression cells, and higher in ABCC5-knockdown cells, compared with the control cells ($p$<0.05, Fig.4). Our results prove a negative correlation between ABCC5 expression and the intracellular concentration of MTA in MCF-7.

## Over-expression of ABCC5 weaken the cytotoxicity of MTA

The cytotoxic effect of MTA was evaluated in ABCC5-slienced MCF-7, ABCC5-overexpressing MCF-7 and MCF-7 cells using the CCK-8 assay. As shown in Fig. 5A, the $IC_{50}$ of MTA was significantly declined after the expression of ABCC5 was silenced ($IC_{50}$=0.06±0.01 and 0.11±0.06 μg/ml for ABCC5 silenced and normal expression MCF-7 cell lines, respectively, $p$=0.02), and the $IC_{50}$ increased to 0.2±0.05 μg/ml if the

expression of ABCC5 was upregulated ($p$=0.003, compared to MCF-7 group). Compared with control cells, there was an obvious right shift of the dose-response curve in ABCC5-overexpression cells, and an inverse shift in ABCC5-knockdown MCF-7 cells. To further investigate apoptosis induced by MTA, the expressions of the cleaved caspase-3 and bax were measured using western blot. As shown in Fig. 5B, the expressions of the cleaved caspase-3 and Bax were significantly down-regulated ($p<0.05$) in ABCC5-overexpression cells, which represent a declined cell apoptosis.

### Tumor growth affected by ABCC5 in mice treated with MTA

The therapeutic effects of MTA were evaluated in mice bearing MCF-7+AdvCtrl or MCF7+AdvABCC5 tumor xenografts. As shown in Fig 6A, MTA treatment resulted in an average 77.7% decrease in tumor volume in mice bearing MCF-7+ AdvCtrl tumor xenografts, whereas a 41.3% decrease in tumor volume was presented in mice bearing MCF7+AdvABCC5 tumor xenografts. By the end of the experiment. The tumor volume in MTA-untreated mice had increased by 4.4-fold compared with the initial tumor volume (150 $mm^3$). The tumor volume in MTA-treated mice increased by 1.3-fold, while the tumor volume in the MTA-treated mice with ABCC5-overexpression had increased by 2.7-fold (AdvABCC5 mice untreated: 665 $mm^3$, AdvABCC5 mice treated with MTA: 195 $mm^3$, AdvABCC5 mice untreated: 703 $mm^3$, AdvABCC5 mice treated with MTA: 412 $mm^3$; AdvCtrl-MTA group vs untreated AdvCtrl mice group: $p<0.01$, AdvCtrl-MTA group vs untreated AdvABCC5 mice group: $p<0.01$, AdvCtrl group vs mice treated with MTA: $p<0.01$). Additionally, the tumor size of AdvABCC5 group was significantly higher than that of AdvCtrl groups when treated with MTA ($p<0.01$). These results showed the over-expression of ABCC5 could reduce the cytotoxic effect of MTA in vivo. (Fig 6B, Fig 6C).

## Discussion

Chemotherapy is the one of the main options for the treatment of nonresectable BC, but the development of resistance to chemotherapy agents has become a critical problem in the clinical practice. Multidrug resistance can be influenced by several factors, and the modulation of the expression and function of drug resistance proteins is an important way among that by which drug resistance is achieved[30], and specially, ABC transporters seems to be critical for emergence of multidrug resistance in cancer[31]. In our study, we have identified that the expression of all multidrug resistance proteins increased in the MCF-7-ADR cell line, and particularly, ABCC5 gene showed highest increase in its expression. This was further confirmed by the ABCC5 overexpression in MCF-7 cell line, in which the cells showed reduced sensitivity to MTA, decreased accumulation of MTA, enhanced efflux of MTA and eventually led to the repression of cell apoptosis. Furthermore, the effect of ABCC5 on MTA resistance was affirmed in an animal study.

It has been reported that the expressions of many proteins, for instance, P-glycoprotein (P-gp), ABC transporters[31], ABCG2 (breast cancer resistance protein, BCRP), lung resistance related protein (LRP) [32], ABCB5[33] and ABCA8[34] and so on were upregulated during multidrug resistance

development in cell lines, which form a unique defense network against multiple chemotherapeutic drugs and cellular toxins. There are many studies implicating the roles of ABC transporters in the efflux of folate and antifolate drugs[10, 35]. ABCC5 has also been shown to be involved in the efflux of many different anticancer drugs, for instance, 6-MP, 6-TG, 5-FU and its metabolites[10-12, 24, 25, 29], and this efflux was often considered to be associated with drug resistance. In addition, plenty of studies presented that the gene polymorphisms of ABC transporters influence the proteins expressions and finally determine the efficacy of some anticancer agents [36, 37] despite some paradoxical results in these studies were shown.

