RESEARCH ARTICLE

Unexpected Lower Expression of Oncoprotein *Gankyrin* in Drug Resistant *ABCG2* Overexpressing Breast Cancer Cell Lines

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Abstract

Background: Development of a multidrug resistance (MDR) phenotype to chemotherapy remains a major barrier in the treatment of cancer. *Gankyrin* (*p28*, *p28GANK* or *PSMD10*) is an oncoprotein overexpressed in different carcinoma cell lines. The aim of this study was to compare Gankyrin expression level in MDR cells (MCF-7/ADR and MCF-7/MX) and non-MDR counterparts (MCF-7). **Methods:** Gankyrin, MDR1 (also known as *ABCB1*; the ATP-binding cassette sub-family B member 1) and *ABCG2* (also known as *BCRP*; the human breast cancer resistance protein) mRNA levels were analyzed by real-time RT-PCR. Western blot analysis was used to detect the protein expression levels of Gankyrin. **Results:** The PCR results showed that the expression of Gankyrin was significantly lower in the *ABCG2* overexpressing cell line MCF-7/MX than in non-resistanct MCF-7 cells. In contrast, there were no significant differences in mRNA expression of Gankyrin in the MDR1 overexpressing cell line MCF-7/ADR in comparison with MCF-7 cells. Similarly, Western blot analysis confirmed lower expression of Gankyrin protein in the MCF-7/MX cell line (26% compared to controls) but not in MCF-7/ADR cells. **Conclusion:** These findings showed that there may be a relation between down-regulation of Gankyrin and overexpression of ABCG2 but without any clear relationship with MDR1 expression in breast cancer cell lines.

Keywords: Multidrug resistance- Gankyrin- PSMD10 protein- breast cancer- MCF-7 Cells

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Introduction

Breast cancer is the most common cause of cancer in women and the second most common cause of cancer death in them (Filipova et al., 2014). Primary breast tumors without metastatic lesions are highly curable with regional treatment. However, most women with primary breast cancer experience subclinical metastases that eventually develop to distant metastases that complicate the curability of the cancer (Morrow and Cowan, 1993; Wong and Goodin, 2009). It seems that understanding of cellular and molecular mechanisms is necessary for chemotherapy selection in breast cancer patient.

Today, there are many reasons that lead to failure of cancer chemotherapy (Krol et al., 2010). One of them is the development of multidrug resistance (MDR) phenotype to chemotherapy which remains as a major barrier in the treatment of cancer. MDR exists against every effective anticancer drugs and can develop by numerous mechanisms, such as decreased drug uptake, increased drug efflux, activation of detoxifying systems, activation of DNA repair mechanisms and evasion of drug-induced apoptosis (Gillet and Gottesman, 2010). During the past four decades, a major goal for cancer biologists is to understanding the mechanisms of MDR that cause simultaneous resistance to different drugs with different targets and chemical structures.

The ATP-binding cassette (ABC) transporter superfamily has an important role in absorption, distribution, and elimination of their substrates (like drugs) that could mediate multidrug resistance (MDR) in cancer cells. The ATP-binding cassette sub-family B member 1 (*ABCB1*, also known as *MDR1* or *P-gp*) and the ATP-binding cassette sub-family G member 2 (*ABCG2*, also known as human breast cancer resistance protein) are the most known members of ABC family which underlay the MDR in different cancer cell types (Bournissen et al., 2009; Bunting, 2002; Liu et al., 2013; Ross et al., 2000; Zhou et al., 2001).

Gankyrin (*p28*, *p28GANK* or *PSMD10*) is an oncoprotein that overexpressed in different carcinoma cell lines (Liu et al., 2013; Zamani et al., 2017). *Gankyrin*

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protein consists of seven ankyrin repeats (Higashitsuji et al., 2005). Typically, function of these ankyrin repeats is mediating specific protein–protein interactions. *Gankyrin* interacts with multiple proteins, for example, it binds to the S6b subunit of the 26S proteasome and enhances the degradation of the tumor suppressor p53 (Nakamura et al., 2007). *Gankyrin*, also binds to retinoblastoma protein (Rb) and induced the phosphorylation and degradation of Rb, suggesting that *Gankyrin* promotes tumorigenicity and cancer cell proliferation (Higashitsuji et al., 2000). In addition, *Gankyrin* acts as an accelerator for cell cycle progression by binding to cyclin-dependent kinase 4 (CDK4) and mouse double minute 2 homolog (MDM2) that counteract the inhibitory function of p16INK4a and p53 (Higashitsuji et al., 2005; Li and Tsai, 2002). This suggests that *Gankyrin* expression is correlated with a malignant phenotype in cancer cells.

