

The Sensitivity of Human Breast Cancer Stem Cells (ALDH+) Against Doxorubicin Treatment is Associated with PCNA and BIRC5 Gene Expressions

Syarifah DEWI^{1,2}, Resda Akhra SYAHRANI³, Mohamad SADIKIN², Septelia Inawati WANANDI^{2,3}

1. Doctoral Program in Biomedical Sciences, Faculty of Medicine Universitas Indonesia
2. Departement of Biochemistry and Molecular Biology, Faculty of Medicine Universitas Indonesia
3. Indonesian Medical Education and Research Institute, Faculty of Medicine Universitas Indonesia

ABSTRACT

Introduction: Breast cancer stem cells (BCSCs) are identified as side populations in breast cancer cells owing stem cell properties and tumorigenic characteristics. Previous studies revealed that breast cancer chemotherapy led to BCSC enrichment which contributed to therapy resistance. Our recent *in vivo* study using Next Generation Sequencing has demonstrated that PCNA - the proliferative gene - and BIRC5 - the anti-apoptosis gene - were under-expressed in human breast tumors after neoadjuvant chemotherapy. This study aimed to verify the role of PCNA and BIRC5 expression in doxorubicin-treated human BCSCs *in vitro* and its association with cell viability. **Method:** Human BCSCs (ALDH+) were treated with 0.25 μ M of doxorubicin for 2, 4, 6, 8, 10, 12, 14 days respectively. Cell viability was measured using trypan blue exclusion assay and the expressions of PCNA and BIRC4 mRNA were determined using qRT-PCR. **Results:** This study demonstrated that the viability of ALDH+ BCSCs decreased after 2 days and increased again after 8 days of doxorubicin treatment, indicating the decrease of doxorubicin sensitivity. Interestingly, PCNA and BIRC5 genes were modulated in line with the modulation of cell viability during doxorubicin treatment of human BCSCs. **Conclusion:** In conclusion, we suggest that the PCNA and BIRC5 expressions play an important role on the BCSCs viability which associated with the sensitivity of doxorubicin treatment.

Keywords: human breast cancer stem cells, cell viability, PCNA, BIRC5, doxorubicin

Breast cancer is the most death-causing cancer among women all over the world due to therapy resistance and disease recurrence. Nowadays, neoadjuvant therapy is established for larger primary and locally advanced breast cancer as a preoperative approach. The advantages of neoadjuvant therapy is to downstaging the tumor, reducing the extent surgery and also evaluating the efficacy of the drugs that will be administered.(1) Yet, classic breast cancer therapy has not considered the presence of side populations, known as breast cancer stem cells (BCSCs), that are characterized by self-renewal, differentiation, and tumorigenic capabilities, similar to normal stem cells.(2)

Current treatments for breast cancer frequently fail to eradicate tumor since they are unable to effectively target BCSC populations. BCSCs have been proved to exhibit significant resistance to conventional chemotherapy. Furthermore, breast cancer chemotherapy may

lead to BCSC enrichment which contributed to therapy resistance. It has been demonstrated that BCSC markers (CD44+/CD24-) were expressed abundantly after neoadjuvant chemotherapy of primary breast cancer patients.(3) Some previous studies have also reported that BCSCs are responsible for therapy resistance, recurrence and metastasis in breast cancer.(4)-(5)-(6) The presence of BCSC populations has been correlated with poor prognosis in breast cancer patients.(7)

Very recently, we have applied Next Generation Sequencing to assess the alteration of p53-pathway gene expressions in breast cancer cells from patients underwent neoadjuvant chemotherapy. We found that seven gene expressions in p53-pathway were significantly down-regulated after neoadjuvant chemotherapy of breast cancer patients, namely ATM, BID, BIRC5, CASP8, CASP9, CDK1 and PCNA.(8) Proliferating cell nuclear antigen (PCNA) is