In our study, we observed that over expression of ABCC5 in MCF-7 cells resulted in increased resistance against high concentrations of MTA. This can be explained by the increased efflux of MTA out of the cells through ABCC5, which consequently led to the decrease in MTA accumulation inside the cells and thus reduced its activity and enhanced resistance. In general, it has been observed that upregulated ABCC5 exports the nucleoside analogs and increase the drug resistance in the range of 2- to 10- fold during in vitro assays.

Clinically, the role of ABC transporters in the intrinsic or acquired resistance is not clearly resolved. Uemura, et al suggested that ABCC11 directly confers the resistance to MTA by enhancing efflux of the intracellular anti-cancer drug in lung cancer[38], which suggested that ABCC11 maybe one of the biomarkers for MTA in the treatment of lung cancer. Oguri et al. found that the paclitaxel could induce the expression of ABCC10 gene, and which then in turn increase paclitaxel resistance by enhancing the paclitaxel efflux [39]. In nasopharyngeal carcinoma cell, the ABCC5 would be induced by paclitaxel through the activation of FOXM1, and the blocking of it could re-sensitize the nasopharyngeal carcinoma cell to paclitaxel [40]. A study has identified that ABCC5 expression is significantly associated with the sensitivity of a panel of non–small-cell lung cancer cell lines to gemcitabine, and inhibition of the transporters' activity by small molecule inhibitors or siRNA knockdown could markedly resensitized the cancer cells to gemcitabine[41]. Moreover, the expression of ABCC5 has presented significantly over-expressed in the non-responding group after neoadjuvant chemotherapy than in the responding group in BC[42]. Nambaru PK et al. reported that ABCC5 efflux the monophosphorylated metabolite of 5-FU when ABCC5 was upregulated in colorectal and BC, which contributed to the 5-FU drug resistance[10]. Other researchers have identified that ABCC5 is expressed and functionally active in pancreatic adenocarcinoma cell lines and contributes to their sensitivities to different drugs [10, 11]. Our study observed a close relationship between ABCC5 expression and cellular sensitivity to MTA in BC cells (R=0.741) too, and this phenomenon was accompanied by significantly decreased accumulation and enhanced efflux of MTA in ABCC5 overexpressing cell lines. In general, a comprehensive profiling of ABC transporters was emphasized and might be promise to drastically improve of outcomes and these transporters may be the new target for anticancer treatment.

# Conclusion

Our results showed that upregulated ABC transporter ABCC5 is positively correlated with MTA resistance in BC based on increased efflux of MTA. Further studies are still required to quantitatively assess the relationship between ABCC5 expression and the MTA dosage, so as to evaluate the ABCC5 expression as a biomarker for dosage optimization of MTA or a new target for BC treatment.

# Abbreviations

MTX: Methotrexate.

MTA: Pemetrexed.

ABC transporter: ATP-binding Cassette Transporter.

ABCC5: ATP-binding Cassette, Sub-family C, Member 5.

AICARFT: Aminoimidazole Carboxamide Ribonucleotide Formyltransferase.

RP-HPLC: Reversed Phase High Performance Liquid Chromatography.

PXR: Pregnane X Receptor.

CAR: Constitutive Androstane Receptor.

GFP: Green Fluorescent Protein

# Declarations

Ethics approval and consent to participate

Informed consents were signed and retrieved from all patients and all the experiments were approved by Research Ethics Committee at Xinhua hospital, affiliated to Shanghai Jiaotong University, school of Medicine.

Acknowledgments and Fundings:

This work was supported by National Institutes of Chinese Natural Science Foundation grants 81202598 (Y.L), 81702588Y.S.L, 81403021(H.S), and 12JC1406800 and 14411950206 (K.J.W); Shanghai Municipal Bureau of Health Grant 20ZR1435400 (Y.L) and Shanghai Collaborative Innovation Center for Translational Medicine(No: TM201710,Y.L.).

Competing interests

The authors declare that there have no any conflicts of interest regarding the content of this article.

Availability of data and material

All the data and materials were available in this paper.

Authors' contributions

Jihui Chen and Zhipeng Wang: manuscript writing and participated in most of the experiments.

