ABCC6P1 pseudogene induces chemotherapy resistance in breast cancer: a new insight into the role of pseudogene in drug resistance

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Research Article

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Abstract

Background: The elevated drug efflux by ABC transports has been considered the primary mechanism of drug resistance in cancer. Recently, non-coding RNAs, such as pseudogenes, have been proposed to be involved in transporter-mediated drug resistance in cancer. The human genome has 22 ABC transporter pseudogenes. Among these pseudogenes, *ABCC6P1*, has co-expression with the ancestral gene in various human tissues.

Methods and Results: In present study, we assessed the effect of *ABCC6P1* pseudogene on *ABCC6* expression and drug resistance. The *ABCC6P1* transfected into MCF-7 cells. In *ABCC6P1*-overexpressing cells, the *ABCC6* mRNA level increased up to 9.45-fold. The results of cell treatment with Doxorubicin, 5FU, Cisplatin, and Paclitaxel showed that the survival of *ABCC6P1*-overexpressing cells was higher than normal cells. Furthermore, uptake of Doxorubicin was lower after *ABCC6P1* overexpression.

Conclusions: In conclusion, our results show that overexpression of *ABCC6P1* pseudogene induces the drug resistance phenotype, possibly through activation of the ancestral gene.

Introduction

Breast cancer accounts for 30% of female cancers, with an increasing incidence rate of 0.5% per year [1]. Surgery and chemotherapy are the predominant therapy options for breast cancer treatment. However, drug-resistant breast tumor is the main challenge for the current treatment protocols [2, 3]. Drug resistance can be associated with various mechanisms, including increased efflux of drugs, DNA repair, and drug metabolism. Although, the elevated drug efflux by ABC transports has been considered the primary mechanism of drug resistance in cancer [3]. ABC transporters are a family of membrane pumps that hydrolyze ATP and use the energy to transport various substrates across the cell membrane [4]. The overexpression of ABC transporters has decreased the drug concentration inside the cell, producing multidrug resistance (MDR). It is well known that the members of the ABCB (*ABCB1*), ABCC (ABCC1-6, 10, 12), and ABCG (*ABCG2*) subfamilies are significant players in the development of cancer drug resistance [5–7].

The genetic mechanisms of ABC transporters upregulation include mutation, chromosomal rearrangement, and gene amplification [3, 8, 9]. Recently, non-coding RNAs (ncRNAs) have been proposed to be involved in transporter-mediated drug resistance in cancer [10, 11]. Accumulating evidence showed that dysregulation of long non-coding RNAs (lncRNAs) could increase the expression of ABC transporters in chemo-resistant cells. The up-regulation of several lncRNAs including XIST, UCA1, FOXD2-AS1, HOTAIR, LUCAT1, FTH1P3, CACS15, KCNQ1OT1, NR2F1-AS1, DANCR, OIP5-AS1, FOXC2-AS1, MALAT1, CASC9, and MRUL has been reported in cancer MDR [12–25]. The regulatory mechanisms of lncRNA are diverse; however, the most common one is that lncRNA competes with the ABC transporter RNA to bind to common miRNAs. Therefore, lncRNAs, as a miRNA sponge, increase the expression of the ABC transporter. This mechanism, known as competing endogenous RNA (ceRNA), has a fundamental role in

cancer development [26]. Nevertheless, none of the lncRNAs ever studied in ABC transporter activation are directly related to the ABC transporter genes.

Processed pseudogenes are a group of lncRNAs that evolved from their original genes [27]. Due to the high similarity to the parental gene, pseudogenes regulate parental gene expression via the ceRNA mechanism [28]. The human genome has 22 ABC transporter pseudogenes that nearly one-half of them are transcriptionally active [11]. One of these pseudogenes, *ABCC6P1*, has co-expression with the parental gene (*ABCC6*) in various human tissues [29].

ABCC6 is a glutathione conjugate pump that can induce resistance to chemotherapeutic drugs [30, 31]. *ABCC6* and *ABCC6P1* have the same regulatory elements, and knockdown of the *ABCC6P1* decreases the *ABCC6* mRNA expression [11]. Moreover, copy number variation and Q378X mutation co-exist in the *ABCC6* and *ABCC6P1* genes in patients with pseudoxanthoma elasticum [32, 33]. The expression of *ABCC6P1* is upregulated in papillary thyroid cancer, and the knockdown of *ABCC6P1* induces cell cycle arrest in cancer cells [34].

