Research Article

Gamze Yıldırım, Meltem D. Kars*, Gökhan Kars and Hamdi Ş. Kılıç

Biomarkers to target and silence stemness of breast cancer stem cell model: silencing MDR1 by siRNA

https://doi.org/10.1515/tjb-2021-0275
Received September 15, 2021; accepted February 24, 2022; published online March 28, 2022

Abstract

Objectives: Aim of the study was to reveal new biomarker genes to target breast cancer stem-like cells (BCSC-like) and then sensitize BCSC-like cells to chemotherapy by silencing MDR1 gene found to be the most suitable target.

Methods: Drug resistance associated genes were screened by cDNA microarray to unveil biomarker genes in drug resistance breast cancer model cells. Drug resistance was then reversed by silencing MDR1 gene in BCSC-like cells. The effect of silencing was monitored by real-time cell proliferation analysis. Differential expressions of MDR1, ALDH1A3, EGFR and BAG4 genes were identified by real-time PCR. P-glycoprotein (P-gp) expression level and its activity were investigated by Western blot and flow cytometry measurements, respectively.

Results: 16 new biomarker genes were identified upon gene expression analysis by cDNA microarray. MDR1 gene was selected as the most potent target gene and silencing of it caused down-regulation of MDR1, ALDH1A3, EGFR, BAG4 expression and P-glycoprotein activity and expression in BCSC-like cells. At the end, silenced BCSC-like cells were found to be more responsive to paclitaxel therapy.

Conclusions: In conclusion, siMDR1 silencing is an effective way to reverse multidrug resistance and malignancy. New biomarker genes revealed in this study require to be investigated to target stemness of BC.

Keywords: ALDH1A3; breast cancer stem cells; EGFR; microarray; paclitaxel; siMDR1.

Introduction

Chemotherapy is a common treatment strategy against breast cancer [1]. However, multidrug resistance (MDR) is encountered frequently in cancer patients treated with chemotherapeutic agents [2]. Breast cancer cells develop resistance to chemotherapy agents by several mechanisms. These molecular mechanisms lead to MDR phenotype. The most known mechanism of drug resistance is increased pumping out of therapeutic agents due to highly expressed ATP binding cassette (ABC) transporter proteins. MDR causes the development of malignancy after exposure to chemotherapy as well [2]. There are several genes and proteins associated with multidrug resistance. One of the remarkable genes is ALDH1A3 that encodes an aldehyde dehydrogenase enzyme whose increased activity has been tightly correlated with metastasis. Cancer stem cells with high ALDH1A3 activity bear invasive characters and drug resistance phenotype [3]. Epidermal growth factor receptor (EGFR) is also associated with MDR. EGFR is a type of receptor having tyrosine kinase activity and functions in most of the cancer types by promoting cell proliferation, differentiation and cell survival [4]. EGFR also promotes AKT pathway which then stimulates cell growth mechanisms. Activated or deregulated EGFR signaling results in drug resistance in breast cancer cells to commonly anticancer drugs such as doxorubicin, daunorubicin, paclitaxel, cisplatin and 5-flouracil as well as ionizing radiation (IR) [5]. The other MDR related protein is Bcl-2 Associate Athanogene 4 (BAG4) which is an anti-apoptotic protein

*Corresponding author: Meltem Demirel Kars, Faculty of Engineering, Department of Biomedical Engineering, Necmettin Erbakan University, Köyceğiz Campus, Konya, 42140, Turkey, Phone: +90 332 325 2024, E-mail: meltemdkars@gmail.com. https://orcid.org/0000-0002-7300-4075
Gamze Yıldırım, Graduate School of Sciences, Department of Nanotechnology and Advanced Materials, Selçuk University, Konya, Turkey. https://orcid.org/0000-0002-6764-7349
Gökhan Kars, Faculty of Science, Department of Molecular Biology and Genetics, Necmettin Erbakan University, Konya, Turkey. https://orcid.org/0000-0002-2507-2305
Hamdi Ş. Kılıç, Faculty of Science, Department of Physics, Selçuk University, Konya, Turkey. https://orcid.org/0000-0002-7546-4243

Open Access. © 2022 the author(s), published by De Gruyter. This work is licensed under the Creative Commons Attribution 4.0 International License.
and is called silencer of death domains. It is generally highly expressed in cancer tissues and provides a mechanism to escape from death [6]. BAG4 protein was defined in radiation resistant HeLa cells and BAG4 has been also linked both to cisplatin resistance in various cancer types [7]. In the current study, ALDH1A3, EGFR and BAG4 genes were found to be correlated with paclitaxel resistance in BCSCs.

