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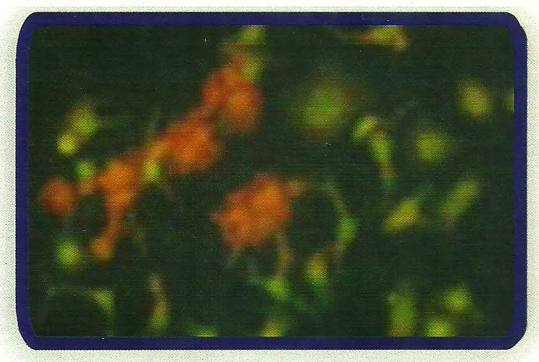

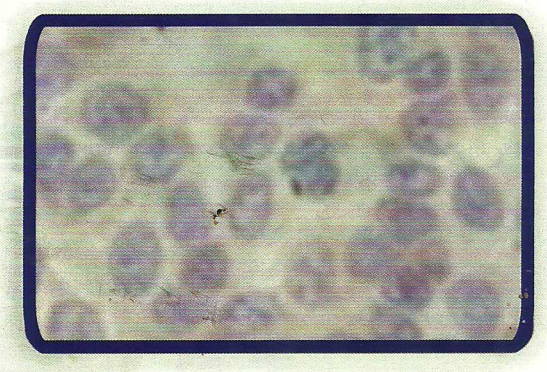

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MCF-7 Resistant doxorubicin are characterized by lamelapodia, strong adhesion on substrate and P-gp overexpression

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Abstract

The prognosis of breast cancer patients is closely associated with the response of tumor cells to chemotherapy agent. Doxorubicin is one of the primary chemotherapeutic agents used for the treatment of breast cancer. Resistance to chemotherapy is believed to cause treatment failure in cancer patients. Furthermore, long time exposure to chemotherapeutic agent induces cancer cells resistance. MCF-7 sensitive cells used as chemoresistance model have overexpression P-gp (P-glycoprotein). Chemoresistance was established by treating MCF-7 cells with 0.5 µg/ml doxorubicin-contained medium for a week. 50% inhibiting concentration (IC_{50}) doxorubicin on MCF-7 cells/DOX were determined using MTT assay. Western blot assay and immunocytochemistry assay was performed to determine the expression of P-gp. Morphological of MCF-7 cell/DOX was changing to become larger and have lamellapodia. IC_{50} value of doxorubicin was 700 nM on MCF-7/DOX and 400 nM on sensitive MCF-7 cells. The MCF-7/DOX sensitivity to doxorubicin was decreased, shown by 1.5 fold higher IC_{50} of doxorubicin on MCF-7/DOX compared to MCF-7 sensitive cells. Treatment doxorubicin to sensitive MCF-7 cells leads to the increasing P-gp expression. The P-gp level expression has strong correlation with the low sensitivity of MCF-7/DOX to doxorubicin.

Key words: doxorubicin, resistance cells, sensitive MCF-7 cell

INTRODUCTION

Protein transporter, such as P-glycoprotein plays a pivotal role in developing of cancer cells resistance. The ABC (ATP Binding Cassete) superfamily includes ± 300 protein as transporters of different compound. Protein of ABC family is a type of adenosine triphosphatase (ATPase) and an energy-dependent trans membrane drug efflux (Loo *et al.*, 2005). These ABC family includes The ABC family ± 300 protein as transporters of different compound (Higgins, 2007) and divided into 7 sub families (Dean, 2001). One of them is MDR (Multi Drug Resistance). P-glycoprotein (P-gp) is a identified ATP-binding cassette transporter, correlated to *multidrug resistant* (*MDR-1*) gene expression (Khrishna *et al.*, 2000), and shown to be an important anticancer agents and play an important function in governing drug disposition (Gottesman *et al*, 2001). P-gp effluxes chemotherapeutic agent extracellular via ATP hydrolysis

Doxorubicin, a chemotherapeutic agent frequently used for the treatment of several cancers, is widely used for the treatment of breast cancer. However, long treatment of doxorubicin induces cardiotoxicity and cancer resistance (Gandhi *et al.*, 2007). Doxorubicin is a P-gp substrate (Coley, 2009). Doxorubin is effluxed by P-gp (Michor *et al.*, 2006), resulting in decrease concentration of doxorubicin intracellular. This mechanism mediates the cancer cell resistance to doxorubicin.