This study hypothesized that increased *ABCC6P1* RNA level could increase *ABCC6* expression levels and eventually lead to drug resistance. We observed that upregulation of the *ABCC6P1* pseudogene increases the *ABCC6* mRNA level. Moreover, we demonstrated that *ABCC6P1* overexpression increases the drug efflux and survival of the cell.

Materials And Methods

Cell culture and ABCC6P1 overexpression

The MCF7 cells were cultured in a DMEM medium with 10% FBS (Biosera, Shanghai, China). The pCDNA3.1 (+) plasmid, which includes the *ABCC6P1* pseudogene, was used for *ABCC6P1* overexpression. First, this vector was transformed into a competent DH5-α cell. The calcium phosphate method was used for transfection. Briefly, MCF-7 cells were plated onto 6-well plates 4–6 hours before transfection. 3–5 µg of plasmid were mixed in HBS (2X) and calcium chloride 2M solutions and filter sterilized. The mixture was sprinkled slowly on the cells and incubated at 37°C. The transfected cells were examined under fluorescence microscopy after 24 and 48 hrs. Cell cultures were treated with 400 µg/ml of Neomycin G418 (Sigma-Aldrich, St. Louis, USA) for three days and harvested for further evaluation.

Real-time PCR

The total RNA was extracted using Trizol reagent (Gibco, Life Technologies, Carlsbad, CA, U.S.A). The cDNA was synthesized using a cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, U.S.A). The PSMB2 gene was used as an internal control gene. Primer sequences are presented in Table 1. The qPCR condition was 95°C for 30 sec, and 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The change of expression unit was calculated using the formula 2-DDCt, and values greater than two-fold were considered significant.

Table 1
The sequence of primers.

Gene	Sequence		Tm	Amplicon (bp)
ABCC6	F	AGGCTTTCCTGCCCTTCTCCAT	62	121
	R	CCAGAGGAACTTGAGTCTACGAC	62	
ABCC6P1	F	ACAGAAAGGCATCCACAGCAT	58	151
	R	GCTGCGTGATCAATCAGGGT	59	
PSMB2	F	ACGGCAGCAGCTAACTTCACA	62	108
	R	TGGCCCTTCATGCTCATCA	59	

Drug sensitivity assay

For the MTS assay, the cells were plated onto 96-well plates at a density of 10000 cells/well and incubated overnight at 37◦C. Then, cells were treated with various concentrations of Doxorubicin (30, 50, 100, 150, 200 and 300 μM), 5FU (0.13, 1.3, 13, 130 and 1300 μM), Cisplatin (0.057, 0.57, 5.7, 57 and 570 μM), and Paclitaxel (2.5, 25, 250 and 2500 μM).

After 24 hours, 20μl of MTS reagent (Abcam Inc, Toronto, ON, Canada, C) was added, and plates were incubated for 4 hours at 37◦C. The drug sensitivity was calculated according to the percentage of cell proliferation.

Quantification of cellular drug uptake

The cells were treated with DOX and, intracellular DOX was measured by BD Accuri C6 Flow Cytometer (BD Biosciences, Dubai, UAE) to estimate the drug efflux. Briefly, 1×105 cells were seeded onto 6-well plates for 24 hours. Then cells were exposed to 1 to 15 μM of DOX at 37°C for 4 hours. Then, cells were washed with ice-cold PBS, trypsinized, and re-suspended in a fresh drug-free medium. Cells were centrifuged at 4°C for 4 minutes, and the pellet was re-suspended in cold PBS buffer. DOX was first excited in 490 nm. Then drug efflux was then measured at 585 nm using a flow cytometer instrument.

Statistical analysis

Relative levels of *ABCC6* and *ABCC6P1* expression were calculated using the calibrator-normalized method. Statistical analyses were conducted by JMP (JMP®, Version 11. SAS Institute Inc., Cary, NC). GraphPad Prism 6.0 (GraphPad Software, La Jolla, USA) was used to calculate the IC50 values from dose-response curves.