The parental MCF-7 cells used here do not express MDRI gene. The parental MCF-7 cells were treated with increasing doses of paclitaxel for approximately two years to develop drug-resistant model cells [2]. As the cells acquired paclitaxel resistance, the cell line (MCF-7/Pac) became unresponsive to paclitaxel therapy and it started to express MDRI and breast cancer stem cell associated genes differentially with respect to parental drug sensitive cell line. In breast cancer stem cell sub populations, highly expressed CD44, ALDH and CD24 are consensus biomarkers. It is known that about two hundred of CD44 positive, CD24 negative and ALDH overexpressing cancer cells are sufficient to initiate tumors [8]. In line with this, the cells used in this study were CD44+, CD24−, ALDH+, P-gp and paclitaxel resistant BCSC-like cells. The method of obtaining BCSC-like cells was patented by our group [9].

Gene silencing has received increased attention in functional genomics research. The method has enabled elucidation of the roles of genes with unknown functions in model organisms [10]. The method of targeting mRNA is named ‘antisense strategy’ and has been widely applied to assess the function of genes. These antisense strategies aim to down-regulate the mRNA encoding the protein to be inhibited [11]. Taking into account the aforementioned arguments, the aim in this study was to reveal target genes for the reversal of stemness of BCSC-like cells.

Materials and methods

Cell lines and culture conditions

Estrogen receptor (ER) positive mammary carcinoma epithelial cells (MCF-7) and ER− BCSC-like cells were used in the present study. Paclitaxel resistant breast cancer cell line was developed from drug sensitive MCF-7 by stepwise dose increments and it was named as MCF-7/Pac by our group [2, 12]. Since MCF-7/Pac cell population exhibited breast cancer stem cell phenotype, BCSC-like cell population was sorted from them. The cells have been selected by flow cytometry from a paclitaxel resistant cell population by using ALDH, CD44 and CD24 as BCSC markers. The method for isolation of chemotherapy resistant BCSC-like cell line was patented by Turkish Patent and Trademark Office with the registration number TR 2013 10837 B and was published by our group [9, 13]. MCF-7 and BCSC-like cell lines were cultured in RPMI640 (Biochrom, Berlin, Germany) solution at 37 °C and in the atmosphere of 5% CO₂.

Analysis of cDNA microarray data

High throughput total genome cDNA microarray screening (Affymetrix, Santa Clara, CA, USA) was performed to figure out differentially expressed genes in Paclitaxel resistant MCF-7 cells and drug sensitive MCF-7 cells previously [16]. After scanning of the gene chip arrays, the statistically significant data were selected from high throughput data sets by independent sample t-test (p-Value <0.05) between duplicate data for drug resistant and sensitive cells. In the context of this work, statistically significant microarray results (p-Value <0.05) were re-analyzed in silico. Significantly altered genes between resistant subline and MCF-7/S were listed and gene trees were constructed from these lists by standard correlation (Figure 1). Upregulated and downregulated genes were selected from gene trees by volcano plot filtering (by excluding the genes differentially expressed between ~2 and 2 folds) to find out new BCSC related target genes.

siRNA transfection

P-gp encoding MDRI gene was targeted to be reversed by siRNA as the most overexpressed gene in BCSC-like population was found to be MDRI according to the results of microarray analysis. First of all, siRNA transfection optimization was achieved by transfecting the cells with Cy3 labeled siRNA (Silencer™, Cy3™-labeled Control, Ambion, Paisley, UK). Then, the cells were observed under confocal microscope (Nikon, Japan, Cy3 is excited at 550 nm and emits at 570 nm) for visualization of the transfected fluorescent siRNA in the cells and for optimization of siRNA transfection protocol. The cells were transfected with Cy3 labeled siRNA (5 pmol) and then they were cultured in 24-well cell view culture plates (Greiner Bio One, Frickenhausen, Germany) for 24 and 48 h to find the optimum incubation time. MDRI siRNA (IDT-Iowa, USA) and siRNA MOCK (non-template siRNA, Invitrogen) oligonucleotide sequences are presented in Table 1. BCSC-like cells were seeded (1 x 10⁶ cells each well) in six well culture plates and incubated for 24 h. Then, optimum amount of lipofectamine and siRNA (25 pmol) solutions were applied to cells and they were cultured for 24 h at 37 °C in the atmosphere of 5% CO₂. The procedure applied in this study was adopted from the report of Dönmez et al. [15].

Analysis of in vitro paclitaxel therapy by a real-time cell analysis system

It was previously demonstrated that the BCSC-like cells used here were resistant to paclitaxel, a microtubule inhibiting anti-cancer drug [13]. The effects of paclitaxel on the proliferation of silenced BCSC-like and siMOCK-treated BCSC-like cells were analyzed using xCELLigence (RTCA system, ACEA Biosciences, San Diego, CA, USA). The method was applied as described previously [13]. In summary, BCSC-like cells were first seeded (10.000 cells/well) and silenced by incubating with siRNA for 24 h on e-plates. After that, paclitaxel was added to the cells at concentrations ranging from 20 to 1.5 µM. Finally, the effect of paclitaxel on siMDRI silenced breast cancer stem cells was compared to the non-silenced drug resistant BCSC-like cells.
Gene expression analysis