Shouhong Gao, KejinWu, Fang Bai, Qiqiang Zhang, Hongyu Wang, Qin Ye, FengjingXu, Hong Sun: experiments execution and data analysis

Yunshu Lu and Yan Liu: manuscript review and editing, and project supervision and administration.

Consent for publication

All authors have reviewed and approved for this publication.

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# Figures

**Fig 1**

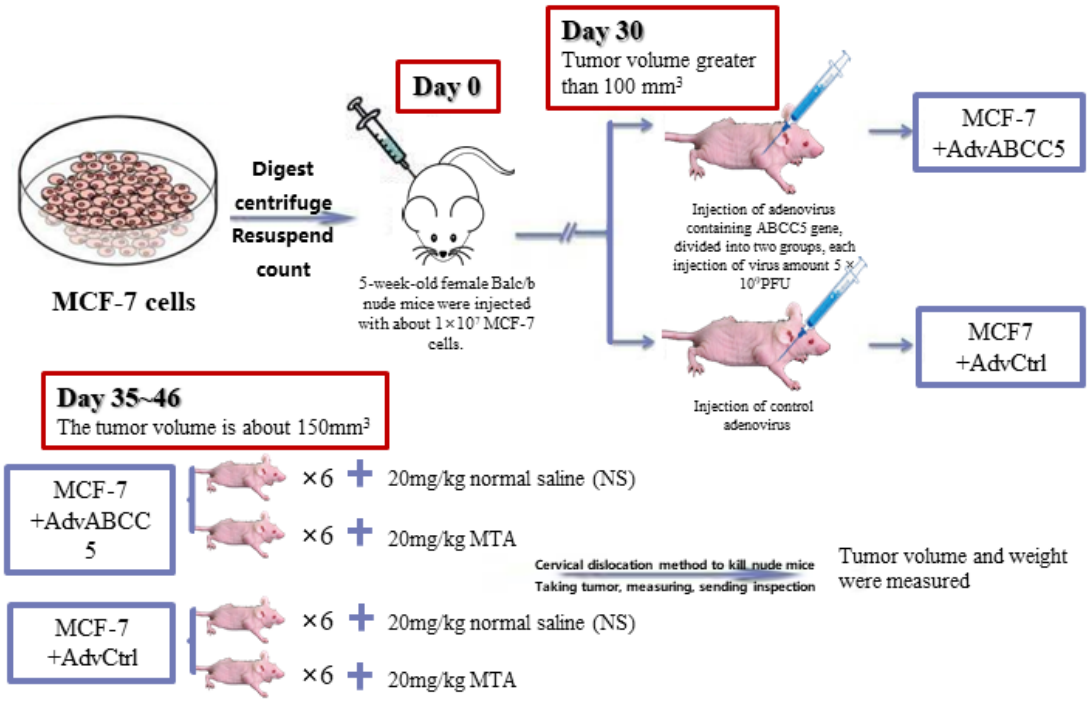
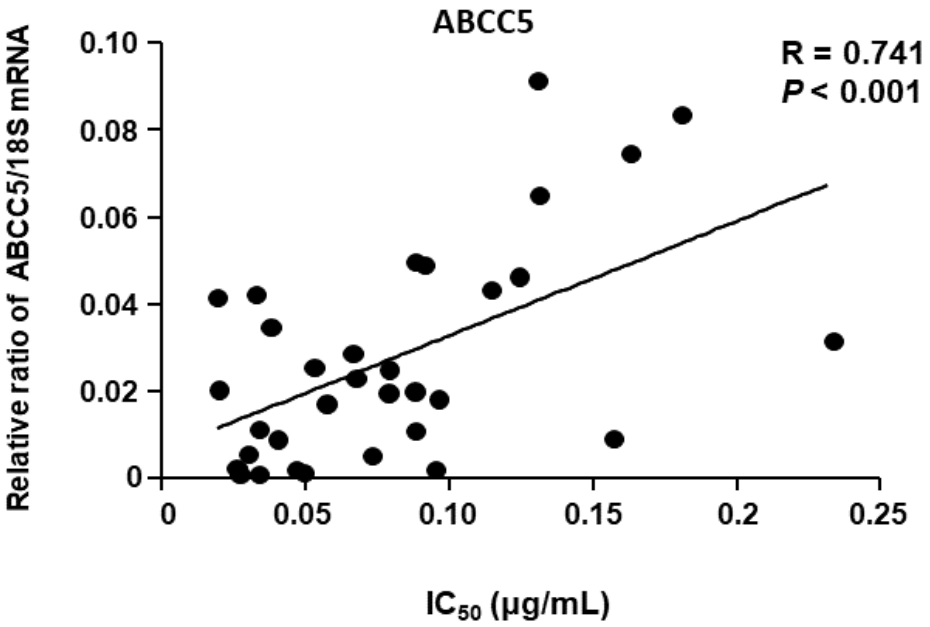