This study was aimed to develop MCF-7 breast cancer cell line resistant to doxorubicin (MCF-7/DOX). The result, then, will be used in researches to develop chemopreventive agent targeting resistant cells. Thus, we need to determine the characteristic of MCF-7/DOX resulted from this study.

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METHODS

Chemicals

Doxorubicin (10mg/5 ml, Ebewe), *universal detection kit* (streptavidin-HRP, second antibody biotinilasi, horse radish serum/*blocking serum*) (*Ultravision plus detection system*, Ref TP 125-HLX, Runcorn, Cheshire, WA71PR, UK; Novostain *Universal Detection kit* NCL-RTU, Novocastra Lab Ltd., Newcastle NE12 8EW, UK).

Cell Lines and drug treatment

MCF-7 cells were obtained from the collection of Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada. The cell line was kindly gift from Prof. Kawaichi, *Nara Institute of Science and Technology* (NAIST), Japan. The resistant cells were originated by growing initial MCF-7 cells with doxorubicin concentration of 0.5 µg/ml. Doxorubicin was added every day for a week. Then, the cells were maintained with 0.1 µg/ml doxorubicin and fresh medium alternately. Check the IC_{50} to ensure that the resistancy . MCF-7 sensitive cells and MCF-7/DOX cells were grown in suspension using Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco), 10,000 units/ml penicillin-10,000 µg/ml streptomycin (Gibco) at 37^{0}C in humidified 5% CO_2.

Cytotoxicity assay

MTT cytotoxicity assay was used to examine the effect of doxorubicin on MCF-7 sensitive cells and MCF-7/DOX cells. MCF-7 sensitive cells and MCF-7/DOX cells were distributed into 96-well plate with the density of $1x10^4$ cells/well, then were incubated in 37°C with 5% CO_2 for 24 hours. Doxorubicin was applied with the concentration of 1, 10, 50, 100, 250, 500, and 1.000 nM. After 24 hours incubation, culture medium was removed followed by PBS washing. Then, 3-[4,5-dimethyl thiazole-2-yl(-2,5- diphenyltetrazoliumbromide)] (MTT) 0.5 mg/ml in PBS was applied, followed by 4 hours incubation in 37°C with 5% CO_2. SDS 10%, in HCl 0.1N as stopper reagent was then applied. Plate was then kept with protection from light overnight, continued with absorbance determination (λ 595 nm) using ELISA reader (Bio-Rad).

Western blot

Cells were harvested, washed with PBS, and lysate for 30 minutes on ice using lysis buffer (20nM Tris HCl, pH 8.0, 5nM EDTA, 1%NP 40, 25 mM NaCl and complete inhibitors of protease. The protein concentration was determined using Bradford assay. After electrophoresis, the proteun was transferred to PVDV membrane. The PVDV membrane was blocked with 5 % skim milk in PBS at room temperature for 1 hour. The membrane then washed with PBS, and incubated with antibody monoclonal *purified mouse anti-human anti*-Pgp (AbCam) 1:50 for 1 hour, the levels of protein were analyzed by enhanced chemiluminescene with an ECL plus Western blotting detection (Amersham, USA).