Results

ABCC6 **expression increases in** ABCC6P1 **- overexpressing cells**

The *ABCC6P1* vector was transfected into MCF-7 cells to increase the level of *ABCC6P1* mRNA. The expression level of *ABCC6P1* and *ABCC6* were measured after treatment. The results indicated that *ABCC6* expression significantly increased in *ABCC6P1*-transfected cells. The *ABCC6* RNA level increased 9.45-fold in *ABCC6P1*-overexpressing cells (Fig. 1).

ABCC6P1 -expressing cells present multi-drug resistance

The results of cell treatment with Doxorubicin, 5FU, Cisplatin, and Paclitaxel show that the survival of *ABCC6P1*-overexpressing cells was higher than normal cells. The IC50 level in normal MCF-7 cells is lower than the IC50 level of transfected cells (Fig. 2). The resistance folds for Doxorubicin (RF = 1.85), Paclitaxel (RF = 2.3), and 5-Fluorouracil (RF = 2.07) were increased after *ABCC6P1* overexpression.

Drug accumulation reduced in ABCC6P1-expressing cells

The results of drug efflux assay showed that the Doxorubicin uptake was reduced in *ABCC6P1* transfected cell. While the MCF-7 cells showed increased uptake of Doxorubicin after treatment in both concentrations (10 μM and 15μM). As shown in Fig. 3 the rate of drug efflux in *ABCC6P1*-expressing cells was significantly higher than control.

Discussion

Despite efforts to investigate the role of pseudogenes in cancer development, the role of these lncRNAs in drug resistance has not yet been determined. Pseudogene function as ceRNA is a promising hypothesis based on the fact that RNA transcribed from active pseudogene can up-regulate target gene expression through sequestration of miRNA activity. Transcribed pseudogenes are good candidates for the ceRNA mechanism because they are non-functional duplicates of ancestral genes and share miRNA-binding sites. In this study, we investigated the role of the *ABCC6P1* pseudogene on drug resistance through a possible effect on *ABCC6* expression.

Our overexpression experiments in MCF-7 cell lines demonstrated that *ABCC6P1* could increase the *ABCC6* mRNA level. Previous studies have shown that knockdown of the *ABCC6P1* decreases the ABCC6 mRNA level and induces cell cycle arrest [11, 34]. *ABCC6* and *ABCC6P1* have similar regulatory elements [11]. We have found 23 common miRNAs of *ABCC6P1* and *ABCC6* in miRNA databases. Therefore, it is possible that the *ABCC6P1* pseudogene traps these miRNAs and enhances *ABCC6* expression.

Our results showed that over-expression of *ABCC6P1* pseudogene is associated with increased cell resistance to doxorubicin, cisplatin, paclitaxel, and 5FU. Since *ABCC6P1* has no protein-coding function and considering the increased expression of *ABCC6* in these cells, it is possible that the induction of drug resistance in *ABCC6P1*-expressing cells is due to the effect of pseudogene on the expression of *ABCC6*. Furthermore, the drug Efflux test results showed that *ABCC6P1*-expressing cells excreted the drug about nine times more than control cells. This may be due to the activation of the pumping mechanism in these cells.

In conclusion, our results show that overexpression of *ABCC6P1* pseudogene induces the drug resistance phenotype, possibly through activation of the ancestral gene. Our results provide new insight into the role of pseudogenes in cancer drug resistance.

Declarations

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval and consent to participate

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Research Ethics Committee at Golestan University of Medical Science (Ethics number: IR.GOUMS.REC.1398.166).

Consent to participate

Not applicable.

Consent to publish

Not applicable.