Most prominent regulators that disrupted in cancer cells are two tumor suppressors, the retinoblastoma protein (RB) and the p53 transcription factor (Sherr and McCormick, 2002). Resistance may develop with loss of genes required for the cell death such as p53 or overexpression of genes that block the cell death (Krishna and Mayer, 2000). On the other hand, the regulation of expression of the multidrug resistance proteins, such as MRP and p53, occurred in MDR cancer cells (Sullivan et al., 2000). Also, *Gankyrin* confers MDR by modulating the expression of MDR1, Bcl-2, and Bax in the cancer cells (Wang et al., 2010). Presumably, there would be an interaction between *Gankyrin* and MDR associated proteins.

In this study, we aimed to more clarify the mechanism of MDR. So, mRNA and protein expression of *Gankyrin* was compared in MDR cells (MCF-7/MX and MCF-7/ADR) compared to non-MDR counterparts (MCF-7). Understanding the mechanism of MDR may provide novel targets for treating MDR tumors and promote screening of suitable patients.

Materials and Methods

Cell lines and cell culture

Three breast cancer cell lines (parental non-resistance cell line MCF-7, mitoxantrone selected cell line MCF-7/MX and doxorubicin (adriamycin) selected cell line MCF-7/ADR) were used for this study. ABCG2 overexpressing cell line MCF-7/MX and MDR1 overexpressing cell line MCF-7/ADR were kindly provided by Professor Herman Lage (Molecular pathology department, Charite Campus Mitte, Berlin, Germany). Breast cancer cell lines were cultured in RPMI 1640 (Gibco, Austria) supplemented with 2 g/L sodium bicarbonate, 2 mM L-glutamine, heat inactivated FBS (Fetal Bovine Serum, Gibco, Austria) 10% (v/v), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37ºC in a humidified atmosphere containing CO_2 5%. In order to maintain the multidrug-resistance phenotype, mitoxantrone (MX) (100 nM) and doxorubicin (DOX) (1 µM) were added to the culture medium of MCF-7/MX and MCF-7/ADR cells, respectively. These cell lines were cultured on MX or DOX free medium for at least 7 days prior to each experiments (Kalalinia et al., 2014; Tsou et al., 2015).

RNA extraction and real-time reverse transcriptase-polymerase chain reaction (Real-Time RT-PCR)

