**Original Article**

**ClC5 is upregulated in paclitaxel resistant breast cancer cells and decreases paclitaxel sensitivity via promoting HMGB1 translocation**

Fengli Sun

2<sup>nd</sup> Department of Oncological Surgery, Cangzhou Central Hospital, Cangzhou 061000, Hebei, People’s Republic of China

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Abstract: The resistance of cancer cells to chemotherapy largely restricts the effective treatment of cancer. Chloride ions transport proteins or channel proteins are closely related with hallmarks of cancer, including unlimited proliferation, cell death resistance and metastasis. The present study was to investigate the effects of ClC-5, a member of voltage-gated chloride channels, on paclitaxel-induced chemoresistance and identify the underlying mechanisms of the action of ClC-5 in breast cancer cell line MCF-7 cells. We firstly established in vitro MCF-7 cells and paclitaxel-resistant MCF-7/PTX cells as cell model with \( IC_{50} \) of 4.78 and 11.92 μM, respectively. Interestingly, we found that ClC-5 expression was dramatically increased in MCF-7/PTX cells compared with in parental cells. Moreover, knockdown of ClC-5 with siRNA further enhanced paclitaxel-induced cell growth inhibition and apoptosis, accompanied by reduced Bcl-2 expression and increased Bax expression. However, overexpression of ClC-5 with adenovirus had opposite results. Additionally, we also found higher expression of high-mobility group box 1 (HMGB1) in MCF-7/PTX cells than in MCF-7 cells. With the increasing concentrations of paclitaxel, HMGB1 was gradually translocated from nucleus to cytoplasm in MCF-7 cells, and this translocation was significantly enhanced by ClC-5 overexpression. Furthermore, inference with endogenous HMGB1 almost abolished the inhibitory effects of ClC-5 overexpression on paclitaxel-induced apoptosis in MCF-7 cells. In conclusion, our data indicate that ClC-5 expression may be a potential mechanism underlying paclitaxel resistance in breast cancer, and inhibition of ClC-5 could improve the efficiency of paclitaxel in the induction of apoptosis.

**Keywords:** Paclitaxel, chemoresistance, ClC-5, HMGB1, apoptosis

**Introduction**

Breast cancer has been a primary physical and mental health in women, and it is also the leading cause of cancer death in women worldwide [1]. Hormone blocking therapy, chemotherapy and monoclonal antibodies are all used to treat breast cancer [2]. Although the survival rate of women diagnosed with breast cancer has been increased with the improvement of treatments, the mortality among breast cancer patients is still high [3, 4]. To note, chemotherapy is one of the most important methods for the treatment of breast cancer, however, the efficacy of therapeutic agents is largely limited by the acquired resistance in breast cancer cells, which represents as the major problem in breast cancer treatment [5]. Therefore, it is necessary to develop novel therapeutic strategies to increase sensitivity of breast cancer cells to chemotherapy.

Paclitaxel is a common antitumor chemotherapeutic agents widely used in the treatment of many cancer, including breast cancer [6]. It disrupts the homeostasis of microtubule assembly, inhibits mitotic progression, and induces cell apoptosis [2]. However, clinical study has shown that nearly most breast cancer patients treated with paclitaxel eventually develop drug resistance that significantly restrains its efficacy [7, 8]. Despite several mechanisms for paclitaxel resistance in breast cancer have been reported, including differential protein expression of ATP-binding cassette transporters, overexpression of multidrug resistant protein 1, mutation of tubulin/microtubule system and alteration in apoptotic pathways [9-12], the mecha-
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Mechanisms of resistance are extremely complicated and have not yet been fully understood.

High-mobility group box 1 (HMGB1) is a highly conserved chromatin-associated protein and has been associated with all hallmarks of cancer, including angiogenesis, unlimited proliferation, cell death resistance, and metastasis [13, 14]. In addition, HMGB1 also plays an important role in regulation of chemoresistance to chemotherapy. In human lung adenocarcinoma, HMGB1-mediated autophagy contributed to docetaxel resistance [15]. Recent study reported that knockdown of HMGB1 reduced drug resistance and inhibited tumor growth of lung cancer cells in vivo [16]. These studies indicate that HMGB1 may be a critical molecular target for decreasing chemotherapy resistance.

