

HDAC2 and PCNA expression is correlated to decreasing of endoxifen sensitivity in human breast cancer stem cells ALDH+

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Abstrak

Latar belakang: Sel punca kanker payudara (breast cancer stem cells/BCSC) adalah subpopulasi sel kanker yang memiliki kemampuan menghasilkan tumor baru dan bersifat seperti sel punca. Penelitian kami sebelumnya menggunakan jaringan kanker payudara mengungkapkan bahwa ekspresi gen histone deacetylase 2 (HDAC2) dan proliferasi sel nuklear antigen (PCNA) ditemukan perbedaan signifikan setelah terapi neoadjuvan hormon dan kemoterapi. Penelitian ini bertujuan untuk menganalisis hubungan antara ekspresi HDAC2 dan PCNA dengan kelangsungan hidup sel punca kanker payudara dengan penanda aldehyde dehydrogenase + (ALDH+) yang diberi perlakuan endoksifen.

Metode: Sampel adalah BCSC primer manusia ALDH+ yang diberi perlakuan endoksifen 4 μ M masing-masing selama 2, 4, 6, 8, 10, 12, 14 hari. Viabilitas sel dilihat dengan menggunakan trypan blue dan ekspresi mRNA HDAC2 dan PCNA ditentukan menggunakan qRT-PCR.

Hasil: Viabilitas BCSCs ALDH+ menurun setelah 2 sampai 4 hari pemberian endoksifen. Pada periode ini juga didapatkan ekspresi mRNA HDAC2 dan PCNA mengalami penurunan. Tetapi setelah pemberian endoksifen selama 8 hari, viabilitas BCSCs ALDH+ mengalami peningkatan dan ditemukan peningkatan yang signifikan pada hari ke-14 pemberian endoksifen. Ekspresi mRNA HDAC2 dan PCNA juga menunjukkan peningkatan mulai pada hari ke-8 dan terus meningkat hingga hari ke-14 pemberian endoksifen. Penelitian ini menunjukkan pola yang sama antara ekspresi mRNA HDAC2 dan PCNA dan viabilitas sel.

Kesimpulan: Induksi endoksifen yang lama menurunkan sensitivitas efek endoksifen pada BCSC manusia dan ekspresi HDAC2 dan PCNA berkorelasi dengan viabilitas BCSC manusia setelah induksi endoksifen. (*Health Science Journal of Indonesia 2019;10(2):77-81*)

Kata kunci: sel punca kanker payudara, viabilitas sel, HDAC2, PCNA, endoksifen

Abstract

Background: Breast cancer stem cells (BCSCs) are subpopulation of cancer cells that has the ability to generate new tumor and similar properties to stem cell. Our previous study using breast cancer patients revealed that gene expression of histone deacetylase 2 (HDAC2) and proliferating cell nuclear antigen (PCNA) were significantly altered after neoadjuvant hormone and chemotherapy. This study aimed to analyze the correlation between HDAC2 and PCNA expressions with the viability of breast cancer stem cells aldehyde dehydrogenase + (BCSC ALDH+) treated by endoxifen.

Method: Samples are human primary BCSCs ALDH+ that treated with 4 μ M of endoxifen for 2, 4, 6, 8, 10, 12, 14 days, respectively. Cell viability was measured using trypan blue exclusion assay and the mRNA expressions of HDAC2 and PCNA were determined using qRT-PCR.

Results: The viability of BCSCs ALDH+ was decreased after 2 days until 4 days-endoxifen treatment. It also demonstrated that mRNA expression of HDAC2 and PCNA were decreased in this period. But after 8 days-endoxifen treatment, the viability of BCSCs ALDH+ was increased. The increasing of viability was higher in 14 days-endoxifen treatment. The mRNA expression of HDAC2 and PCNA also showed increasing begin on 8 days and continued to increase until 14-days endoxifen treatment. We found a similar pattern between HDAC2 and PCNA expression and cell viability

Conclusion: Prolonged endoxifen treatment decrease sensitivity of endoxifen effect in human BCSC and the expression of HDAC2 and PCNA are correlated to human BCSCs viability after endoxifen treatment. (*Health Science Journal of Indonesia 2019;10(2):77-81*)

Keywords: human breast cancer stem cells, viability, HDAC2, PCNA, endoxifen

Epigenetic modifications play an important role in regulating biological processes. Histone deacetylation is one of epigenetic modifications that regulated by an enzyme family of histone deacetylases (HDACs). This enzyme will remove acetyl group from histones and resulting in a non-permissive chromatin conformation that suppress some genes transcription activities. Cancer is one of the diseases that could be affected by epigenetic alteration. Some studies reported that an increase in histone deacetylation causes increased cell proliferation, cell migration, angiogenesis and invasion by reducing transcription of tumor suppressor genes.^{1,2}

Proliferating cell nuclear antigen (PCNA) is known as a molecular marker for proliferation because of its role in DNA replication. This protein is found overexpressed in cancer cells that have high proliferation.³ PCNA has proven correlated to worse disease progression and prognosis in cancer.