**Figure 1**

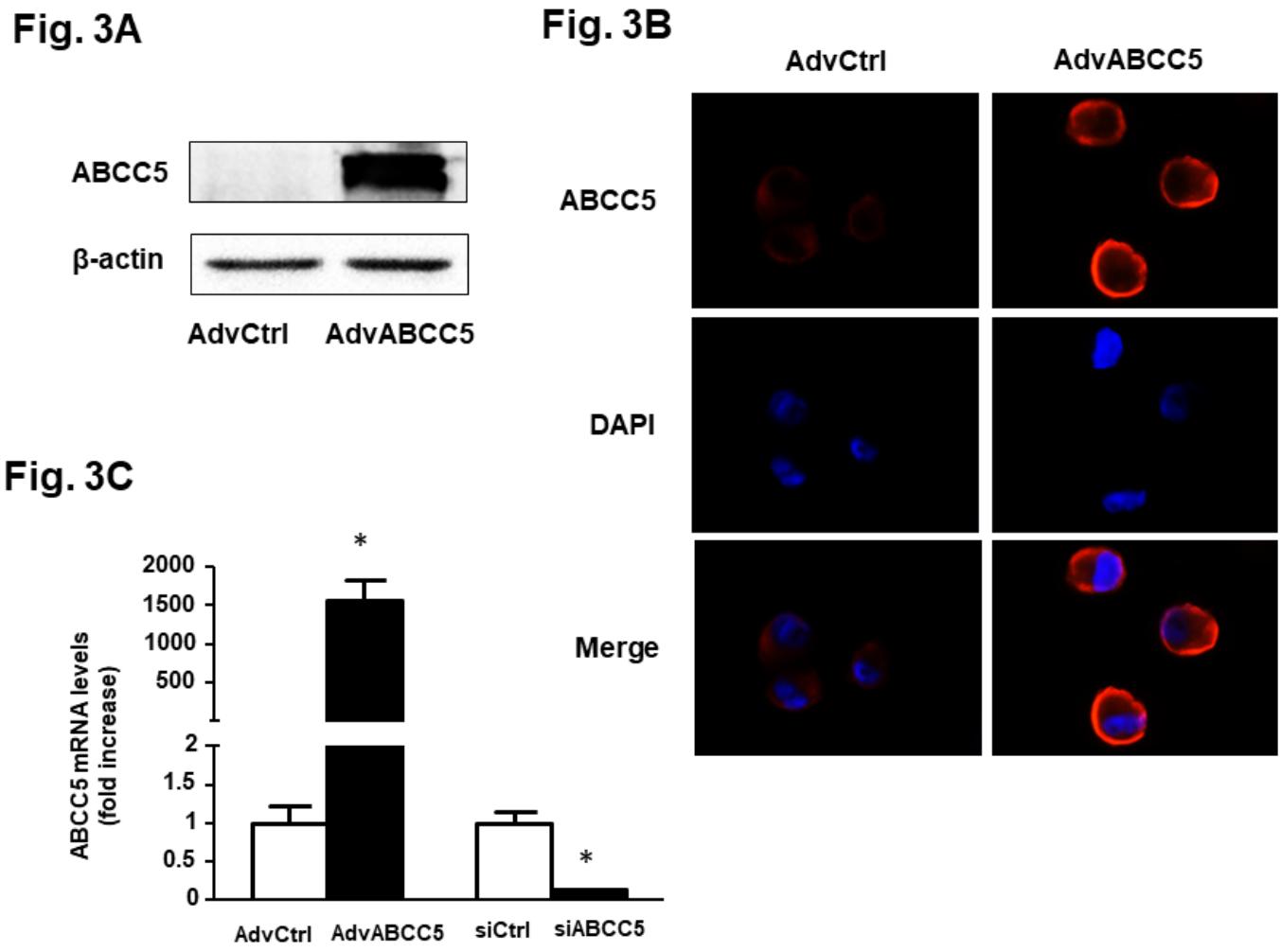
schematic diagram of animal study to evaluate the influence of ABCC expression on the efficacy of MTA

