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Resistance to doxorubicin correlated with dysregulation of microRNA-451 and P-glyoprotein, caspase 3, estrogen receptor on breast cancer cell line

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ABSTRACT

Doxorubicin (Dox) has been used widely in breast cancer therapy. One of the problems in chemotherapy is the development of resistance to chemotherapy that lead to metastasis and relapse aggressiveness of cancer. MicroRNAs (miRNAs) are small non-coding RNA that regulate protein expression and play role in carcinogenesis, as well as cancer chemotherapy resistance. miR-451 is classified as tumour suppressor miRNA, that binds to messenger RNA (mRNA) of MDR1, and leads disruption of P-glycoprotein (Pgp) expression. The study aimed to investigate the association between miR-451 and Pgp related with Dox resistance mechanism. *In silico* analysis was conducted to predict the binding affinity between miR-451 and mRNA of MDR1. The MCF-7 cell line was used as wild type model, while MCF-7/Dox was used as a model of resistance. qPCR was conducted to calculate miR-451 expression and immunocytochemistry was used to observe Pgp expression. miRNA was down-regulated in both on MCF-7 and MCF-7/Dox. On the other hand, Pgp expression was detectable in the cytoplasmic and cytoplasmic membrane in MCF-7/Dox. The Pgp expression was higher in the MCF-7/Dox compared to MCF-7. In conlusion, the over expression of Pgp is associated with the resistance to MCF-7/Dox.

ABSTRAK

Doxorubisin (Dox) adalah salah satu kemoterapi yang telah digunakan secara klinis dalam terapi kanker payudara. Salah satu masalah dalam kemoterapi adalah perkembangan resistensi terhadap kemoterapi yang akan mengarah pada metastasis dan kekambuhan agresivitas kanker. MicroRNAs (miRNAs) merupakan untai RNA kecil yang dapat mengatur ekspresi protein dan berperan dalam karsinogenesis, serta resistensi obat kemoterapi. miR-451 diklasifikasikan sebagai miRNA penekan tumor, yang berikatan dengan *messenger RNA* (mRNA) MDR1 yang, dan menyebabkan gangguan ekspresi P-glycoprotein (Pgp). Tujuan dari penelitian ini adalah untuk memahami hubungan antara miR-451 dan Pgp terkait dengan mekanisme resistensi Dox. Analisis *in silico* dilakukan untuk memprediksi afinitas pengikatan antara miR-451 dan mRNA MDR1. Sel line MCF-7 digunakan sebagai *wild-type* sedangkan MCF-7/Dox, digunakan sebagai model resistensi. Kuantifikasi ekspresi miR-451 diukur menggunakan qPCR dan imunositokimia digunakan untuk mengamati ekspresi Pgp. miRNA-451 ditemukan mengalami penurunan ekspresi baik pada sel MCF-7 dan MCF-7/Dox. Dalam penelitian ini, Pgp ditemukan terekspresi dalam membran sitoplasma dan sitoplasma sel MCF-7/Dox dan MCF-7. Ekspresi PgP ditemukan terekspresi lebih tinggi pada MCF-7/Dox dibandingkan dengan MCF-7 *wild-type*. Dapat disimpulkan, tingkat ekspresi Pgp ini berkaitan dengan resistensi MCF-7/Dox.

Keywords:
doxorubisin
microRNA-145
resistance
MCF-7
MCF-7/Dox

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INTRODUCTION

Cancer is a genetic disease as result of the accumulation of genetic alterations which causeless regulation in the cell and the cellular environment.[1,2] The certain genotype and phenotype complexity in cancer furthermore can be utilized as tumour biomarker which useful for tumour diagnosis, prognosis, and risk prediction of relapse.[3] The high incidence and mortality in breast cancer patients are caused by the difficulty of early diagnosis and the low level of successful treatment which also lead to high risk of the side effect of therapies.[1]

Doxorubicin (Dox) is one chemotherapy used clinically in cancer.[4-7] Resistance to chemotherapy, such as Dox, causes tumour progressiveness by forming certain adaptation to the environment, both in tumour genotype and phenotype. This condition can encourage massive cellular proliferation and escape from anticancer drug treatment.[5,8] One of the causes of the resistance to chemotherapy in cancer is related to ATP-binding cassette transporter (ABC-transporter), a drug transporter. MDR1 gene express Pgp which has a function in efflux pump mechanism in drug resistance.[9-11]

MicroRNAs (miRNAs) are 18-24 long nucleotide that play an important role in protein expression regulation by binding to the messenger RNA (mRNA) of genes which led to post-transcriptional inhibition. Several studies indicate expression related to miRNA in cases of resistance to cancer.[9,11] In the previous study, microRNA 451 (miR-451) has been identified as negative post-transcriptional regulator of MDR-1 and Pgp so that it could lead to the proliferation inhibition and apoptosis in some breast cancer cell lines.[12]

Regulation of miRNA in drug mechanism associated with resistance can be used potentially as a biomarker for resistance.[3,13] miRNA profiling analysis in the cancer cells is a potential method to know the correlation of miRN as to various phenotypic complexity in particular cancer cells. In this study, we investigated the mechanism of resistance to Dox in the cancer cells. We developed Dox resistance cell line in MCF-7 breast cancer cell line as a cell model to investigate the expression of miR-451 and Pgp protein which expressed by MDR1 gene.