Chloride ions are the most common and abundant anions in organism regulating various physiological and pathological processes [17]. Voltage-gated chloride channels have been implicated as being important for a wide variety of cellular and intracellular functions, including regulation of cell volume, proliferation, migration and differentiation [18, 19]. CIC-5, a member of the voltage-gated chloride channel family, has been suggested to be primarily expressed in early and recycling endosomes in proximal tubule cells of the kidney [20, 21], and to be closely related to cell cycle [22]. Previous studies indicate that CIC-5 is found to be also expressed in human glioma and leukemic cells [23, 24]. Nevertheless, the expression pattern and the functional role of CIC-5 in breast cancer cells are poorly understood. In this study, we found that CIC-5 was expressed in breast cancer cell line MCF-7 cells and was increased in paclitaxel-resistant MCF-7/PTX cells, which promotes us to speculate that the upregulation of CIC-5 may play a potential role in the development of drug resistance in breast cancer cells. Our results suggest that inhibition of CIC-5 is a promising target of therapeutic approaches to increase the sensitivity of breast cancer cells to chemotherapy.

Materials and methods

Materials and reagents

PRMI-1640 medium and fetal bovine serum (FBS) were purchased from Hyclone Corporation (Logan, Utah, USA). Streptomycin and penicillin were obtained from Invitrogen (Carlsbad, CA, USA). Paclitaxel was from Nanjing Sike Pharmaceutical (Nanjing, China). Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

The human breast carcinoma cell line MCF-7 was obtained from the Cell Bank of Chinese Academy of Medical Science (Shanghai, China). The paclitaxel-resistant MCF-7/PTX cells were established from the parental MCF-7 cells according to an intermittent stepwise selection protocol as described previously [25]. All cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 μg/mL streptomycin and 100 U/mL penicillin in a humidified incubator with 5% CO₂ at 37°C. MCF-7/PTX cells were also constantly cultured in 0.4 μM paclitaxel until at least two weeks before experiment.

Cell viability assay

Cell viability was determined with the Cell counting Kit-8 (CCK-8) assay kit (Yiyuan Biotechnology, Guangzhou, China) as previously described [2]. MCF-7 or MCF-7/PTX cells were seeded at a density of 4×10³ cells per well in 100 μL RPMI 1640 medium overnight and treated with paclitaxel for different concentrations (1, 2, 4, 8 or 16 μM) for 48 h. In another treatment, MCF-7 cells were transfected with CIC-5 siRNA or adenovirus for 48 h before paclitaxel for another 48 h. 20 μL CCK-8 was added into each well and incubated for 2 h at 37°C. The absorbance was read at 450 nm with a microplate reader (BioTek, Santa Barbara, CA, USA). IC₅₀ values of DDP were calculated using the GraphPad 5.0 software.

Extraction of cytoplasmic and nuclear proteins

Cytoplasmic and nuclear proteins were extracted with NE-PER extraction reagents according to manufacturer’s instructions (Pierce Biotechnology, Rockford, IL, USA). Nuclear translocation of HMGB1 was determined by western blotting with HMGB1 antibody (1:500, Abcam, San Diego, USA).

Western blotting

Western blotting analysis was performed as previously described [16]. Briefly, cell proteins...
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RNA isolation and real-time quantitative polymerase chain reaction analysis (qRT-PCR)

Total mRNA was isolated from MCF-7 or MCF-7/PTX cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. First-stand cDNA was synthesized with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). qRT-PCR was performed on a LightCycler 2.0 Real-Time PCR System (Roche Applied Science, Indianapolis, IN, USA) using PrimeScript RT Master Mix Perfect Real Time kit (TaKaRa DRR036A, Shiga, Japan) for 32 cycles (95°C for 10 seconds, 56°C for 1 min) after an initial 3 min incubation at 95°C. The mRNA expression in each sample was normalized to 18s RNA expression using the 2^{-ΔΔCT} method. Specific primers for ClC-5 were synthesized by Invitrogen: 5’-GTGAGGGAGAAATCCAGA-3’ and 5’-TTGATGCAGCTCA-3’.