Cells were seeded at a density of 5.0×10^5 cells/well in a six-well plate and were incubated at 37ºC in a humidified atmosphere containing CO_2 5%. When the cells were approximately 80% confluent, total RNA was extracted from human breast cancer cells using a TriPure Isolation Reagent Kit according to the protocol provided by the manufacturer (Roche, Germany). In order to prevention of DNA contamination, RNA was treated with ribonuclease-free DNase that provided by RNA Isolation Kit. Immediately following extraction, the total RNA concentration of each sample was determined using a NanoDrop 1,000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The purity of the RNA samples were determined by the ratio of absorbance at 260 and 280 nm and the ratio of absorbance at 260 and 230 nm with acceptable ratios in the range of 1.8-2.2. All primer pairs bonded to separate exons that resulted in avoiding false positive results arising from amplification of contaminating genomic DNA. The primers were synthesized by Metabion company (Germany), purified by HPLC, and quality controlled via mass spectrometry. The sequences of primers were as the following: *Gankyrin* (NM_002814.3), forward, 5′-AGCAGCCAAGGGTAACTTGA-3′ and reverse, 5′-TACTTGCTCCTTGGGACACC-3′ (Ortiz et al., 2008); MDR1 (NM_000927.4), forward, 5′-CCCATCATTGCAATAGCAGG-3′ and reverse, 5′-TGTTCAAACTTCTGCTCCTGA-3′ (Zhao et al., 2011), ABCG2 (NM_001257386.1), forward, 5′- TATCAATGGGATCATGAAACCTGG-3′ and reverse, 5′-GCGGTGCTCCATTTATCAGAAC-3′ and β-actin (NM_001101.3), forward, 5′-TACTGAAGTGTGACGTGGACATC-3′ and reverse, 5′-CAGGAGGAGCAATGATCTTGATCT-3′ (Kalalinia et al., 2012; Kalalinia et al., 2014). The primer pairs resulted in amplified products of 155 bp for *Gankyrin*, 140 bp for ABCG2, 158 bp for MDR1 and 156 bp for β-actin. One step real-time RT-PCR was performed according to the protocol of One Step SYBR® PrimeScriptTM real-time RT-PCR Kit II (Takara, Japan) and Stratagene 3,000 p sequence detector system. RT-PCR reactions (10 µl) were performed on 300 ng mRNA by starting with cDNA synthesis step at 42 °C for 5 min, followed by amplification step which was performed with a denaturation step at 95 °C for 10 min and 40 amplification cycles at 95 °C for 5 s, 60 °C for 30 s, 72 °C for 30 s. Amplification of a single product for each primer set was confirmed by the dissociation curve analysis (melting curve analysis). Standard curves were prepared for target (Gankyrin, ABCG2 and MDR1) and reference (β-actin) genes. Relative expression levels for gene of interest were normalized to that of the β-actin by the MxPro-Mx3,000P software. In all experiments, No-template controls (NTC) were considered to test genomic DNA contamination of the enzyme/primer mixes. The results were expressed as the target/reference ratio of the drug resistance cell's samples divided by the target/reference ratio of the

parental cell's samples (Bustin et al., 2009; Kalalinia et al., 2012; Kalalinia et al., 2014).

Western blot analysis

Cells were seeded at a density of 5.0 × 10^5cells/well in a six-well plate till the cells were approximately 80% confluent. For preparing total cell lysates, cells were lysed with Nonidet-P40 (NP-40) lysis buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 10% glycerol (v/v), 1% nonidet P-40, 2 mM EDTA (v/v) and cocktail proteinase inhibitor), homogenized by sonication on ice and centrifuged at 15,000 g for 20 min to remove cell debris. The total protein concentration in the cell lysate was measured by Bradford assay. Cell lysate (60 µg) were loaded in 15% SDS-PAGE, then electrophoresed at 110 V using precooled electrophoresis running buffer containing 25 mM Tris–HCl, 190 mM glycine, 0.1% SDS (w/v) that has pH 8.0 at room temperature for 60 min or until the front dye reached the bottom of the gel. After protein transferring to PVDF membrane, the membranes blocked with 2% (w/v) bovine serum albumin (BSA) (Sani et al., 2015). To detect specified proteins, PVDF membranes were probed with *Gankyrin* rabbit polyclonal antibody (Santa Cruz, US) at a dilution rate of 1:250 or β-actin mouse monoclonal antibody (Sigma Co. Germany) at a dilution rate of 1:5,000 in 2% (w/v) BSA in PBS for overnight at 4 °C. The PVDF membranes were washed with PBS for 15 min and incubated at room temperature for 2 h with goat anti-rabbit IgG-HRP (Abcam, US) at a dilution rate of 1:2,000 or anti-mouse IgG (Sigma Co. Germany) at a dilution rate of 1:7,000, respectively. Detection was performed using the enhanced chemiluminescence reagent using G-Box gel documentation system (Syngene, UK). Densitometry of western blot bands was measured by ImageJ 1.46r software (NIH, USA).

Statistical analysis

Three independent experiments were performed in each steps. Statistical significance (P-Value) was calculated by SPSS version 16.0 using one-way Analysis of variance (ANOVA) and Tukey's post-hoc. All the data are expressed as mean ± SEM, and $P<0.05$ was assumed to be statistically significant.