Approximately 70-80% of breast cancer has overexpression of estrogen receptor (ER). Breast cancer with hormone receptor (ER) positive has a good response to anti-estrogen or aromatase inhibitor as first line drug. Endoxifen is one of hydroxylated tamoxifen metabolit (4-hydroxy-N-desmethyl-tamoxifen) and significantly more potent than tamoxifen in its ability to bind to ER, and in suppression of ER-dependent breast cancer proliferation.⁵

Breast cancer is a heterogenous disease which consists of various type of cells including cancer stem cells. Breast cancer stem cells are known as a minor population among breast cancer cells that have capability to self renew, promote tumor growth and differentiate into all cell types in a tumor.⁶ Our recent study investigated gene expression profiles of stem cell and p53 in advanced breast cancer using next generation sequencing has reported that expression HDAC2 and PCNA is significantly altered after neoadjuvant hormone and chemotherapy.^{7,8} This study aims to investigate the mRNA expression of HDAC2 and PCNA in human BCSC ALDH+ during endoxifen treatment and its correlation to cell viability.

METHODS

Samples are primary culture of human BCSCs ALDH+ which obtained from previous study that has isolated the cancer stem cells by ALDH1 marker (flowcytometer). The cells were grown in serum free medium DMEM F12 with 1% penicillin/streptomycin

and 1% amphotericin B and incubated in 5% CO₂ at 37°C.

Cytotoxic Assay

Approximately 10³ cells/well (96-microwell-plate) of human BCSCs ALDH+ were incubated in DMEM F12 with 5% CO₂ at 37°C and after 24 hours, the medium was replaced with 100 µL of fresh medium for control and fresh medium containing various concentration of endoxifen (0.1, 0.5, 1, 5, 10 and 20 µM) for treatment group. Endoxifen-treated cells were incubated in 24 hours and the relative viable cells number was determined by MTS assay method (Promega®). After 24 hours, the medium culture was replaced with 120 µL fresh medium containing MTS:PMS ratio=20:1 and incubated in 1-4 hours. After the brown colour appears, the absorbance was read at 490 nm using microplate reader (Varioskan®). The 50% cytotoxic concentration (CC50) was determined from the dose-response curve. We determined the endoxifen concentration for the cells treatment not higher than CC50 concentration.

Endoxifen treatment

Approximately 10⁵ human BCSCs ALDH+ were plated each well in 12 well-plate and incubated overnight. Medium was replaced by endoxifen-treated medium 4 µM (according to cytotoxic assay result) for treatment group and only complete medium DMEM F12 for the control group. Treatment was conducted during 2nd, 4th, 6 th, 8th, 10th, 12 th and 14th day and every 2 days the medium was refreshed and the cell number was counted both in endoxifen-treated and control group to determine the cell viability using trypan blue exclusion method. We collected the cells and isolated the total RNA using Tripure isolation reagent kit (Roche®) according to the manufacturer's instructions.

Quantitative RT-PCR

The concentration of RNA was measured using spectrofluorometer (Varioskan, Thermo Scientific). One-step RT-PCR was carried out using ECO48® real time qPCR system (PCRmax). The RT PCR was performed in 20 µL volume with a 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, 60°C for 30 seconds, 72°C for 30 seconds, and melting curve incubation. We used 18sRNA as housekeeping gene. The primer sequences for quantitative RT-PCR are shown in table 1. The relative expression is produced by comparing Ct value of treatment group to control group and it is calculated by Livak formula.⁹

Table 1. Primer sequences for HDAC2, PCNA and 18sRNA

No.	Gene	Primer Sequence	Product
1.	HDAC2	F: 5'- CCA TAA AGC CAC TGC CGA AG -3' R: 5'- CAC AGC TCC AGC AAC TGA AC -3'	199 bp
2.	PCNA	F: 5'- CTT CCC TTA CGC AAG TCT CAG -3' R: 5'- TTG AGT GCC TCC AAC ACC TT -3'	189 bp
3.	18sRNA	F: 5'- AAA CGG CTA CCA CAT CCA AG -3' R: 5'- CCT CCA ATG GAT CCT CGT TA -3'	155 bp

Ethical Declaration

This study has been approved by the Health Research Ethics Committee Faculty of Medicine Universitas Indonesia – Cipto Mangunkusumo Hospital number 390/H2.F1/ETIK/2014.