*Corresponding author : Septelia Inawati Wanandi, Departement of Biochemistry and Molecular Biology, Faculty of Medicine Universitas Indonesia, Indonesia Email: septelia.inawati@ui.ac.id

known as a molecular marker for cell proliferation, thereby it has an important role in replication. The function of PCNA is indispensable for the maintenance of genomic integrity and propagation in actively growing cells.(9) BIRC5 or survivin is a member of the inhibitor of apoptosis (IAP) protein family that suppresses caspase-9 activity leading to apoptosis inhibition. This protein is highly expressed in most cancers and is associated with a poor clinical outcome. (10) Our previous study has demonstrated that BIRC5 was highly expressed in BCSCs after oxidative stress induction and led to high cell survival.(11)

The present study aimed to verify the role of PCNA and BIRC5 expressions on the sensitivity of human BCSC against chemotherapy. We analysed the decrease of BCSC sensitivity against doxorubicin by determining cell viability in association with PCNA and BIRC5 expressions in human BCSCs (ALDH+) after repeated exposure to doxorubicin treatment. The result of this study is consistent to our previous next generation sequencing analysis, suggesting that PCNA and BIRC5 could be used as markers for determining chemotherapy sensitivity, particularly in BCSCs.

MATERIALS AND METHODS

Cell culture

Human BCSCs (ALDH+) were grown in serum free DMEM F12 medium with 1% penicillin/streptomycin and 1% amphotericin B and incubated in 5% CO₂ at 37°C.

Cytotoxic Assay

MTS assay was performed to determine cytotoxicity of human BCSCs. About 1x10³ human BCSCs (ALDH+) were plated triplicate in each well of 96-well plate and grown in non-serum DMEM/F12 medium supplemented with 1% penicillin/streptomycin and 1% amphotericin at 37°C and 5% CO₂. After 24-incubation, cells were treated with various concentrations of doxorubicin (0.1, 0.5, 1, 5, 10 and 20 µM) for 24 hours. As controls, cells were grown without doxorubicin under standard conditions for 24 hours. Afterwards, medium was replaced with 100 µl fresh DMEM/F12 medium containing 20 µl MTS/PMS (20:1) and incubated for 1 hour. After the brown colour has appeared, the absorbance was read at 490 nm using microplate reader (Varioskan®, Thermo Fisher Scientific USA). The absorbances of doxorubicin-treated cells were normalized to that of control cells to calculate the percentage of cell viability.

Doxorubicin treatment

Approximately 1x10⁵ cells were plated each well in 12 well-plate and incubated overnight. Afterwards, medium was replaced with fresh DMEM/F12 medium containing doxorubicin of 0.25 µM for 2, 4, 6, 8, 10, 12, and 14 days respectively. As controls, cells were grown in DMEM/F12 without doxorubicin for 2, 4, 6, 8, 10, 12, and 14 days respectively. After incubation, cells were harvested and counted using trypan blue exclusion assay and automated cell counter (Luna®, Logos Biosystems Korea). The number of viable doxorubicin-treated cells was normalized to that of the respective control cells to calculate the percentage of cell viability. Total RNA and isolated from the cells using Tripure® isolation reagent kit (Roche) according to the manufacturer's instructions.

Quantitative RT-PCR

The concentration of total RNA was measured using spektrofotometer (Varioskan, Thermo Fisher Scientific USA). One-step quantitative RT-PCR was carried out using ECO48® real time qPCR system (PCRmax UK). The RT PCR was performed in 20 µL volume with 100 ng total RNA. The primer sequence for quantitative RT-PCR are listed in table 1. PCR cycles are 45°C incubation for 5 minutes initially, followed by a 3-minute incubation at 95°C, then 40 cycles of 95°C for 5 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and melting curve incubation.

Relative expression was relatively calculated using Livak formula(12) with 18S rRNA gene as a reference gene and normalized to its respective control.

RESULTS

Cytotoxic Assay

Cytotoxicity of doxorubicin treatment on human BCSCs (ALDH+) was determined using MTS assay. The 50% cytotoxicity concentration (CC50) was calculated using cytotoxicity curve generated by logarithmic regression equation ($y = -9.572\ln(x) + 62.291$). As shown in Figure 1, CC50 value of doxorubicin on human BCSCs was 3.63 µM. Based on this data, we used doxorubicin concentration lower than CC50 for all experiments.