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Figures

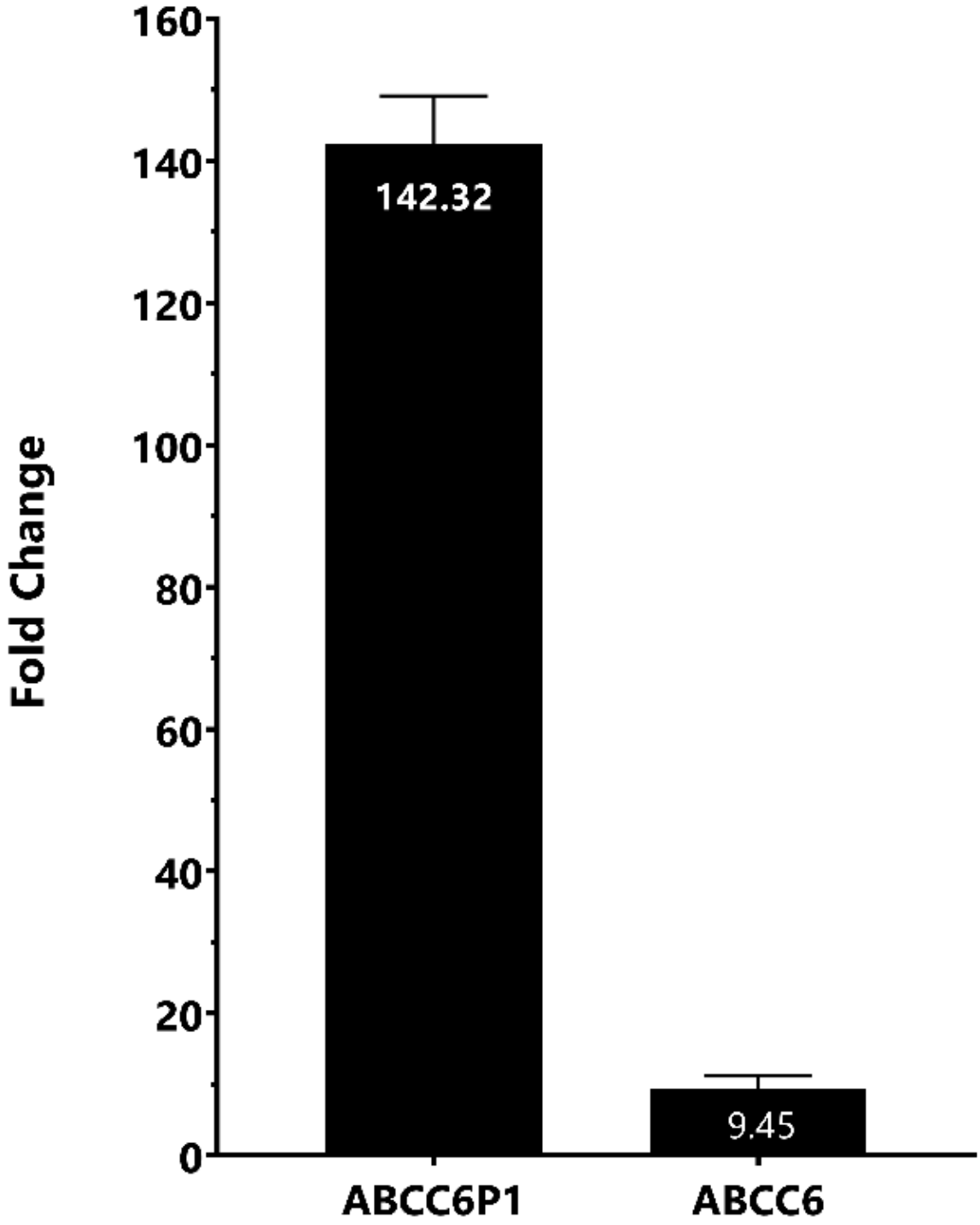
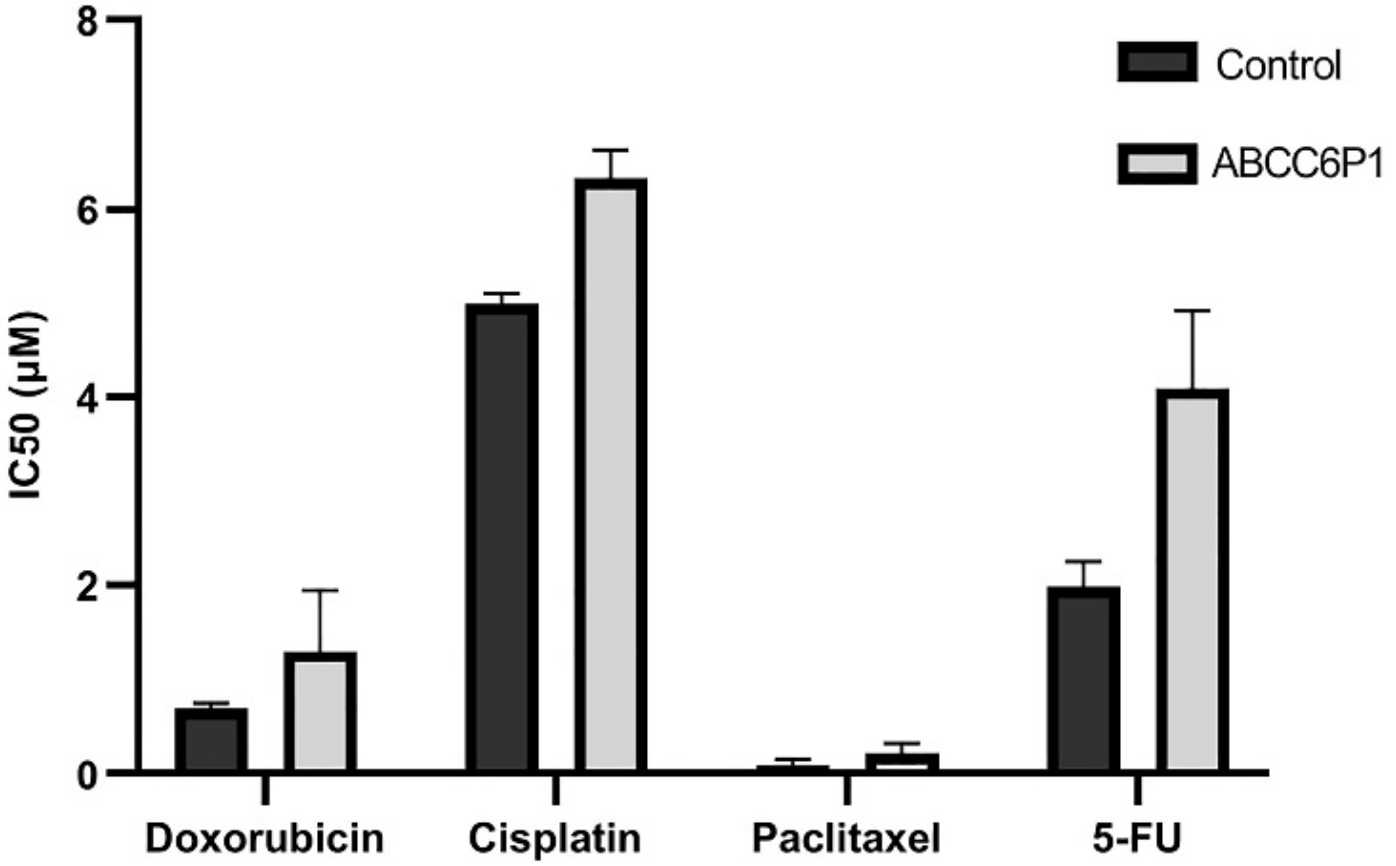