Results

Comparison of ABCG2 expression in breast cancer cell lines

In order to confirm the overexpression of ABCG2 in mitoxantrone resistance cell line MCF-7/MX compared with parental non-resistance cell line MCF-7, the of ABCG2 mRNA expression was evaluated by Real time RT-PCR. The results showed that ABCG2 mRNA level in resistant cell line was about 838.75 times more than its expression level in MCF-7 (Figure 1).

Comparison of MDR1 expression in breast cancer cell lines

To confirm the overexpression of MDR1 in doxorubicin-selected human breast cancer cell line MCF-7/ADR compared with parental non-resistance cell line MCF-7, the of MDR1 mRNA expression was evaluated by Real time RT-PCR. The results indicated that MDR1 mRNA level was remarkably higher (about 95.86

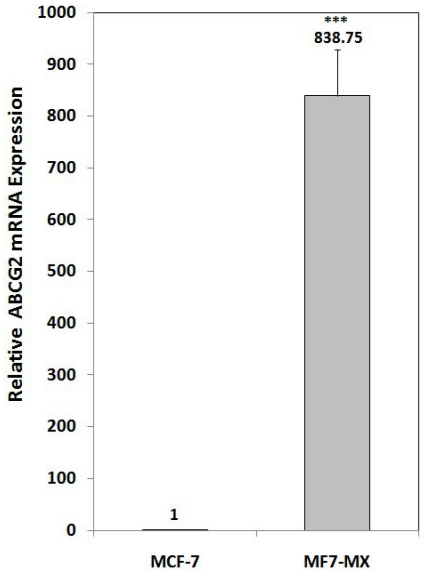

Figure 1. The Expression of *ABCG2* at mRNA Level in MCF-7 Derived Breast Cancer Cell Lines. *ABCG2* mRNA level is compared between drug resistance cell line MCF-7/MX and its parental non-resistance MCF-7 cells. Real-time RT-PCR analysis was performed on total RNA extracted from cells. Values were normalized to the *β-actin* content of samples and expressed as mean ±SEM (n = 3). Statistical significance was calculated by one-way Analysis of variance (ANOVA) and Tukey's post-hoc; ***$p < 0.001$ compared with the non-resistance parental cell line.

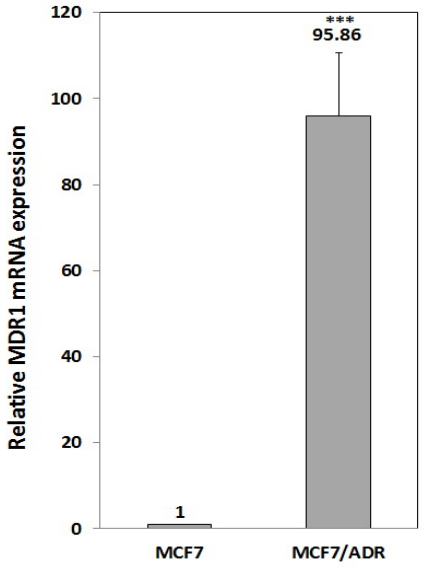

Figure 2. The Expression of *MDR1* at mRNA Level in Breast Cancer Cell Lines. *MDR1* mRNA level is compared between drug resistance cell line MCF-7/ADR with MCF-7 cells. Real-time RT-PCR analysis was performed on total RNA extracted from cells. Values were normalized to the *β-actin* content of samples and expressed as mean ±SEM (n = 3). Statistical significance was calculated by one-way Analysis of variance (ANOVA) and Tukey's post-hoc; ***$p < 0.001$ compared with the non-resistance parental cell line.