Immunocytochemistry

Coverslips (Iwaki) were placed in 24-well plate (Iwaki). Then MCF-7 sensitive cells and MCF-7/DOX were seeded ($5x10^4$ cells/well). After 24 hours incubation, cells were treated with doxorubicin for 18 h. Culture medium was removed and cells were washed in PBS, fixed using cold methanol and added with H_2O_2 blocking solution. Cells were added with prediluted blocking and incubated with monoclonal antibody monoclonal *purified mouse anti-human anti*-Pgp (AbCam) 1:100 overnight. Then, cells incubated with biotinylated universal secondary antibody, streptavidin-peroxidase and stained with substrate solution DAB. Cells were counterstained with Mayer Haematoxylin. Coverslips, then, were fixed with ethanol and xylol and moved onto object-glass. After that, mounting media were added and coverslips were covered by other coverslips. Protein expression was observed using light microscope (Nikon YS100). Cells with positive P-gp expression appear in brown/dark color, while cells with negative protein expression appear in blue/violet color. Immunocytochemistry analysis used provided evidence for localization of P-gp.

Analysis

Cytotoxicity assay Linear regression between concentration and percentage of cell viability giving the equation $y = Bx + A$ were used to calculate IC_{50} value, the concentration that inhibits 50% cell proliferation.

Statistical analysis. Statistical analysis was done using SPSS 16 software. T-test was used to evaluate the significance of the differences

between groups. $p>0.05$ was considered as the significant difference.

RESULTS

Morphological on MCF-7/DOX

Doxorubicin with concentration of 0.5µg/ml exposed to MCF-7 sensitive cells for a week lead to morphological changing (Figure 1). The MCF-7/DOX cells were found to be larger than MCF-7 sensitive cells. It was also observed that MCF-7/DOX have a lamellapodia and filopodia. The MCF-7/DOX cells were attached tightly to the surface compared to MCF-7 sensitive cells.

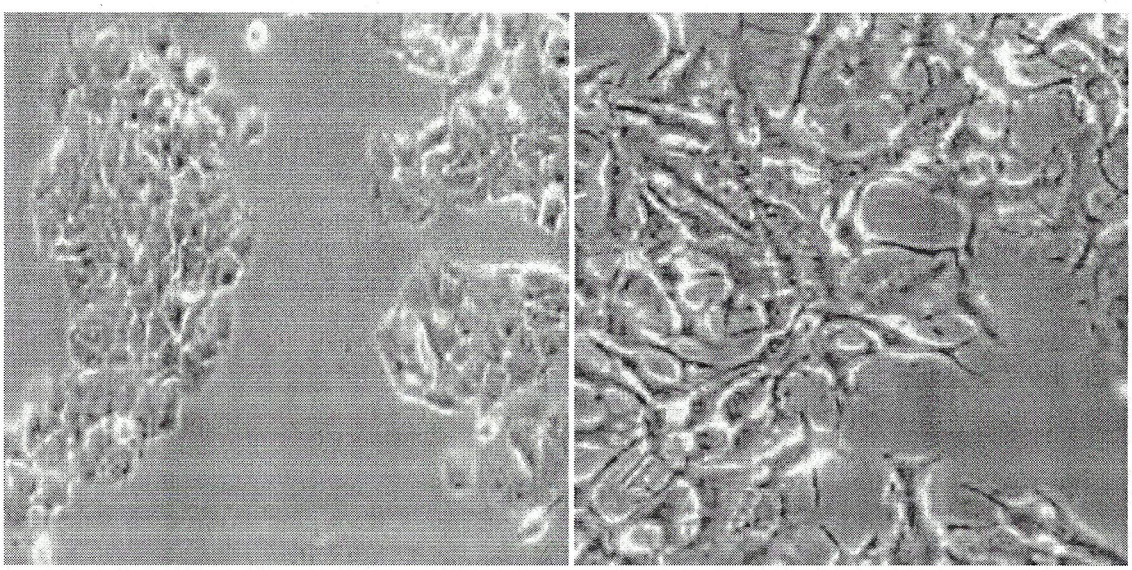
Doxorubicin – induced resistant on MCF-7 sensitive cells.