RNA isolation from siMDR1 and siMOCK treated BCSC-like cells and un-transfected control cells were performed by combining two RNA isolation protocols as follows. First, total RNA was extracted from cells \((1 \times 10^6)\) by TRI Reagent (Sigma, MO, USA). Then, the aqueous RNA phase was collected and diluted in 70% ethanol. Finally, the RNA suspension was purified using Qiagen RNA isolation kit (Manchester, UK). After the final elution of RNA samples, the quality of RNA samples was determined by agarose gel electrophoresis (Biorad, California, USA) and nanodrop (Thermo Scientific, Massachusetts, USA) measurements. Following RNA extraction, the effect of MDRI silencing on the expression level of BCSC associated genes were investigated by reverse transcription polymerase chain reaction (RT-PCR). The specific primers for detecting the genes of interest were designed by utilizing Primer3 and NCBI BLAST programs (Table 2).

![Gene tree constructed during the analysis of microarray gene expression data.](Image1)

When we compare the drug resistant cells and drug sensitive cells, the upper part of the tree demonstrates the gene clusters of which expression levels are low (blue) in sensitive cells and high in (yellow-orange-red) in drug resistant cells. The bottom part of the tree demonstrates the gene clusters of which expression levels are low (blue, yellow) in drug resistant cells and high in (orange-red) in drug sensitive cells.

### Table 1: Oligonucleotide sequences of siRNA and control (MOCK).

| sRNA      | Oligonucleotide sequence                  |
|-----------|------------------------------------------|
| MDRI sense| 5'GGAGUGAGUUGGUGUUUUAUdTdT3'             |
| MDRI antisense | 5'AUCAAACCAACACAUCC dTdT3'    |
| MOCK sense | 5'GGAGUUGUGUUGGUGUUUUAUdTdT3'           |
| MOCK antisense | 5'AUCAAACCAACACAUCC dTdT3'    |

Figure 1: Gene tree constructed during the analysis of microarray gene expression data.
cDNA was synthesized from total RNA by reverse transcription using GAPDH, MDRI, EGFR, ALDH1A3 and BAG4 specific primers (Revert-aid RT enzyme, Fermentas, Ontario, Canada). Gene expression analysis was performed by a real-time thermal cycler (ABI-7500 system, Thermo Scientific, ABD). The reaction mixture consisted of SYBR Green master mix (Biorad, California, USA), cDNA and primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) encoding gene was used as internal reference. The $2^{-\Delta\Delta CT}$ analysis method was applied to calculate the expression levels of the target genes in silenced and non-silenced cells [16].

### Evaluation of expression levels of P-gp in BC cells

The P-gp was selected as a representative protein for confirmation of RT-PCR gene expression analysis. For this, total protein was extracted from cells and Western blot analysis was applied for the target protein P-gp. Total lysate of silenced and MOCK control BCSC-like (5 × 10^6) cells were prepared in RIPA cell lysis buffer (Amresco, USA) as previously mentioned [13]. The protein concentration of the cell lysate was determined as previously described [13]. Protein lysates were analyzed on SDS-PAGE and transferred onto 0.45 µm nitrocellulose membrane for 5 h using BioRad Electro Blot System. The membrane was hybridized with antibodies (Rabbit Anti-Human GAPDH antibody, 1:200 dilution; Rabbit Anti-Human-Pgp antibody, 1:1000; ABCAM). Then membrane was hybridized with secondary antibody (HRP conjugated anti-Rabbit for 1 h). After washing, the detection buffer ECL substrate (Abcam, USA) was added the membrane was analyzed under chemiluminescent gel imaging system and detection buffer ECL substrate (Abcam, USA) was added. The band densities of P-gp were normalized by the band densities of GAPDH for drug resistant and silenced BCSC-like cells. The results revealed that in addition to MDRI, ALDH, CD44, CD24, EGFR, SNAI2, SMAD3 and WNT-3, 16 cancer stem cell associated genes were differentially expressed in MCF-7/Pac cells (containing BCSC-like cells) with respect to drug sensitive MCF-7 cells (Table 3) [13, 14]. According to the results, MDRI gene is the most overexpressed gene (95 folds) in BCSC-like populated drug resistant cells. Additionally, MUC18, AKT3, RAB3B, WNT5A, LPL, CAPR, MAP3K12.