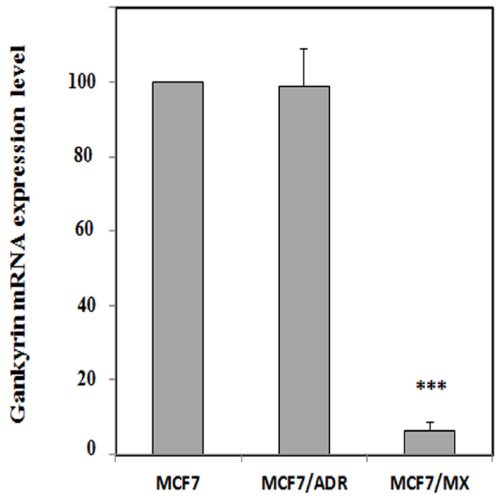

Figure 3. The Expression of *Gankyrin* at mRNA Level in MCF Derived Breast Cancer Cell Lines. Total RNA of MCF-7, MCF-7/ADR and MCF-7/MX cells were extracted and subjected to Real-time RT-PCR analysis. Values were normalized to the *β-actin* content of samples and representative of three independent experiments is presented as mean ± SEM. Statistical significance was calculated by one-way Analysis of variance (ANOVA) and Tukey's post-hoc; ***$p < 0.001$ compared with the control.

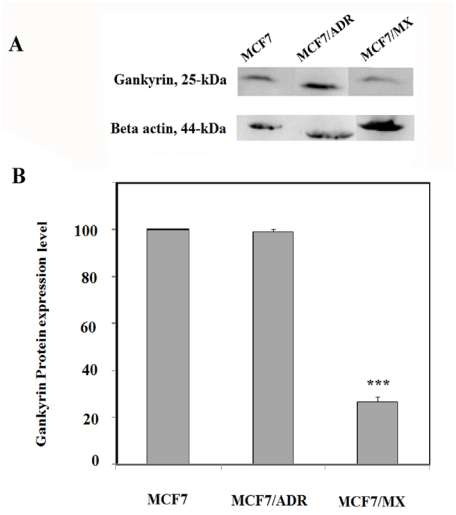

Figure 4. The Expression of *Gankyrin* at Protein Level in MCF-7 Derived Breast Cancer Cell Lines. Total protein of MCF-7, MCF-7/ADR and MCF-7/MX cells were extracted and subjected to Western blotting analysis (A, B). Band density was analyzed using ImageJ software, version 1.46r, and expression levels were normalized to *β-actin* protein level. Representative of three independent experiments is presented as mean ± SEM. Statistical significance was calculated by one-way Analysis of variance (ANOVA) and Tukey's post-hoc; ***$p < 0.001$ compared with the control.

times) in MCF-7/ADR cell line than its expression level in MCF-7 (Figure 2).

Expression of Gankyrin in breast cancer cell lines at mRNA level

Expression of *Gankyrin* in human breast carcinoma cell lines MCF-7, MX selected MDR cell line MCF-7/MX (ABCG2-overexpressing cell line) and ADR selected MDR cell line MCF-7/ADR (MDR1-overexpressing cell line) were detected by Real time RT-PCR. Our result showed lower expression of *Gankyrin* in MCF-7/MX cells (6% to the control level) in compare with MCF-7 cells (Figure 3). On the other hand, difference between mRNA expressions of *Gankyrin* in MCF-7/ADR cells in compare with MCF-7 cells was not significant (Figure 3).

Expression of Gankyrin in breast cancer cell lines at protein level

The gene expression of the *Gankyrin* at protein level in human breast cancer cell lines MCF-7 and its resistance cell line MCF-7/ADR and MCF-7/MX were studied by western blotting analysis. Western blot analysis revealed that *Gankyrin* was down-regulated in MCF-7/MX cell line, in which the level of *Gankyrin* protein was 26% in MCF-7/MX cells in compared with MCF-7 cells (Fig. 4A and B). Protein expression level analyzing also displayed that differences between the *Gankyrin* expression was not significant between MCF-7/ADR and MCF-7 cells (Figure 4A and B).

Discussion

Doxorubicin and mitoxantrone, two of the most effective anticancer drugs, are commonly used against breast cancer, while unfortunately many of breast cancer cells exhibit resistance to them (Doyle et al., 1998; Heidemann et al., 2002; Prados et al., 2012). MDR phenotype occurred by different mechanisms in cancer cells is a kind of the simultaneous development of resistance to a diversity of anticancer drugs (Doyle and Ross, 2003). On the other hand, *Gankyrin* is defined as an oncoprotein and its overexpression has been found to be associated with human cancer cells compare to normal cells via various mechanisms (Bunting, 200; Dawson et al., 2002; Higashitsuji et al., 2000; Ortiz et al., 2008; Park et al., 2001). Different studies showed that *Gankyrin* could be a key point in the regulation of important cell cycle regulator protein and may contribute to the establishment of MDR. The aim of this study was to explore mRNA and protein level of *Gankyrin* in MDR cells (MCF-7/MX and MCF-7/ADR) and non-MDR counterparts (MCF-7).