Cancer cell resistant to chemotherapeutic agent was signed to reduce sensitivity. The alteration of doxorubicin sensitivity on MCF-7/DOX cells, exposed to doxorubicin in sub toxic concentration for a week, was compared to MCF-7 sensitive cells. The cell viability was determined by MTT assay. IC_{50} value of doxorubicin on sensitive MCF-7 cells and MCF-7/DOX cells were 400 nM and 700 nM, respectively (Figure 2). The sensitivity of MCF-7/DOX to doxorubicin were decrease to 1.5 fold compared to MCF-7 sensitive cells.

Expression of P-gp on MCF-7 sensitive cells and MCF-7/DOX

To clarify whether the induction of P-gp expression on MCF-7 cells resistance by doxorubicin 0.5 µg/ml for a week, an western blot assay for whole protein samples extracted from MCF-7 sensitive cells and MCF-7/DOX cells was carried out. As shown in figure 3, treated by doxorubicin increased level of P-gp. On MCF-7/DOX cells showed higher expression of P-gp than MCF-7 sensitive cells.

MCF-7 sensitive cells expressed relatively undetected Pgp, while MCF-7/DOX were obviously overexpressed Pgp in their cell membrane. All these results, level expression of P-gp and site of P-gp in membran cells, corelation with decreasing sensitivity of doxorubicin on MCF-7/DOX.



(A) (B)

Figure 1. MCF-7 sensitive cells (A) and MCF-7/DOX (B) cells' morphology (300x magnifications). This phenomenon showed morphological changes on MCF-7/DOX after doxorubicin exposure for a week.

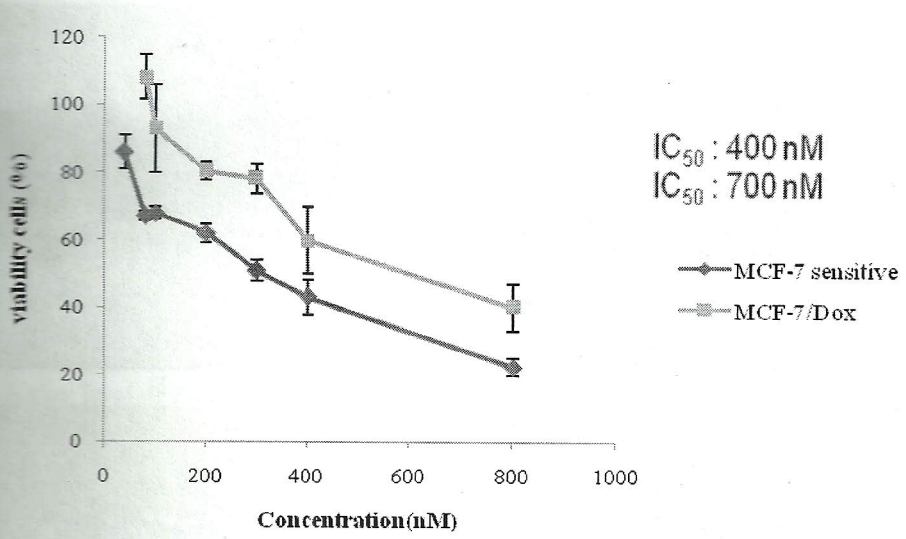


Figure 2. **The cytotoxic effect of doxorubicin on MCF-7 sensitive cells and MCF-7/DOX cells, showing a reduce sensitivity of doxorubicin on MCF-7/DOX cells. Giving IC_{50} value of 400 nM on MCF-7 sensitive and 700 nM on MCF-7/DOX cells.**