Knockdown of ClC-5 or HMGB1 using siRNA

Endogenous ClC-5 or HMGB1 mRNA in MCF-7 cells was knocked down using siRNA. The stealth human ClC-5 (5’-GCACTTCCATCATCATT-3’) or HMGB1 (5’-CCCAGATGCTTCAGTCAACTT-3’), siRNA duplex oligoribonucleotides was designed and synthesized by Invitrogen. Negative siRNA, ClC-5 siRNA or HMGB1 siRNA was transfected into MCF-7 cells with Hiperfect Transfection Reagent (Qiagen, Carlsbad, CA, USA) according to the manufacturer’s instructions for 48 h.

Adenovirus infection

CIC-5 adenovirus was purchased from Hanbio Medical Biotechnology (Shanghai, China). Human CIC-5 cDNA was amplified and cloned into pCMV-Tag2 (Invitrogen) and then transfected into HEK293A cells with Hiperfect Transfection Reagent to generate recombinant adenovirus. The titers of virus were assayed using by p24 ELISA kit (Cell Biolabs, San Diego, CA, USA). An adenovirus bearing LacZ was obtained from Clontech (Mountain View, CA, USA) and used as a negative control. MCF-7 cells were transfected with LacZ or ClC-5 adenovirus for 48 h.

Analysis of apoptosis by flow cytometry

MCF-7 cell apoptosis was determined by Epics Profile II flow cytometer (Beckman Coulter, Fullerton, CA, USA) using the Annexin V-propidium iodide (PI) apoptosis detection kit (Bio-Vision, Milpitas, CA, USA) according to the manufacturer’s instructions. Briefly, the cells were digested with trypsin and suspended at a density of 5×10^6 cells/100 μL in binding buffer containing 5 μL of Annexin V and 5 μL of PI. After incubation at room temperature for 20 min in dark, cells were subjected to flow cytometry for analysis of apoptosis with MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA).

Statistical analysis

Values were presented as mean ± SEM. The significance between groups was determined by one-way analysis of variance (ANOVA) or the unpaired two-tailed Student’s t test using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). P<0.05 indicated significant difference.

Results

ClC-5 is upregulated in paclitaxel resistance of MCF-7 cells

To determine whether ClC-5 is implicated in paclitaxel resistance of breast cancer cells, we used MCF-7 cell line and paclitaxel-resistant cell line MCF-7/PTX as cell models, and then the cells were treated with increasing concentrations of paclitaxel for 48 h. Cell viability assay showed that the IC_{50} value of paclitaxel in MCF-7 cells and MCF-7/PTX was 4.78 μM and 11.92 μM, respectively (Figure 1A). We next examined the expression of ClC-5 by qRT-PCR.
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in MCF-7 cells and its corresponding paclitaxel- resistant cells. The results revealed that the mRNA expression of CIC-5 was increased in paclitaxel-resistant MCF-7/PXT cells compared with the parental cells (Figure 1B). Moreover, in accord with the results from qRT-PCR, higher protein expression of CIC-5 was also found in MCF-7/PXT cells than in parent MCF-7 cells (Figure 1C). These results indicate the changes of CIC-5 expression may be associated the paclitaxel resistance in breast cancer cells.

Interference with endogenous CIC-5 sensitizes the effects of paclitaxel on apoptosis in MCF-7 cells