Gankyrin was recognized in normal breasts while it was found to be overexpressed in breast cancers, and also promoted the metastasis in this kind of cancer cells (Gao et al., 2014; Kim et al., 2013). In MCF-7 breast cancer cells, down-regulation of *Gankyrin* was allied with a decrease of cell proliferation and tumorigenicity (Kim et al., 2013). A previous study reported that *Gankyrin* is a critical regulator for breast cancer metastasis that highlighted the potential effect of *Gankyrin* as a therapeutic target for tumor metastasis (Zhen et al., 2013). In 2009, Li et al., tried to investigate the roles of *P28/GANK* in multidrug resistance phenotype of gastric cancer cells. They showed that down-regulation of *P28/GANK* by transfection of siRNA vector of *P28GANK* into human vincristine-resistant gastric adenocarcinoma cell line *SGC7901/VCR*, could enhance the sensitivity of these cells towards anticancer

drugs. Also, downregulation of *P28GANK* could down-regulate the expression of MDR1 and finally decreased the capacity of cells to efflux adriamycin. Similarly, one year later Wang et al., (2010) reported that transfection of cisplatin induced MDR osteosarcoma cell line Saos-2/CDDP with the expression vector of *P28GANK* resulted to resistance to both P-glycoprotein (P-gp)-related and P-gp-nonrelated drugs. On the other hand, blocking of *P28GANK* expression by *P28GANK*-specific small interfering RNA (siRNA) increased the cell sensitivity to various chemotherapeutic drugs. They showed that *P28GANK* gene could significantly up-regulate the expression of MDR1 and Bcl-2 and down-regulate the expression of Bax. In a similar study, Wang and Cheng (2014) studied the effects of *Gankyrin* silencing on the cisplatin resistance of ovarian cancer drug-resistant cell line SKOV3/DDP. They showed increased expression of *Gankyrin* in SKOV3/DDP cells that its silencing could increase the cisplatin sensitivity of SKOV3/DDP by downregulation of the mRNA expressions of MDR1, increasing the intracellular levels of cisplatin and promoting cell apoptosis. In this study, we confirmed that MDR1 expression level in MCF-7/ADR cell line was remarkably higher than non-resistance MCF-7 cells, while there were no significant differences between *Gankyrin* gene expressions in MDR1 resistance and parental cell lines. These results proposed that there is no clear relationship between *Gankyrin* expression and multidrug resistant phenotype in breast cancer MCF-7/ADR cell line.

Chen et al., (2009), tried to understand the mechanisms of arsenic resistance in two arsenic-resistant liver cancer and gastric cancer cell lines (HepG2/AS and SGC7901/AS, respectively). Their results showed increased expression of three ABC transporter proteins, *ABCB1*, *ABCC1* and ABCC2 in two arsenic-resistant cell lines. Interestingly, *Gankyrin* was upregulated in HepG2/AS, while it was down regulated in SGC7901/AS in compare with their non-resistance cell lines. In similar way, we observed significant lower mRNA expression of *Gankyrin* in MCF-7/MX cells (6% to the control level) that caused to down-regulated protein expression of *Gankyrin* in this resistance cell line (26% to the control level).

In conclusion, our findings showed that there is no clear relationship between *Gankyrin* and MDR1 expression in drug resistance breast cancer cell line MCF-7/ADR, while there is a marked relevance between down-regulation of *Gankyrin* and higher expression level of MDR associated transporter proteins, *ABCG2*, in MCF-7/MX breast cancer cells. The results proposed that further molecular investigation in cellular and animal models is essential for clarifying the role of *Gankyrin* in tumorigenesis and MDR phenotype.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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