RESULTS

Cytotoxic Assay Analysis

The cytotoxicity of endoxifen on primary human breast cancer stem cells (ALDH⁺) was determined by calculation of CC50 using several endoxifen concentrations (0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 20 μ M). The curve of CC50 for endoxifen was created based on endoxifen concentration against the absorbance in log10 (figure 1). We got the CC50 of endoxifen on human breast cancer stem cells ALDH⁺ is 6 μ M. Based on the cytotoxic assay result and optimization trial, we determined the endoxifen concentration is 4 μ M for BCSC induction.

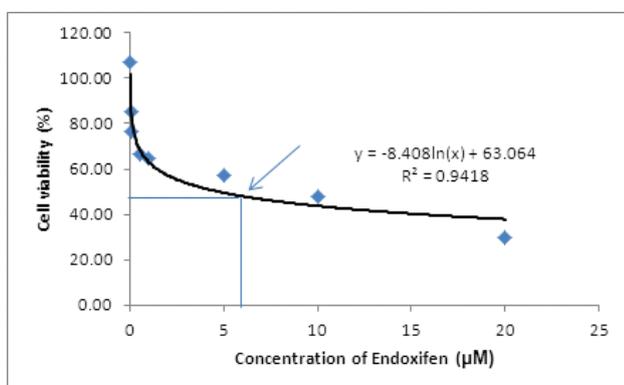


Figure 1. Cytotoxic concentration 50 of endoxifen treatment in human BCSC ALDH⁺. It shows the CC50 of endoxifen is 6 μ M.

Cell Viability

The number of cell viability was shown as the ratio (%) of live cell number in endoxifen group divided by live cell number in control group. Cell viability in endoxifen group appears to decrease in 2nd-day (51%) and persistent until 6th-day (58%), but the cell viability found significantly increased after 8th-day (82%) and more increased until 14th-day (99%) compare to control group (figure 2).

The mRNA expression of HDAC2 after endoxifen treatment

HDAC2 expression was decreased after 2 day-treatment of endoxifen (0.790) compare to control group and it still decreased in 6 day-treatment (0.502). The expression was significantly increased in 10 and 14 day-treatment (1.429 and 2.633) compare to 2 day-treatment groups (figure 3).

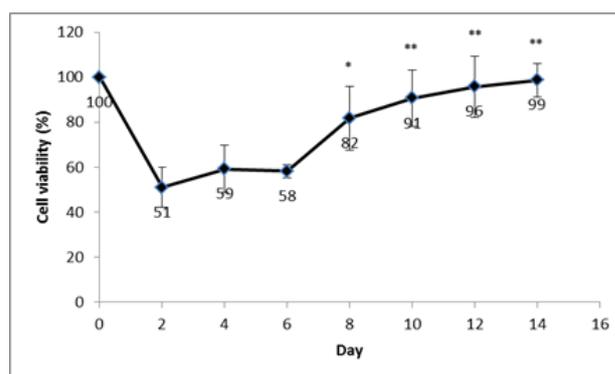


Figure 2. Cell viability of human BCSC ALDH⁺ treated by endoxifen 4 μ M. Cell viability is ratio (%) of live cells in endoxifen treatment group compare to live cells in control group during 14 days treatment. Significance compared to 2-day group (* p <0.05 and ** p <0.001).

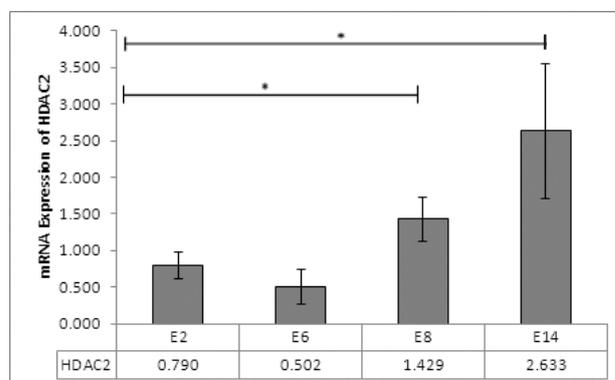


Figure 3. The mRNA relative expression of HDAC2 in human BCSC ALDH⁺ during 14 days treatment of endoxifen 4 μ M. Significance compared to 2-day treatment (E2) group (* p <0.05).

The mRNA expression of PCNA after endoxifen treatment

PCNA expression also decreased after 2 day-treatment of endoxifen (0.767) compare to control group and it still decreased in 6 day-treatment (0.447). The expression was significantly increased in 10 and 14 day-treatment (1.777 and 3.843) compare to 2 day-treatment groups (figure 4).