To examine whether CIC-5 is involved in paclitaxel resistance in breast cancer cells, we knockdown CIC-5 expression by CIC-5 siRNA in MCF-7 cells and determined its sensitivity to paclitaxel. Firstly, the efficiencies of siRNA and CIC-5 expression were determined. Western blotting showed that CIC-5 siRNA at 20 nM significantly decreased endogenous CIC-5 expression more than 85% (Figure 2A). Moreover, MCF-7 cells were transfected with CIC-5 siRNA and negative siRNA, and then treated with paclitaxel for 48 h. As shown in Figure 2B, cell viability was significantly suppressed in MCF-7 cells transfected with CIC-5 siRNA in the presence of paclitaxel, compared with that transfected with negative siRNA (Figure 2B). To answer whether the inhibition of cell viability was induced by apoptosis, quantitative analysis of apoptosis by flow cytometry was performed. The Annexin V/PI double staining showed that apoptotic cells were dramatically increased in MCF-7 cells transfected with CIC-5 siRNA from 19.71% to 30.66% in the presence of 5 μM paclitaxel (Figure 2C and 2D). Moreover, we measured the expression of Bcl-2 and Bax, two Bcl-2 family members known to be important in the regulation of apoptosis. We found that the protein expression of Bcl-2 was decreased, while the expression of Bax was increased after paclitaxel treatment. As expected, inhibition of CIC-5 further augmented the effect of paclitaxel on Bcl-2 and Bax expression (Figure 2E).

Overexpression of CIC-5 inhibits paclitaxel-induced apoptosis

To further confirm the role of CIC-5 in regulating paclitaxel resistance in MCF-7 cells, we determined the effects of CIC-5 upregulation on paclitaxel-induced apoptosis. Efficiency of adenoviral infection was detected by western blot. Infection of CIC-5 adenovirus significantly increased CIC-5 expression and the peak increase was observed at 50 MOI, while Lacz produced no effect (Figure 3A). Moreover, paclitaxel treatment resulted in a significant decrease of MCF-7 cell viability, whereas overexpression of CIC-5 was associated with increased cell viability (Figure 3B). Similarly, the paclitaxel-induced apoptosis in MCF-7 cells in-
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Infected with ClC-5 adenovirus was remarkably inhibited compared with paclitaxel treatment alone, with a decrease of apoptosis from 19.76% to 9.66% (Figure 3C and 3D). In addition, low levels of Bcl-2 and high levels of Bax expression were observed after paclitaxel treatment for 48 h. However, these alterations were dramatically inhibited by ClC-5 upregulation. These data suggest that overexpression of ClC-5 decreases paclitaxel sensitivity in breast cancer cells.

Paclitaxel increases HMGB1 levels in cytoplasm of MCF-7 cells

Given that HMGB1 has been suggested to a critical factor to promote chemoresistance in chemotherapy, we next investigated whether...
HMGB1 is also involved in paclitaxel resistance of MCF-7 cells. Western blotting showed that HMGB1 expression in MCF-7/PTX cells was remarkably higher than that in MCF-7 cells (Figure 4A), indicating that high levels of HMGB1 may contribute to paclitaxel resistance. Furthermore, MCF-7 cells and MCF/PTX cells were treated with increasing concentrations of paclitaxel for 48 h and HMGB1 location was detected by western blotting. The results showed that the cytoplasmic HMGB1 amounts in MCF-7 cells were increased by paclitaxel in a concentration-dependent manner, while the nuclear HMGB1 was gradually decreased. However, both cytoplasmic and nuclear HMGB1 amounts in MCF-7/PTX were not changed after paclita-
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The results suggest that paclitaxel promotes HMGB1 translocation from nucleus to cytoplasm in MCF-7 cells.

**CIC-5 decreases sensitivity of MCF-7 cells to paclitaxel via promoting HMGB1 translocation from nucleus to cytoplasm**

The above results indicate that HMGB1 translocation to cytoplasm in MCF-7 may contribute to paclitaxel resistance, so we next tested the effects of CIC-5 on HMGB1 translocation. We found that the increased HMGB1 amounts in the cytoplasm after paclitaxel treatment were enhanced by CIC-5 overexpression, while the nuclear HMGB1 amounts were further decreased (Figure 5A and 5B). To further investigate whether the inhibitory effect of CIC-5 on paclitaxel-induced apoptosis in MCF-7 cells is resulted from its capacity to enhance HMGB1 translocation, we co-transfected with HMGB1 siRNA and CIC-5 adenovirus in the presence of paclitaxel, and then examined the apoptotic cells. Western blot analysis of interference efficiency showed that the HMGB1 expression was decreased to nearly 90% at 10 nM as compared to control group (Figure 5C). Following inhibition of HMGB1, the inhibitory effect of CIC-5 on apoptosis under paclitaxel treatment was abolished (Figure 5D), indicating that HMGB1 may be a critical molecular target for CIC-5 in attenuation of paclitaxel sensitivity in MCF-7 cells.