Fig.2




## Figure 2

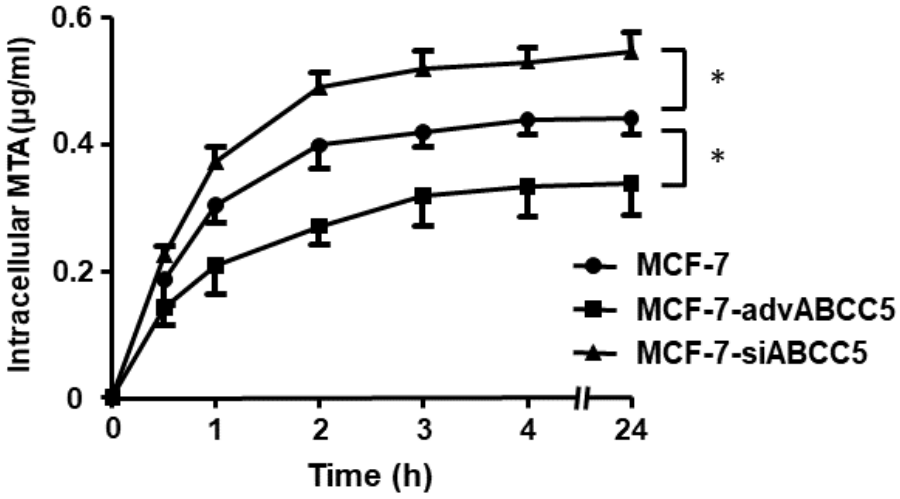
Correlation of IC50 values of MTA and expression of ABCC5 mRNA in primary cell lines from patients (pearson's correlation test). The dot represents means of three cell viability assays performed in triplicate. The 18S rRNA was used as an endogenous control.




## Figure 3

Assessment of transduction of ABCC5 adenovirus in MCF-7 cells FIG.3A: ABCC5 expression was determined by western blot analysis after transduction with recombinant ABCC5-specific adenoviruses (AdvABCC5; multiplicity of infection (MOI) = 1000) or AdvCtrl(MOI = 1000) in MCF-7 cells for 48 hours (n = 4), *p<0.05 vs AdvCtrl. FIG.3B: ABCC5 expression was determined by Immunofluorescence microscopy after transduction with recombinant AdvABCC5 or AdvCtrl. FIG.3C: Transductions of recombinant AdvABCC5 or siABCC5 regulate the expression of ABCC5. Data represent means ± SD (n=3), *p<0.05.

**Fig. 4**




* $p<0.05$

## Figure 4

Intracellular concentration-time curve of MTA influenced by the expression of ABCC5 in MCF-7 cells Data represents means ± SD (n=3), *p<0.05. The cells were incubated at 37°C with 50 μM(21.37 μg/mL) MTA for 0, 0.5, 1, 2, 3, 4 and 24 h. After washing three times with cold PBS (0.1 M, pH 7.4), the cells were resuspended in RPMI-1640 (0.2 mL) and then homogenized. The MTA was quantified in the supernatant after centrifugation.

Fig. 5A

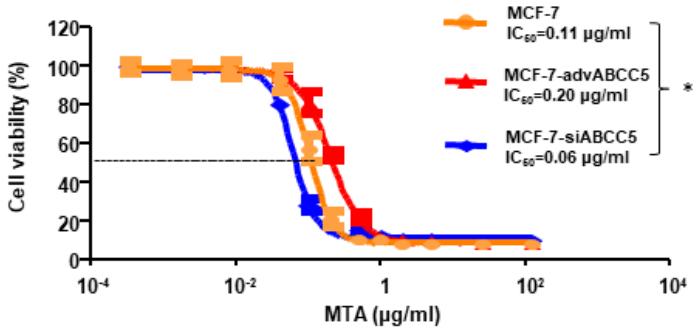
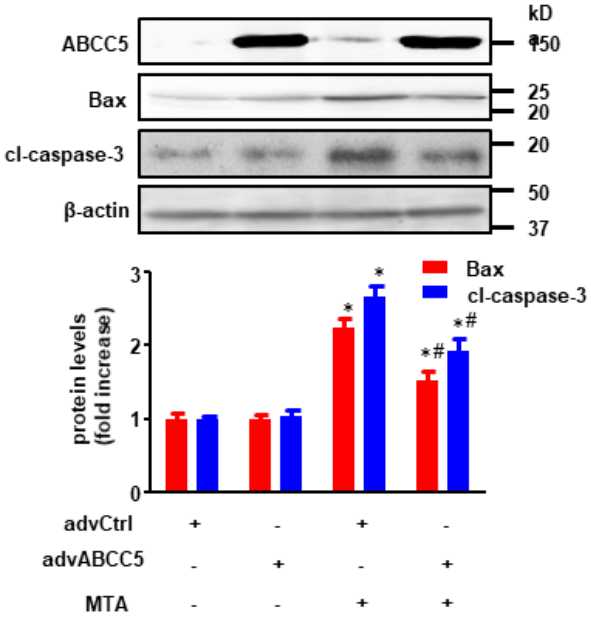