### Confirmation of downregulation of P-gp activity by Rhodamine-123 assay

Rhodamine-123 (Sigma Aldrich, M0, USA) is a fluorescent compound that is effluxed out of the cell by an active P-gp. Rhodamine is generally used to determine the activity of P-gp through flow cytometry [2]. Briefly, drug sensitive MCF-7 cells, silenced and MOCK treated BCSC-like cells were incubated with 1 µM rhodamine in serum free media for 10 min at 37 °C. Then, the cells were centrifuged and washed in PBS. Finally, fluorescent cells were counted and fluorescent values were measured by a flow cytometer (FACS ArihIII, Becton Dickinson, NJ, USA.). The accumulation of rhodamine results in high fluorescent value (y axis of flow cytometry histogram) which is the designation of drug accumulation in the cells. The decrease in mean fluorescent value indicates that P-gp exports rhodamine out of the cells. The fluorescent activity ratio (FAR) demonstrates the degree of reversal of drug resistance. FAR is calculated by dividing the mean fluorescent value of inhibited drug resistant cells by the mean fluorescent value of untreated drug resistant cell. When the FAR value is higher than 1.10, it shows that the drug resistant cells experience reversal of drug resistance or decrease in P-gp activity [2].

### Statistics

Duplicate set of high throughput microarray results were firstly analyzed by Student’s t-test (p-Value <0.05) and then filtered by Volcano plot (by excluding genes with fold change values between –2 and 2 folds) to select the genes that were differentially expressed in drug resistant cells with respect to drug sensitive cells. The analyses regarding toxicological assessments, gene expression analysis and flow cytometry measurements were repeated at least three times and subjected to Student’s t-test (p-Value <0.05).

### Results

#### Whole genome cDNA analysis

Whole genome cDNA microarray expression analysis results revealed that in addition to MDRI, ALDH, CD44, CD24, EGFR, SNAI2, SMAD3 and WNT-3, 16 cancer stem cell associated genes were differentially expressed in MCF-7/Pac cells (containing BCSC-like cells) with respect to drug sensitive MCF-7 cells (Table 3) [13, 14]. According to the results, MDRI gene is the most overexpressed gene (95 folds) in BCSC-like populated drug resistant cells. Additionally, MUC18, AKT3, RAB3B, WNT5A, LPL, CAPR, MAP3K12,
MAP4K4, MAP4K1, STAT6B, BAG4, MAPKKK4 genes were found to be overexpressed about two to 25 folds more in BCSC-like cell populated cells compared to sensitive BC model. On the other hand, MAP3K3, MAPKAPK2, MAP3K1, VMP1 were down regulated about three to six folds. In the present study, 16 differentially expressed genes were disclosed which will provide insight into studies for targeting stemness of BC.

**siRNA transfection**

siRNA transfection process was optimized by using Cy-3 labelled fluorescent siRNA and by visualizing it inside BCSC-like cells under a confocal microscope. A 24 h incubation period was found to be suitable for transfection (Figure 2). Accordingly, the antisense MDR1 RNA transfection was applied for 24 h for all assays including...
cell proliferation, gene expression, P-gp activity and expression.

Cell proliferation assays

Assessment of the effect of chemotherapy on BCSC-like cells was conducted by a Real Time Cell Analysis system. The responses of siMDR1 silenced and siMOCK treated BCSC-like cells to paclitaxel were evaluated in various time periods by treating the cells with different drug concentrations (Table 4 and Figure 3). According to the results, the antiproliferative effect of 1.25 μM paclitaxel application on siRNA silenced BCSC-like cells was much more pronounced than that on control-MOCK cells. On the other hand, 20 μM paclitaxel resulted in similar anti-proliferative effect on both siMDR1 and siMOCK treated BCSC-like cells are clearly figured out as time and dose-dependent way (Figure 3). The IC50 values computed at the logarithmic phase of cell proliferation are listed in Table 4. Regarding the results, it can be deduced that BCSC-like cells become sensitive to chemotherapy after MDR1 silencing.

Gene expression analysis upon MDR1 silencing

The 2^ΔΔCT method was used to examine changes in gene expression levels. The results demonstrated that siRNA silencing of the MDR1 gene dramatically down-regulated the expression levels of MDR1, ALDH1A3, EGFR and BAG4 genes. Indeed, MDR1, ALDH1A3, EGFR and BAG4 gene expression levels decreased about 94, 99, 99 and 50% respectively. There was not any significant alteration in gene expression levels in mock treated BCSC-like cells (Figure 4A). The whole genome cDNA microarray analysis was previously performed by our group to evaluate differential gene expressions between paclitaxel sensitive MCF-7 and paclitaxel resistant MCF-7 cells [17]. These results showed that ALDH1A3, EGFR and BAG4 expression levels were 16.79, 33.39 and 2.05 folds higher respectively in drug resistant cells when compared to drug sensitive cells (Table 3). Here, RT-PCR results evidenced that sensitization of drug resistant BCSC-like cells by siMDR1 reverses the cells to the drug sensitive form by silencing MDR1 gene.