**Discussion**

Paclitaxel is one of the most effective chemotherapy that used to treat breast cancer [6]. However, drug resistance is increasing in the development of breast cancer after paclitaxel treatment. The mechanisms of drug resistance are still not fully understood although numerous studies have been reported. Identification of an effective novel strategy to overcome drug resistance has long been a goal of oncologists. In the present study, we demonstrated for the first time that CIC-5 expression was increased in paclitaxel-resistant MCF-7/PTX cells as compared with the parental cells. Moreover, inhibition of CIC-5 promoted paclitaxel-induced apoptosis in MCF-7 cells, whereas overexpression of CIC-5 was associated with reduced cell apoptosis induced by paclitaxel. The results suggest that upregulation of CIC-5 may play a survival role to facilitate paclitaxel resistance in breast cancer cells.

Several studies have reported on the expression and function of voltage-gated chloride channels in different cancer cells [23, 24, 26]. For example, CIC-3 has been extensively investigated, which is found to be upregulated in breast [27], glioma [28], and cervical tumors [29], and plays an important role in regulating the migration and invasion of glioma [30] and nasopharyngeal cancer cells [31]. Different from the other members of voltage-gated chloride channels, the expression and function of CIC-5 in cancer cells remain obscure. Previous
studies reported that CIC-5 expression was detected in human glioma cells [22, 24]. Moreover, CIC-5 was also found to be expressed in different human leukemic cell lines [23]. In addition to these studies, there is no more information related to CIC-5 in tumor. Here, we revealed a novel role of CIC-5 in breast cancer cells that mediated their chemoresistance to paclitaxel.

Accumulating evidences suggest that HMGB1 expression is implicated in the regulation of genomic stability, autophagy and tumor growth [13, 14, 32]. Upregulation of HMGB1 expression has been observed in many tumors, including stomach, prostate, lung, liver and breast cancer [33]. In this study, we found that the expression of HMGB1 was obviously increased in paclitaxel-resistant breast cancer cell line
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MCF-7/PTX cells compared with the parental cells, which is in accordance with the previous study that HMGB1 expression was significantly higher in cisplatin-resistant lung cancer cell line A549/DDP than in A549 cells [16]. These results indicate that upregulation of HMGB1 expression may be associated paclitaxel resistance in MCF-7 cells. Normally, HMGB1 is expressed both in cytoplasm and nucleus of cell with the potential of extracellular release upon certain stimulation [33, 34]. Our results showed that paclitaxel promoted HMGB1 translocation to cytoplasm, with paclitaxel increasing HMGB1 amounts in cytoplasm while decreasing HMGB1 amounts in nucleus. This enhanced translocation may contribute to chemotherapy resistance.

Moreover, HMGB1 has been proposed to exhibit carcinogenic effects that associated with increased chemoresistance, proliferation and invasiveness [16, 35, 36]. These studies, together with our findings led us to explore the possibility that whether CIC-5 reduces sensitivity of MCF-7 cells to paclitaxel via regulation of HMGB1. Our results revealed that overexpression of CIC-5 dramatically augmented paclitaxel-induced HMGB1 translocation to cytoplasm, further indicating the translocation of HMGB1 from nucleus to cytoplasm is likely a survival mechanism in response to paclitaxel.

In summary, these findings are first to our knowledge to clarify the critical role of CIC-5 in paclitaxel-induced chemoresistance and unveil the underlying mechanisms that CIC-5 regulates HMGB1 translocation, suggesting CIC-5 could be a potential target for overcoming paclitaxel resistance in breast cancer.

Disclosure of conflict of interest

None.

Address correspondence to: Fengli Sun, 2nd Department of Oncological Surgery, Cangzhou Central Hospital, No. 16 Xinhua West Road, Yunhe District, Cangzhou 061000, Hebei, People’s Republic of China. Tel: +86 18931769256; E-mail: sunflczch@126.com

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