Cell Viability

Percentage of cell viability was determined by comparing the number of viable doxorubicin-treated cells with that of control cells. Figure 2 demonstrates that BCSC viability after 2-day doxorubicin treatment decreased (66%), however

started to increase on day 8 (81%) until reached 92% viability on day 14.

Relative mRNA expression of PCNA and BIRC5 after doxorubicin treatment

To analyze the role of PCNA and BIRC5 on the sensitivity of BCSCs against doxorubicin, we examined the relative expression of PCNA and BIRC5 mRNA following 2-, 6-, 10-, and 14-day doxorubicin treatment. Compared to each respective controls, PCNA mRNA expression was significantly decreased after 2-day treatment (0.787-times; $p=0.040$), but slightly increased on day 10 (1.337-times; $p=0.236$) and day 14 day of treatment (1.437-times; $p=0.169$) (Fig. 3). PCNA relative expressions in BCSCs treated with doxorubicin for 10 and 14 day was significantly

higher compared to that with 2-day treatment ($p=0.016$ and $p=0.011$ respectively, One-way ANOVA test). Relative expression of BIRC5 mRNA in human BCSCs treated with doxorubicin was also modulated in a similar manner to PCNA expression. It was decreased after 2-day treatment of doxorubicin (0.846; $p=0.089$), but started to increase on day 10 (1.257; $p=0.140$) and day 14 of treatment (1.530; $p=0.094$) compared to each respective controls (Fig. 4). Although there were no significant differences compared to controls, the increase of BIRC5 relative expression in BCSCs after 10- and 14-day doxorubicin treatment was significantly higher compared to 2-day treatment ($p=0.027$ and $p=0.001$, One-way ANOVA test).

Table and Figures

Table 1. Primer sequence of PCNA, Survivin and 18S rRNA

No.	Gene	Primer Sequence	Product
1.	PCNA	F: 5'- CTT CCC TTA CGC AAG TCT CAG -3' R: 5'- TTG AGT GCC TCC AAC ACC TT -3'	189 bp
2.	Survivin	F: 5'- AGG ACC ACC GCA TCT CTA CA -3' R: 5'- GTT CCT CTA TGG GGT CGT CA -3'	186 bp
3.	18S rRNA	F: 5'- AAA CGG CTA CCA CAT CCA AG -3' R: 5'- CCT CCA ATG GAT CCT CGT TA -3'	155 bp