MATERIALS AND METHODS

In silico binding analysis of miR-451 to mRNA MDR1 (ABCB1)

In silico bioinformatics analysis method was used to analyze the binding. Sequence database was obtained from miRBase (http://www/.mirbase.org), while sequence mRNA target was obtained from Genebank (http://www.ncbi/genbank). Interaction analysis was conducted through UTRdb database software online (http://utrdb.ba.itb.cnr.it/) and UTRscan (http://itbtolls.ba.itb.cnr.it/utrscan/). RNAhybrid free online software (http://bibiserv.techak.uni-bielefeld.de/rnahybrid/) was used to calculate the minimum free energy of binding sequence miRNA-mRNA.

MCF-7 cell resistance Dox (MCF/Dox) induction

MCF-7 cell line was obtained from Stem Cell and Cancer Institute, Jakarta, Indonesia. The cell was cultured in Dulbecco modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and incubated in 5% CO_2 at 37°C.

The resistance cell line was produced by the stepwise increased dose of Dox treatment to the cell every 2 or 3 days. IC_{50} of MCF-7 wild-type was determined first as a basis concentration of Dox concentration which would be administrated to the cells. Then, 1, 2, and 5% from IC_{50} Dox concentration were

gradually administrated, respectively.

MTT assay

Cytotoxic test was conducted by MTT assay method using MCF-7 wild type and MCF-7/Dox to determine the IC_{50} which would be used to determine the level of resistance of MCF-7/Dox cells compared to MCF-7 *wild-type* cells. MCF-7 line cells were grown on a 96-well plate and incubated for 24 h at 37^{0}C and 5% CO_2. The media was removed from the well plate, and the prepared test solution were put into the well (100 µL/well) then incubated again for 24 h at 37^{0}C and 5% CO_2. The test solution was removed and the well plate was filled with 100 µL MTT 0.5 mg/mL. After incubation for 4 h, add 100 µL stop solution (SDS 10%, and 0.01 M HCl) to dissolve formazan crystals and incubate again for 18 h at room temperature in the absence of light. The absorbance of each was measured at a 550-600 nm wavelength using micro plate reader (Bio-Rad Model 680 XR).

Expression of ER, caspase 3 and Pgp.

The expression level of Pgp, ER and caspase 3 were determined using immunohistochemistry. Immunohistochemistry was conducted using primary antibody anti-human monoclonal antibodies Dako Envision System™ (USA) for ER antibody, caspase 3 and Pgp. Secondary antibody, labelled with biotin (Biotinylated Universal Secondary Antibody) Trek-Avidin HRP marker, was also used according to the manufacturer's protocols.

Expression of miR-451.

Isolataion of total RNA of wildtype MCF-7 and MCF-7/Dox was conducted using miRCURY™ RNA Isolation Kit – Cell & Plant (Exiqon Inc, USA). The RNA was then subjected to cDNA synthesis using miRCURY LNA Universal RT microRNA PCR (Exiqon Inc, USA). The both protocols were conducted according to the manufacturer's protocols.

Quantitative real-time PCR had been done using miRCURY SYBR Green Master Mix (Exiqon Inc, USA) and hsa-miR-451 primer-specific LNA (locked nucleic acid) according to the manufacturer's protocols. The results of the qPCR were then analyzed using GeneEx 6 Multi-D software. The relative expression was calculated using the Livak method by comparing the cycle quantification value (Cq) of wildtype MCF-7 or MCF-7/Dox.

RESULTS

miR-451 targets mRNA MDR1 (ABCB1).

RNA hybrids software was used to investigate the interaction between miR-145 and mRNA of MDR1. FIGURE 1 shows the binding position of miR-451 to the 3'UTR coding region of MDR1's mRNA. The binding locations were on base pair 4246 to 4266 and 4334 to 4718 with minimum free energy of the binding was -6.10 kJ/mol

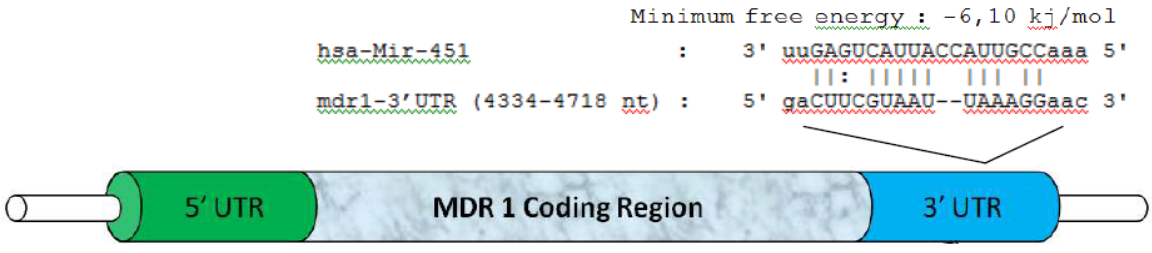

FIGURE 1. miR-451-mRNA MDR1 interaction prediction through in-silico analysis.