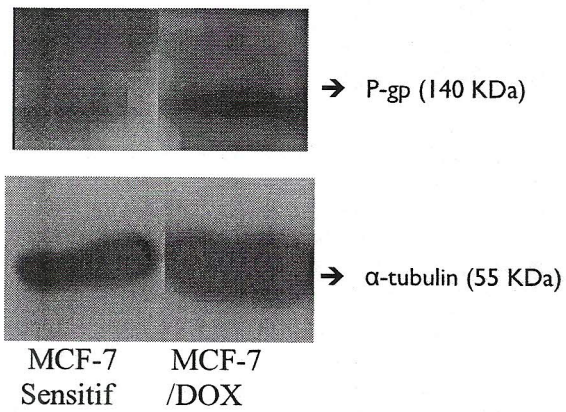


Figure 3. **The effect of expression of P-gp by doxorubicin exposure 0.5 µg/ml doxorubicin for a week on MCF-7 sensitive cells. Doxorubicin induce expression of P-gp was detected by western blotting on MCF-7/DOX.**

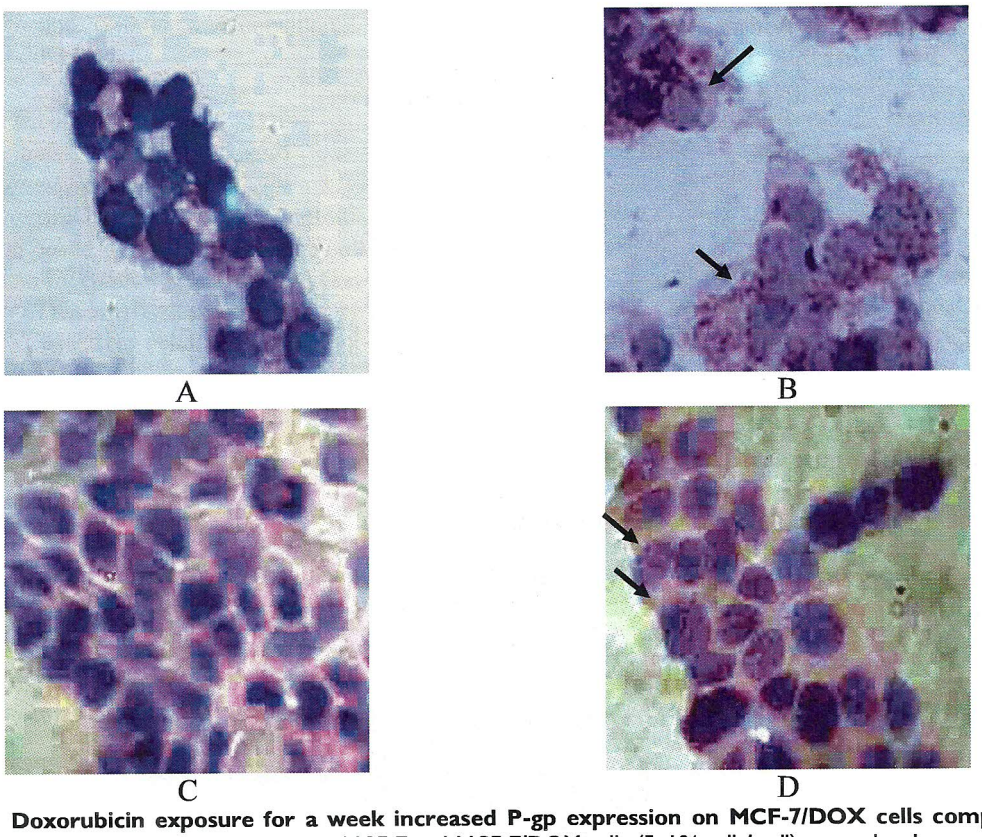


Figure 3. Doxorubicin exposure for a week increased P-gp expression on MCF-7/DOX cells compared to MCF-7 sensitive cells. Sensitive MCF-7 and MCF-7/DOX cells ($5x10^4$ cells/well) were plated on coverslips in 24-well plate and 24 h after plating, the cells were treated with agents for 20 h. (A). MCF-7 sensitive cells without staining antibody anti P-gp,(B). MCF-7 sensitive cells staining antibody anti P-gp (C). MCF-7/DOX cells without staining antibody anti P-gp, (D). MCF-7/DOX cells staining anti P-gp. Original magnification was 400x. Cells expressed P-gp showed brown color in membrane.