Evaluation of P-gp expression level and drug efflux activity

According to the Western blot analysis, the representative protein expression, P-gp, was down-regulated by siRNA silencing of MDR1 (according to Image J densitometry analysis) (Figure 4B). P-gp down-regulation was also examined and revealed by functional analysis through measuring P-gp activity by flow cytometry. When the mean fluorescent values of histograms were analyzed, it was observed that MCF-7 drug sensitive cells accumulated about 5.2 folds more fluorescence than drug resistant BCSC-like/MOCK cells (Figure 5). Additionally, FAR value for the siRNA silenced BCSC-like cells was calculated as 1.26. This clearly demonstrates reversal of MDR1 expression and P-gp activity in BCSC-like cells. The flow cytometry histograms for each cell line were given as supplementary materials (S1A, B and C).

Discussion

Breast cancer is one of the most widespread cancer diseases worldwide. Chemotherapy resistance in breast cancer is still an important difficulty encountered in clinic [18]. Drug resistance against paclitaxel has been reported to develop in nearly 90% of metastatic breast cancer patients [19]. Over-expression of MDR1 gene coding for P-gp plays a significant role in drug resistance by exporting drugs and thereby preventing them reaching their intracellular targets [20]. In this study, the aim was to reveal new marker genes to target stemness of BCSC-like cells based on whole genome cDNA microarray results. And following, the mostly overexpressed MDR1 gene was downregulated by
Figure 3: Real-time cell proliferation analysis of (A) BCSC-like (MOCK) cells and silenced cells treated with 1.25 µM paclitaxel, (B) BCSC-like (MOCK) cells treated with 1.25–20.0 µM paclitaxel, (C) siRNA silenced BCSC-like cells treated with 1.25–20.0 µM paclitaxel.

Figure 4: (A) results of gene expression analysis for MDR1, ALDH1A3, BAG4 and EGFR genes by $2^{-\Delta\Delta CT}$ method. (B) Western blot analysis for P-gp/GAPDH and densitometry values of the normalized membrane blot bands 1. BCSC-like (resistant) 2. BCSC-like (silenced).
siRNA mediated silencing technique. Breast cancer stem-like cells that were ALDH positive, CD44 positive, CD24 negative, P-glycoprotein positive, ER (estrogen receptor) negative were used in this study. The cell line used in the current work was previously developed and established from drug sensitive MCF-7 cells and was validated by in vitro chemotherapy, toxicological tests and pharmacogenomics analysis [13, 21]. High throughput gene expression analysis results exhibited that BCSC-like cell containing drug resistant cells differentially expresses CSC associated genes significantly when compared to drug sensitive parental MCF-7 cells. For instance, MDR1, EGFR, MUC18, AKT3, RAB3B, SMAD3, WNT5A, ALDH1A3, SNAI2 genes were expressed between nine to 95 folds more in the drug resistant cell population that was crowded by BCSC-like cells with respect to drug sensitive MCF-7 model cell. As a result of this, these genes may be proposed as target genes to reverse drug resistance and to target stemness of BC. In addition, LPL, CAPR, WNT3, MAP3K12, MAP4K4, MAP4K1, STAT6B, BAG4, MAPKKK4 genes were found to be upregulated in drug resistant BC cells about two to five folds. On the other hand, MAP3K3, CD24, MAPAPK2, MAP3K1 and VMP1 were significantly downregulated in drug resistant BC. In concordance with our results, overexpression of BAG4, MDR1, EGFR, AKT3, SMAD3, ALDH1A3, SNAI2, WNT3, STAT6B, CAPR genes were declared to be closely associated with cancer development, proliferation and drug resistance in many studies [22–27]. SMAD3 induces stemness in breast cancer (BC) cells and inhibition of SMAD3 results in blockade of the SMAD3 associated TGF-β signal pathway and cancer stem cell (CSC) features [22]. In normal breast tissue, Wnt signaling controls important cellular events such as cell fate determination, proliferation, migration and tissue homeostasis. Noncanonical signaling is activated by Wnt4, 5A, 5B and Wnt 11. Wnt5A is known to be one of the regulators of macrophage mediated malignant invasion in BC [23]. Small transcription repressor 2 (SNAI2) is highly associated with epithelial to mesenchymal transition (EMT) in BC and was reported as one of the CSC markers [24]. A tumor suppressor, α-catenin (cadherin associated protein-alpha, CAPR) is known as one of the components of the E-cadherin and catenin complex and it takes role in maintaining the stability of the intercellular adherent junctions [25]. STAT6 can act as both transcription factor and signaling molecule. STAT6 is highly associated to IL-4 and IL-13 signal pathway, and also has role in polarization of the T helper cells in immune system. STAT6B is one of the isoforms of STAT6 and has about 110 amino acids deletion at the N-terminal region but the product is intact concerning its biological functions [26]. It was reported that STAT6 might be a sensitive marker for prostatic stromal lesions [27]. As an exception, function of MUC18 (CD146) in cancer development took little attention such that only a group declared that it was a new biomarker that promoted angiogenesis in vitro and in vivo [28]. In line with this, we also found that MUC18 was overexpressed in BC cells with CSC and metastatic features. RAB3B which is an oncogene expressed on cell junctions was found to be upregulated in liver cancer stem cells according to the RNA seq analysis in a report in 2019 [29]. Additionally, RAB3B was reported as a new prognostic marker for extracellular vesicle biopsies of prostate cancer patients [30]. Similarly, we observed 24 folds increase in RAB3B gene expression level in BC for the first time. Based on these results, we also propose RAB3B as a biomarker in BCSC. Manupati et al. asserted that LPL was an effector of CD44 signaling and might be a potential target in BCSCs to inhibit progression [31]. We also found that LPL was overexpressed by drug resistant BC cells five folds more than drug sensitive BC cells. So, here we propound that LPL may be considered as target to combat BC in future studies. Mitogen activated protein kinases (MAPKs) are the members of protein kinases family. They phosphorylate their own serine and threonine residues or that of on their target proteins. MAPKs modulate the genes related with cell growth, metabolism, cell survival, stress response, apoptosis, migration, cellular differentiation and immune defense [32]. MAP kinase cascade has important role in cell proliferation and migration. In the present work, we displayed that while MAP3K12, MAP4K4, MAP4K1, MAPKKK4 were upregulated, MAP3K3, MAPAPK2 and MAP3K1 were down regulated by development of drug resistance in BC cells. Activation of MAP kinases may exhibit anti apoptotic or pro apoptotic effect and may regulate tumor progression. Based on these discussions, MAP cascade seems to be modulated in favor of drug resistance in BC model.
We found VMP1 gene expression to be significantly downregulated (circa 6.5 folds) in drug resistant BC cells with respect to sensitive MCF-7 model. It was declared that VMP1 was associated with regulation of autophagy and inhibition of VMP1 results in blockage of autophagosome formation [33]. In our case, down regulation of VMP1 may have resulted in resistance to cell death through autophagy. Hence, we may propose that there may be a cross regulation between autophagy and apoptosis in BCSC-like model cells.