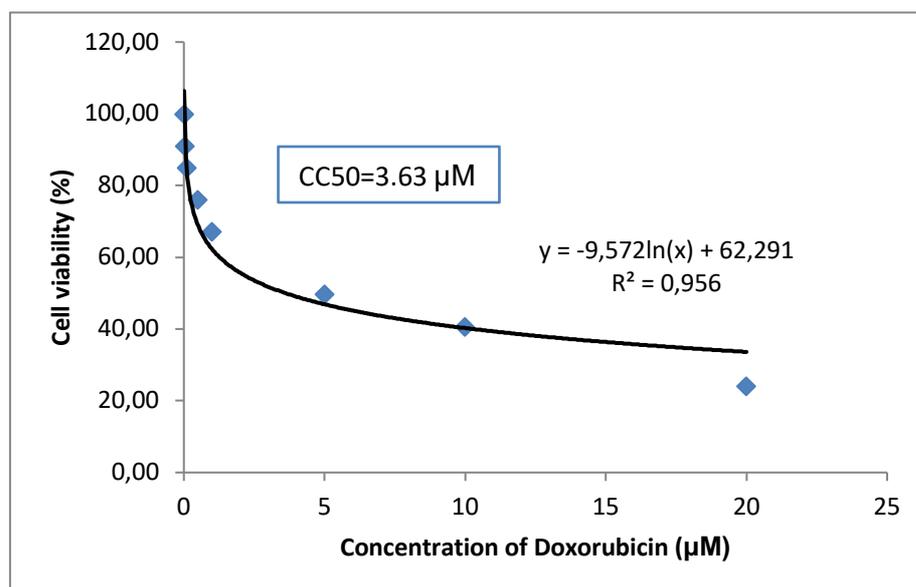


Figure 1. Cytotoxic assay of doxorubicin-treated BCSCs. MTS assay was performed to determined cytotoxicity of human BCSCs. About 1×10^3 human BCSCs (ALDH+) were plated triplicate in each well of 96-well plate and grown in non-serum DMEM/F12 medium supplemented with 1% penicillin/streptomycin and 1% amphotericin at 37°C and 5% CO₂. After 24-incubation, cells were treated with various concentrations of doxorubicin (0.1, 0.5, 1, 5, 10 and 20 µM) for 24 hours. As controls, cells were grown without doxorubicin under standard conditions for 24

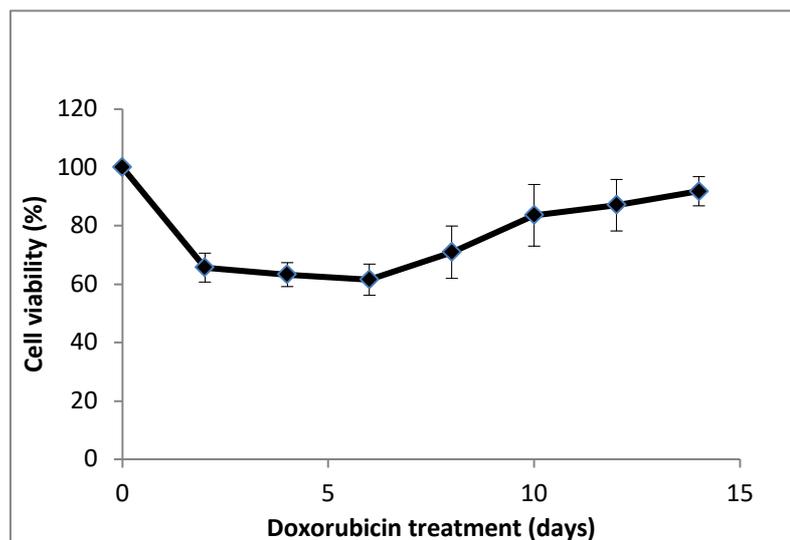


Figure 2. Viability of doxorubicin-treated BCSCs. Approximately 1×10^5 cells were plated in each well of 12 well-plate and incubated overnight. Afterwards, medium was replaced with fresh DMEM/F12 medium containing doxorubicin of $0.25 \mu\text{M}$ for 2, 4, 6, 8, 10, 12, and 14 days respectively. As controls, cells were grown in DMEM/F12 without doxorubicin for 2, 4, 6, 8, 10, 12, and 14 days respectively. After incubation, cells were harvested and counted using trypan blue exclusion assay and automated cell counter (Luna). The number of viable doxorubicin-treated cells was normalized to that of the respective control cells to calculate the percentage of cell viability. Significant differences are considered at $*p < 0.05$ (One-way ANOVA test) compared to cell viability after 2-day doxorubicin treatment. Significant differences compared to each respective controls are considered at $##p < 0.01$ and $\#p < 0.05$ (Student's t-test).