Figure 1

Expression of *ABCC6* and *ABCC6P1* after plasmid transfection. The expression level was measured by qPCR using the calibrator normalized method and the *PSMB2* as control. The *ABCC6* mRNA level significantly increased (~9 fold) after *ABCC6P1* pseudogene overexpression.



	Doxorubicin (μM)	*Cisplatin (μM)*	*Paclitaxel (μM)*	*5FU (μM)*
MCF-7	0.7±0.05	5.0±0.11	0.1±0.05	1.98±0.26
MCF-7 (ABCC6P1)	1.3±0.64	6.33±0.29	0.23±0.1	4.1±0.83
Resistance fold	1.85	1.2	2.3	2.07

Figure 2

Multidrug resistance of ABCC6P1-expressing cells. The IC50 values of MCF-7 and MCF7-*ABCC6P1* cells and to Doxorubicin, Cisplatin, Paclitaxel, and 5-Fluorouracil was measured using MTT assay. The IC50 of *ABCC6P1* cells was higher in all drugs. The resistance fold for Doxorubicin, Paclitaxel, and 5-Fluorouracil was significantly higher in MCF7-*ABCC6P1* cells (RF> 1.5)

Figure 3

Drug accumulation assay to assess intracellular drug concentration. (A) Drug efflux/retention was measured using doxorubicin and flow cytometry. (B) The concentration of doxorubicin in the *ABCC6P1*-expressing cells was significantly higher than in the control. All experiments were performed in triplicate. Extra., Extracellular doxorubicin concentration; Intra., intracellular doxorubicin concentration; SSC, side

scatter; FSC, forward scatter; FL3, forward light scatter; V1-L and V10-L, percent of extracellular doxorubicin accumulation; V1-R, and V10-R, percent of intracellular doxorubicin.