In recent years, increasing knowledge on the mechanisms of endogenous RNA interference opened a door for the usage of siRNAs as innovative nucleic acid therapeutics for the treatment of cancer and multi-drug resistance. The research and development of siRNA drugs has taken 20 years and three siRNA drugs have been approved by the FDA since 2018 [34]. Furthermore, there has been intensive research on the treatment of cancer with siRNA-based drugs, which is quite promising. In this regard, after revealing potential marker genes, we applied siRNA mediated silencing method to downregulate MDR1 gene. In this context, we figured out the effect of MDR1 silencing on both proliferation of BCSC-like cells and cancer stem cell related genes. Eventually, we intended to propose a mechanism to reverse drug resistance in BCSC-like cells. RTCA results clearly demonstrated that silencing of the MDR1 gene and its consequences together caused two folds sensitization of BCSC model cells to paclitaxel treatment. Similarly, Li et al. successfully down-regulated MDR1 gene expression by about 50% in leukemia cell line models through transfection with 5-triphosphate modified-siMDR1 [35]. They also observed a decrease in cell viability in response to in vitro doxorubicin therapy. They proposed that down-regulation of MDR1 gene expression by siRNA might be a good strategy to combat drug resistance in myeloid leukemia [35]. Dönmez et al. also reported that siRNA silencing might be an effective MDR down-regulation strategy that restores the concentration of the drug inside resistant cells and increases the efficiency of doxorubicin in doxorubicin-resistant MCF-7 cell line [15]. Based on Western blot and flow cytometry analyses, we demonstrated that siMDR1 down regulated P-gp expression and also its function in BCSC-like cells. The decreased drug efflux most probably resulted in the decrease in cell viability after paclitaxel therapy.

Here, we also tested the effect of MDR1 down-regulation on the expression levels of important multiple proliferative genes which showed pronounced expressions in BCSCs. Strikingly, down-regulation of MDR1 gene resulted in down-regulation of ALDH1A1, EGFR and BAG4 genes. So, our results indicated that inhibition of MDR1 led to the suppression of genes that play role in proliferation of BCSC-like cells. We can simply conclude that apoptosis was induced by restoring the concentration of paclitaxel within cells and reducing the expression of the anti-apoptotic BAG4 gene. In accordance with our results, Li et al. declared that MDR1 silencing resulted in increased apoptosis by releasing cytochrome C from mitochondria and increasing cleavage of caspase-3 and caspase-9 [35]. As drug resistance develops, cell membrane is also modulated; synthesis of sphingolipids, ceramide and cholesterol are elevated. These lipids are embedded in the membrane leading to decreased permeability (decreased influx). Increased expression of EGFR and CD44 membrane receptors was regarded as the outcomes of these changes in the membrane [36]. In parallel to this finding, it could be concluded that MDR1 gene down-regulation resulted in suppression of membrane changes which led to a decrease in EGFR expression. Changxing et al. showed that silencing of MDR1 gene by nano-carrier siRNA system and paclitaxel treatment exhibited synergistic anti-proliferative and gene suppression effects in colon cancer stem cell models [37], which is in concordance with our results. Li et al. recently published a gene correlation table using the Breast Cancer Gene-Expression Miner v4.5. The bioinformatics data consisted of gene expression, correlation and prognostic modules of 4908 breast cancer patients. The table and the additional results demonstrated that high expression levels of EGFR, CD44 and ALDH1A3 were correlated in triple negative breast cancer cells, known to be resistant to chemotherapy. Silencing of EGFR by inhibiting gamma amino butyric acid type A receptor in triple negative breast cancer patients resulted in down regulation of CD44, ALDH1A1 and hindrance of stemness of breast cancer. In parallel to our discussions, they also argued that inhibition of EGFR enhanced the sensitivity to chemotherapeutic agents [38].