Fig. 5B




## Figure 5

ABCC5 affects the cell viability treated with MTA FIG.5A: Dose-response curves for MCF-7, MCF-7-advABCC5 and MCF-7-siABCC5 cell lines. At least five drug concentrations were used to determine IC50 values. Experiments at each concentration were performed in triplicate. The presented data represent the mean of three independent experiments. Data are shown as means ± SD (n=3, * $p<0.05$). FIG. 5B: The transductions of ABCC5 regulate the cell apoptosis and the expression of Bax and caspase after treatment with MTA. The experiments were repeated for three time in triplicate. An equal amount of cell lysate (20 μg of protein/lane) was analyzed using beta-actin as the loading control(*$p<0.05$, compared with non-MTA treatment group; #$p<0.05$, compared with AdvCtrl group).

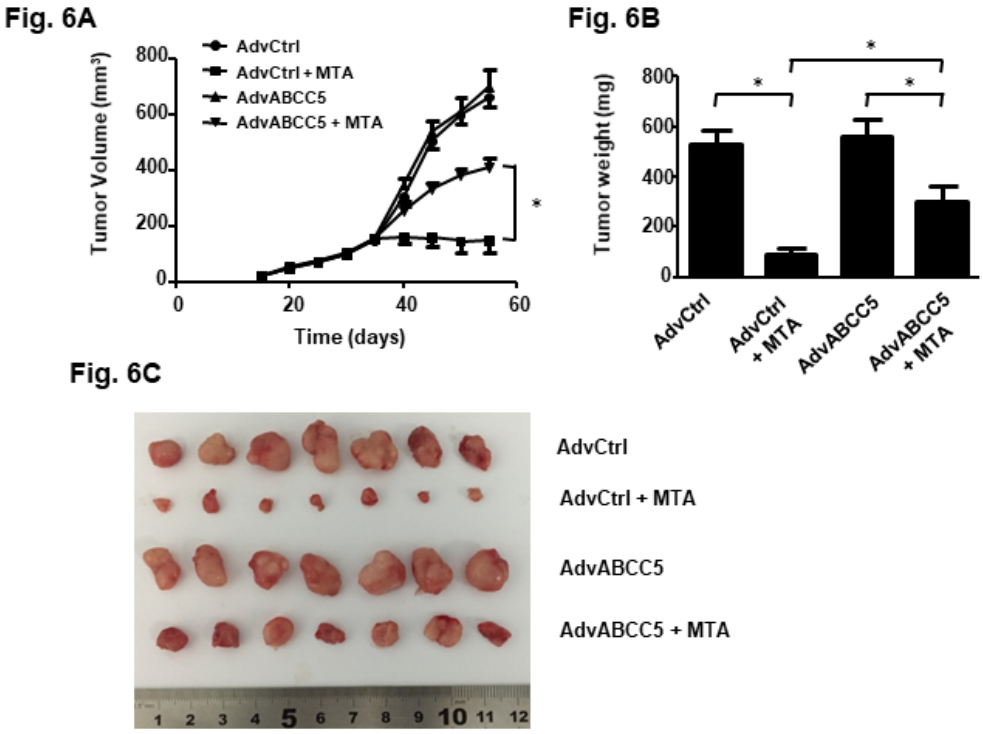



**Figure 6**

Overexpression of ABCC5 in mice bearing subcutaneous breast cancer xenografts reduces antitumor activity of MTA Tumors size were measured with a caliper (FIG.6A). The excised tumors were weighed at the end point (FIG.6B) and data are presented as mean ± SD (n=6), *p<0.05. Images of excised tumors of each group are shown (FIG.6C).

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable.docx
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- OriginaldataMicetumorxenograftsdata.xlsx
- OriginaldataInvitrodata.xlsx

- OriginaldataClinicaldata.xlsx