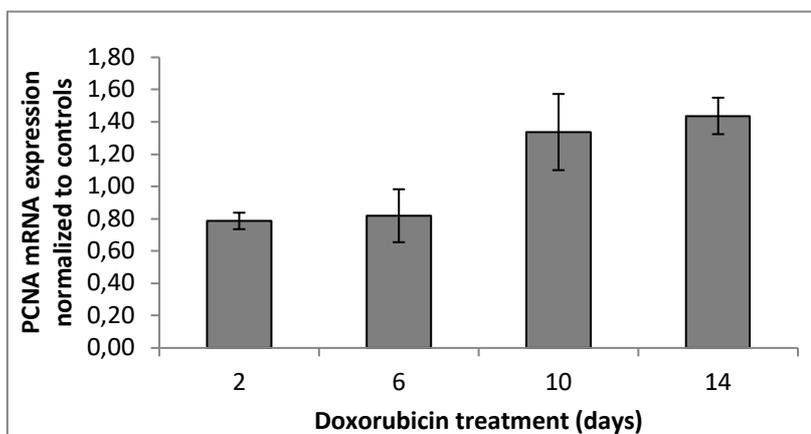


Figure 3. Relative expression of PCNA mRNA in doxorubicin-treated BCSCs. Human BCSCs (ALDH+) were treated with doxorubicin $0.25 \mu\text{M}$ and incubated for 2, 6, 10, and 14 days respectively. As controls, cells were grown without doxorubicin for 2, 6, 10, and 14 days respectively. After incubation, cells were harvested for total RNA isolation. Quantitative RT-PCR was performed using 100 ng total RNA to determine PCNA mRNA relative expression levels in human BCSCs, as described under Materials and Methods. The expression was relatively calculated using Livak formula with 18S rRNA gene as a reference gene and normalized to its respective control. All values are means \pm SE, $n = 6$. Significant differences are considered at $*p < 0.05$ (One-way ANOVA test) compared to 2-day doxorubicin treatment. Significant differences compared to each respective control are considered at $\#p < 0.05$ (t test).

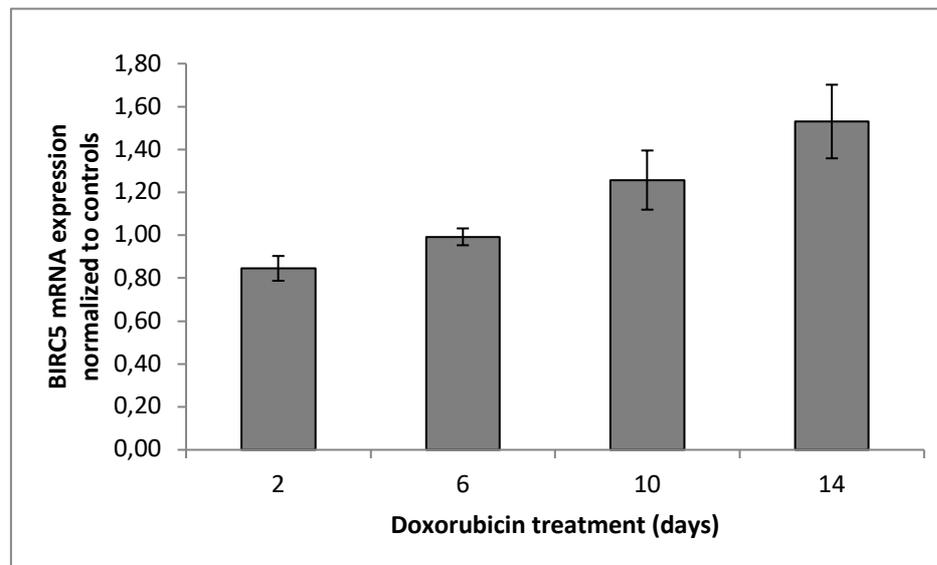


Figure 4. Relative expression of BIRC5 mRNA in doxorubicin-treated BCSCs. Human BCSCs (ALDH+) were treated with doxorubicin 0.25 μ M and incubated for 2, 6, 10, and 14 days respectively. As controls, cells were grown without doxorubicin for 2, 6, 10, and 14 days respectively. After incubation, cells were harvested for total RNA isolation. Quantitative RT-PCR was performed using 100 ng total RNA to determine BIRC5 mRNA relative expression levels in human BCSCs, as described under Materials and Methods. The expression was relatively calculated using Livak formula with 18S rRNA gene as a reference gene and normalized to its respective control. All values are means \pm SE, n = 6. Significant differences are considered at **p<0.01 and *p<0.05, (One-way ANOVA test) compared to 2-day doxorubicin treatment.