To conclude, siRNA mediated silencing strategy could successfully be applied to target BCSC-like cells and may be proposed as a future chemotherapy strategy. To our knowledge, this is the first report demonstrating that silencing of the MDR1 gene results in down-regulation of survival/death-related genes such as EGFR, ALDH1A3 and BAG4. Considering that chemotherapy drugs targeting the MDR1 gene are in the research and development phase, the outcomes of this research will encourage and inspire those studies. As a future prospect, the CSC marker genes, revealed by microarray analysis, need and deserve to be further investigated for the purpose of targeting BCSCs.

Research funding: The project was funded by Selcuk University (project number: 15201105).

Author contribution: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest.
Informed consent: Not applicable.
Ethical approval: Not applicable.

References

1. Krishan A, Fitz CM, Andriths I. Drug retention, efflux, and resistance in tumor cells. Cytometry 1997;29:279–85.
2. Kars MD, Iseri OD, Ufuk G, Ural AU, Arpac F, Molnar J. Development of rational in vitro models for drug resistance in breast cancer and modulation of MDR by selected compounds. Anticancer Res 2006;26:4559–68.
3. Rodriguez-Torres M, Allan AL. Aldehyde dehydrogenase as a marker and functional mediator of metastasis in solid tumors. Clin Exp Metastasis 2016;33:97–113.
4. Lo HW, Hung MC. Nuclear EGFR signalling network in cancers: linking EGFR pathway to cell cycle progression, nitric oxide pathway and patient survival. Br J Cancer 2006;94:184–8.
5. Steelman LS, Chappell WH, Akula SM, Abrams SL, Cocco L, Manzoli L, et al. Therapeutic resistance in breast cancer cells can result from deregulated EGFR signaling. Adv Biol Regul 2020;78:100758.
6. Takayama S, Reed JC. Molecular chaperone targeting and regulation by BAG family proteins. Nat Cell Biol 2001;3:237–41.
7. Mariotto E, Viola G, Zanon C, Aveic S. A BAG's life: every connection matters in cancer. Pharmacol Ther 2020;209:107498.
8. Enciso-Benavides J, Alfaro L, Castañeda-Altamarino C, Rojas N, González-Cabeza J, Enciso N, et al. Biological characteristics of a sub-population of cancer stem cells from two triple-negative breast tumour cell lines. Helinovy 2021;7:e07273.
9. Kars MD. Method of isolation of chemotherapy resistant breast cancer stem cells. Turkish Patent Institute; TR: 2013;10837 B: 2020.
10. Kurreck J. Antisense technologies. Improvement through novel chemical modifications. Eur J Biochem 2003;270:1628–44.
11. Gewirtz AM. On future’s doorstep: RNA interference and the pharmacopoeia of tomorrow. J Clin Invest 2007;117:3612–4.
12. Kars MD, Iseri OD, Gündüz U, Molnar J. Reversal of multidrug resistance by synthetic and natural compounds in drug-resistant MCF-7 cell lines. Chemotherapy 2008;54:194–200.
13. Kars MD, Yıldırım G. Determination of the target proteins in chemotherapy resistant breast cancer stem cell-like cells by protein array. Eur J Pharmacol 2019;848:23–9.
14. Iseri OD, Kars M, Arpac F, Atalay C, Pak I, Gündüz U. Drug resistant MCF-7 cells exhibit epithelial-mesenchymal transition gene expression pattern. Biomed Pharmacother 2011;65:40–5.
15. Dönnmez Y, Gündüz U. Reversal of multidrug resistance by small interfering RNA (siRNA) in doxorubicin-resistant MCF-7 breast cancer cells. Biomed Pharmacother 2011;65:85–9.
16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402–8.
17. Kars MD, Iseri OD, Gündüz U. A microarray based expression profiling of paclitaxel and vincristine resistant MCF-7 cells. Eur J Pharmacol 2011;657:4–9.
18. Hu Y, Yague E, Zhao J, Wang L, Bai J, Yang Q. Sabutoclax, pan-active BCL-2 protein family antagonist, overcomes drug resistance and eliminates cancer stem cells in breast cancer. Cancer Lett 2018;423:47–59.