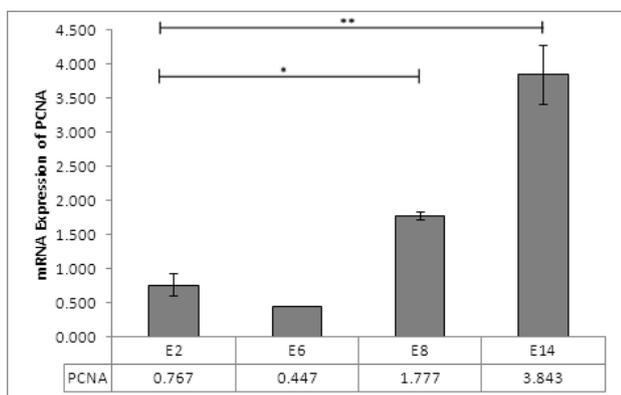


Figure 4. The mRNA relative expression of PCNA in human BCSC ALDH⁺ during 14 days treatment of endoxifen 4 μ M. Significance compared to 2-day treatment (E2) group (* $p < 0.05$ and ** $p < 0.001$).

DISCUSSION

According to the cytotoxic assay result, we got the CC50 of endoxifen on human breast cancer stem cells ALDH⁺ is 6 μ M. We also performed the optimization trial with several concentrations that lower than CC50. We determined the endoxifen concentration to BCSC treatment is 4 μ M, because among the below CC50 concentration, this concentration gave the best inhibition towards BCSC proliferation.

In this study, we demonstrated that the cell viability of human BCSCs was decreased after endoxifen treatment in the early period (2-day until 6-day treatment) however it would increase in late period (8-day until 14-day treatment). The mRNA expression of HDAC2 and PCNA also have the same pattern as cell viability after endoxifen treatment. It was decreased in the early period (2-day until 6-day treatment), but the expression would increase after 8-day until 14-day treatment. It showed us that the expression of HDAC2 and PCNA contributed to the human BCSCs cell viability.

Breast cancers are comprised of a highly heterogeneous population of cells, including the small population possess the ability to regenerate

tumors in vivo. Another study was proved that histone deacetylases (HDACs) play essential roles in the cancer stem cells phenotype.¹⁰ Histone deacetylation is one of genetic modification that regulated by HDACs. Histone deacetylases (HDACs) regulate the expression and activity of numerous proteins involved in cancer initiation and cancer progression. By removal of acetyl groups from histones, HDACs create a non-permissive chromatin conformation that prevents the transcription of genes that encode proteins involved in tumorigenesis. In addition to histones, HDACs also deacetylate a variety of other protein targets including transcription factors and other abundant cellular proteins involved in control of cell growth, differentiation and apoptosis.¹¹ There are eighteen isoenzymes of HDACs that have already known but only class 1 HDAC (HDAC 1, 2, 3 and 8) that reported involved in cancer.¹²

Endoxifen was proved to have better antiestrogen capacity compare to tamoxifen through ER α degradation and blocking ER activity.⁵ Some evidence suggests that ER α signaling has the potential to contribute to epigenetic alteration. Estrogen stimulation is shown to induce several histone modifications at the ER α target gene promoters such as acetylation, phosphorylation and methylation through interaction with histone modifying enzymes.¹³ One mechanism of drug resistance is overexpression of efflux transporters such as ATP binding cassette subfamily B member 1/ABCB1 (P-glycoprotein), where its expression is also found high in breast cancer stem cells.¹⁴

Proliferating cell nuclear antigen (PCNA) is a cofactor of DNA polymerases that coordinate several functions in the replication process. Some studies reported that HDAC1 interacts with PCNA and HDAC1 and 2 are associated with newly replicated DNA.¹⁵ Another study demonstrated that HDAC inhibitors inhibit cell cycle progression and kill cancer cells by triggering DNA damage during DNA replication.¹⁶

The increasing of cell viability after endoxifen treatment showed that the sensitivity of this treatment was decreased. It is showed by the viability in 8-day treatment was begin increased (figure 2). The decreasing of sensitivity treatment is early mechanism of drug resistance. In this study has revealed the high expression of HDAC2 and PCNA is associated with decreasing of endoxifen effect. It showed by the expression of HDAC2 and PCNA were begin increased in 8-day until 14-day treatment

(figure 3 and 4). Other study reported that HDAC2 overexpression correlated with the metastasis, progression and the increased Ki67, multidrug resistance protein expression in breast cancer.¹⁷ HDAC2 will remove acetyl groups from histones and creates a closed chromatin structure that prevents the transcription of genes involved in growth arrest, differentiation, and apoptosis.¹¹ The expression of PCNA is correlated with high cytological grading and poor prognosis in renal carcinoma.¹⁸ PCNA enhances the processivity of DNA polymerase ϵ which conducted DNA replication. Beside DNA synthesis, DNA polymerase ϵ is involved in DNA damage revision, so it will increase the ability of cancer cells to avoid apoptosis.¹⁹

In conclusion, prolonged endoxifen therapy can cause decreasing in endoxifen effect which can lead to resistance. The increasing of HDAC2 and PCNA expressions correlated to the decreasing of endoxifen effect in human BCSC that showed by the increase of cell viability in late group of endoxifen treatment.

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