DISCUSSION

Breast cancer stem cells could be identified based on several surface antigen markers such as CD44 \square CD24 \square \square or ALDH1+.(13)-(14) High expression of ALDH1 is correlated with poor prognosis. Some studies reported that high number of BCSC was shown to be capable of tumorigenesis, metastasis, and drug resistance.(6):(15) This study was performed to analyse the role of PCNA and BIRC5 gene expression in response to repeated exposure of doxorubicin especially in ALDH1+ breast cancer stem cells.

Result of cell viability percentage shows the decreasing of the BCSC viability in the early of doxorubicin treatment (until day 6), suggesting that BCSCs were still sensitive to doxorubicin therapy. However, this study shows that the sensitivity of BCSC against doxorubicin began to decrease after 8-day doxorubicin treatment and reached 92% viability on day 14.

The present study indicated that the expressions of PCNA and BIRC5 were modulated in line with the modulation of human BCSC viability during doxorubicin treatment for 14 days. Both genes were expressed lower than control on day 2 and 6, but significantly higher on day 10 and 14 of doxorubicin treatment.

Therefore, we confirm that PCNA and BIRC5 gene expressions involved in the sensitivity of BCSC against doxorubicin by regulating the cell viability, which is consistent to our previous study using next generation sequencing.⁸

PCNA is a responsible factor for DNA replication and repair and contribute to cell proliferation. Cell growth/proliferation is a requirement for cancer progression, so PCNA plays important role in cancer progression at both primary and metastatic sites. PCNA phosphorylation is stimulated by epidermal growth factor (EGF). When EGF binds the receptor (EGFR), it will lead the PCNA phosphorylation on tyrosine residue 211 (pY211). The phosphorylated-PCNA (pY211-PCNA) was found frequently in human breast cancer, and the levels of pY211-PCNA were correlated with poor overall survival.(16) On the other hand, PCNA also acts as an antiapoptotic activity through interaction with proteins of the Gadd45 family (Gadd45, MyD118, and CR6), which are contributed in growth control, apoptosis, and DNA repair. PCNA interaction in these protein will inhibit their activities.(17) PCNA has also been widely used as a tumor marker, and its expression is correlated poor prognosis.(18)

BIRC5 (Survivin) is an inhibitor of apoptosis protein and is expressed in a lot of malignancies,

including breast cancer.(19) Overexpression of BIRC5 gene in cancer may overcome cell cycle checkpoints to facilitate aberrant progression of transformed cells through mitosis, suggesting the involvement of this gene in tumor aggressiveness and therapy resistance.(20) Its expression levels correlate with more aggressive disease and poor clinical outcome. Khan et al reported higher expression of BIRC5 as a critical factor for radioresistance in head and neck squamous cell carcinoma (HNSCC) cell lines.(21) A study reported that reducing the mRNA levels of BIRC5 to approximately 40%, results in a concomitant reduction of OCT4 and NANOG mRNA. It suggesting that BIRC5 plays a role in pluripotency.(22)

PCNA and BIRC5 are genes that involved in p53 signalling pathway. The p53-binding sites are present in PCNA gene promoter.(23) Induction of PCNA transcription is regulated by p53 protein in response to DNA damage (radiation or other stresses) to stimulate DNA repair. Low and moderate levels of p53 positively stimulate transcription of PCNA, while high levels of p53 inhibit PCNA expression.(24) BIRC5 is a target of p53 protein for its action and downregulation. P53 protein may induce apoptosis by antagonizing the anti-apoptotic activity of BIRC5. The promoter of BIRC5 gene has a p53 binding element, thereby it may give the possibility of p53 binding to BIRC5 promoter either alone or in combination with other protein to repress BIRC5 expression.(25) Based on our results, we suggest that BCSCs modulated PCNA and BIRC5 gene expressions that regulate cell viability via proliferation and anti-apoptosis mechanism in response to prolonged exposure to doxorubicin .

In the presen study, we conclude that the PCNA and BIRC5 expressions play an important role on BCSCs viability which associated with the sensitivity of doxorubicin treatment. Hence, PCNA and BIRC5 expressions should be considered as markers for determining chemotherapy response and sensitivity in particular to breast cancer stem cells.

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