19. Yang LT, Leong Y, Song WS, Yang H, Wang W, Wang M. Efficient and tumor-specific knockdown of MTDH gene attenuates paclitaxel resistance of breast cancer cells both in vivo and in vitro. Breast Cancer Res 2018;20:113.
20. Li Y, Gao X, Yu Z, Liu B, Pan W, Li N. Reversing multidrug resistance by multiplexed gene silencing for enhanced breast cancer chemotherapy. ACS Appl Mater Interfaces 2018;10:15461–6.
21. Phil LTH, Sari IN, Yang YG, Lee SH, Jun N, Kim KS. Cancer stem cells (CSCs) in drug resistance and their therapeutic implications in cancer treatment. Stem Cell Int 2018;2018:5416923.
22. Wang N, Weng J, Xia J, Zhu Y, Chen Q, Hu D, et al. Sipa1 enhances SMAD2/3 expression to maintain stem cell features in breast cancer cells. Stem Cell Res 2020;49:102099.
23. Mukherjee N, Panda CK. Wnt/β-Catenin signaling pathway as chemotherapeutic target in breast cancer: an update on pros and cons. Clin Breast Cancer 2020;20:361–70.
24. Zhang JY, Luo Q, Xu JR, Bai J, Mu LM, Yan Y, et al. Regulating stem cell–related genes induces the plastic differentiation of cancer stem cells to treat breast cancer. Mol Ther Oncolytics 2020;18:396–408.
25. Sun Y, Zhang J, Ma L. α-catenin. A tumor suppressor beyond adherens junctions. Cell Cycle 2014;13:2334–9.
26. Hebenstreit D, Wirsberger G, Horejs-Hoeck J, Duschl A. Signaling mechanisms, interaction partners, and target genes of STAT6. Cytokine Growth Factor Rev 2006;17:173–88.
27. Guner G, Bishop JA, Bezzerra SM, Taheri D, Zahavi DJ, Mendoza Rodríguez MA, et al. The utility of STAT6 and Ahd1 expression in the differential diagnosis of solitary fibrous tumor versus prostate-specific stromal neoplasms. Hum Pathol 2016;54:184–8.
28. Jiang T, Zhuang J, Duan H, Luo Y, Zeng Q, Fan K, et al. CD146 is a coreceptor for VEGFR-2 in tumor angiogenesis. Blood 2012;120:2330–9.
29. Tsunodomi R, Yoshihara K, Kimura Y, Nishiyama M, Matsukuma S, Tokumitsu Y, et al. Cancer stem-like phenotypes including immune surveillance and its responsible genes in induced liver cancer stem-like cells. Ann Oncol 2019;30:i59–60.
30. Weng J, Xiang X, Ding L, Wang AL, Zeng Q, Sethi G, et al. Extracellular vesicles, the cornerstone of next-generation cancer diagnosis? Semin Cancer Biol 2021;11:139–5.
31. Manupati K, Yeeveralli R, Kaushik K, Singh D, Mehra B, Gangane N, et al. Activation of CD44-Lipoprotein lipase axis in breast cancer stem cells promotes tumorigenesis. Biochim Biophys Acta (BBA) - Mol Basis Dis 2021;1867:166228.
32. Mohan CD, Liew YY, Jung YY, Rangappa S, Preetam HD, Chinnathambi A, et al. Brucine D modulates MAPK signaling cascade to exert multi-faceted anti-neoplastic actions against breast cancer cells. Biochimie 2021;182:140–51.
33. Ishq M, Ojha R, Sharma AP, Singh SK. Autophagy in cancer: recent advances and future directions. Semin Cancer Biol 2020;66:171–81.
34. Kim YK. RNA therapy: current status and future potential. Chonnam Med J 2020;56:87–93.
35. Li D, Gale RP, Liu Y, Leи B, Wang Y, Diao D. S’-Triphosphate siRNA targeting MDRI reverses multi-drug resistance and activates RIG-I–induced immune-stimulatory and apoptotic effects against human myeloid leukemia cells. Leuk Res 2018;58:23–30.
36. Singh MS, Tammam SN, Shetab BMA, Lamprecht A. MDR in cancer: addressing the underlying cellular alterations with the use of nanocarriers. Pharmacol Res 2017;126:2–30.

37. Changxing L, Zhao G, Liu J, Ma N, Chivukula P, Perelman L. Novel biodegradable lipid nano complex for siRNA delivery significantly improving the chemosensitivity of human colon cancer stem cells to paclitaxel. J Contr Release 2009;140:277–83.

38. Li X, Wang H, Yang X, Wang X, Zhao L, Zou L, et al. GABRP sustains the stemness of triple-negative breast cancer cells through EGFR signaling. Cancer Lett 2021;514:90–102.

Supplementary Material: The online version of this article offers supplementary material (https://doi.org/10.1515/tjb-2021-0275).