DISCUSSION

Prolonged exposure of chemotherapeutic agent on cancer cells leads to decreased sensitivity to it. This research was conducted to develop doxorubicin resistance MCF-7 (MCF-7/DOX). The results, then, will be used in other research to develop chemoprevention agents that target resistant cells. Thus, we need to determine the characteristic of MCF-7/DOX resulted from this study.

After treatment with doxorubicin as mentioned in material and methods, MCF-7/DOX sensitivity to doxorubicin were decreased. MCF-7/DOX were 1.5 fold more resistant to the cytotoxic effect of doxorubicin compared to MCF-7 sensitive cells. The sensitivity of MCF-7/DOX to doxorubicin was lower than of MCF-7/DOX developed by Lukyanova *et al.* (2009). Lukyanova *et al.* (2009) was successes in developing 16 fold resistant to doxorubicin compared to the initial MCF-7 cells. This difference may be due to the method divergence in resistance development. Lukyanova *et* al., (2009) induced MCF-7 resistant cells using doxorubicin with 0.1 to 32 µg/ml doxorubicin and test the IC_{50} every two months value until cells were 16 fold more resistant to the cytotoxic effect doxorubicin as compared with the initial MCF-7 cells, while we only used 0.5 µg/ml doxorubicin for a week. Thus, the sensitivity of MCF-7/DOX developed in this study was different to MCF-7/DOX developed by Lukyanova *et al.*

MCF-7/DOX cells were strongly attached to the underlying substrate, as described by Lukyanova *et al.* (2009). The strongly adhesion might be caused by overexpression of the number of microtubules. Besides the strong attachment to the substrate, MCF-7/DOX cells showed to have bigger lamellapodia and filopodia compared to the MCF-7 sensitive cells. These changes were also observed by Lukyanova *et al.* (2009). The morphological changes on MCF-7/DOX are probably due to the Rac-1 activity. Rac-1 induces the lamellapodia and filopodia formation compiling the cell cytoskeleton. Acivation of Rac-1 in response to extracellular signaling will lead to Pak1 acitvation. Pak1

phosphorylates the myosin light chain, protein involved in cell motility. Pak1 also depolymerizes F-actin through LIM kinase and cofilin (Yang *et al.*, 1998), resulting in lamellipodia and filopodia outgrowth. Ruffling lamellopodia is associated with the creation of new substrate contact (Rottner *et al.*, 1999), resulting in stronger adhesion to the underlying surface (Lukyanova *et al.*, 2009). These possibilities should be explored more.

The level of Pgp overexpression was confirmed by western blot assay. The MCF-7/DOX gave higher level of P-gp expression than MCF-7 sensitive cells. This finding indicated that treatment 0.5 µg/ml doxorubicin for a week induce P-gp overexpression. P-gp is an intergral plasma membrane protein and to be a major contributor to drug resistance (Kano *et al.*, 2011). And based on immunocytochemisty assay, P-gp has been localized in membrane cells to do its fuction as substrate transporter. The Western blot and immunocytochemisty' results were correlated with IC_{50} value of doxorubicin on MCF-7/DOX. That mean, treatement doxorubicin 0.5 µg/ml for a week can be used as breast cancer resistant to doxorubicin. Further study on resistant induction of doxorubicin and localization P-gp on plasma membrane need to be conducted in order to know more about its molecular mechanism.

CONCLUSION

From this study, exposure of doxorubicin 0.5 µg/ml (sub toxic concentration) for a week enhances P-gp expression correlated with the low sensitivity of doxorubicin on MCF-7/DOX.

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