Dox resistance MCF7 cells

The gradual concentration of Dox was administrated to the MCF-7 cell line at given period for making the Dox resistance MCF-7 cells. The detail of Dox treatment to the cells was shown in TABLE 1, while the MCF-7 parental induction resistance proses to get MCF-7/Dox is shown in FIFURE 2.

TABLE 1. Resistance induction proses on MCF7 parental to get MCF7/Dox. Freezing process and subculture was done routinely before treatment.

Treatment description	Time (week)								
	1	2	3	4	5	6	7	8	9
Cell viabiliy (%)	75	25	50	75	30	45	60	75	10
Treatment	1%	Rec	Rec	2%	Rec	Rec	Rec	5%	X
		IC_{50}			IC_{50}			IC_{50}	

Note: Rec = recovery; X = cell death

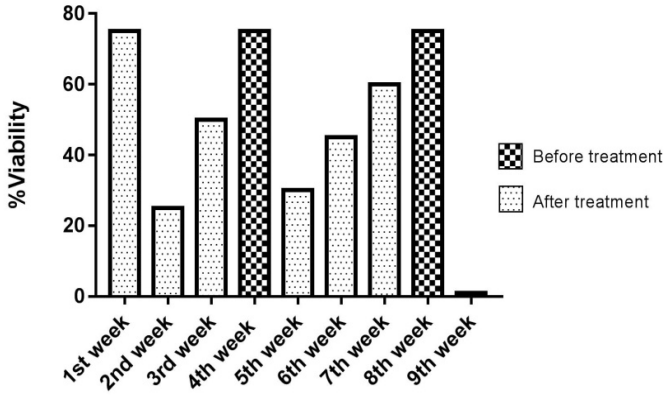

FIGURE 2. MCF-7 parental induction resistance process to get MCF-7/Dox

Originally, the *wild-type* MCF7 was epithelial like cells, but after the exposure of Dox, the morphology of cell was changed (FIGURE 3A and B). The change of the morphological stucture of the lamellipodia in the cytoplasm component was observed in the Dox-treated cells after 5 weeks of the Dox treatment (FIGURE 3B). The change was as a form of adaptation to the cell adhesion due to the effect of Dox treatment.

The resistance of MCF-7/Dox was proven as presented the change of the IC_{50} value after 5 weeks Dox treatment. The IC_{50} of MCF-7/Dox increased 7.5 fold higher (IC_{50}: 15.160 µg/mL) compared with that MCF-7 wildtype (2.017 µg/mL).

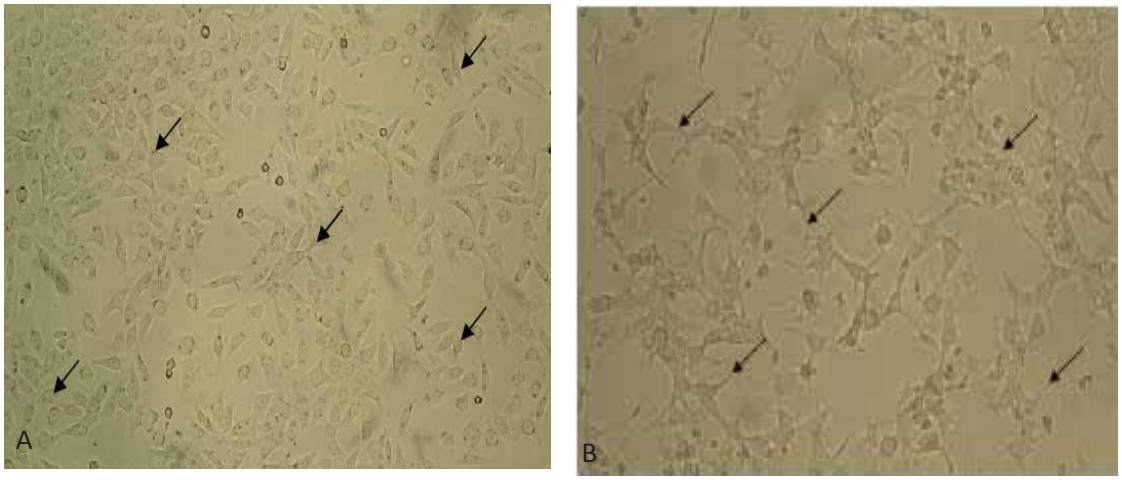

FIGURE 3. A. Parental cell of MCF-7 wildtype (100x); B.MCF-7/Dox treatment periodically for 5 weeks. Arrow marker showed lamellipodia (100x)

Expression of Pgp, caspase 3, estrogen receptor level

Immunohistochemistry assay was conducted to observe the Pgp, caspase 3, and estrogen receptor expressions levels. The labelled binding between antibody and antigen produced brown colour on the cytoplasm of the cells indicating that the cell was Pgp positive cell. The observation showed that the expression level of Pgp positive cell on MCF-7/Dox was higher than MCF-7 wild type.

Caspase 3 expression observation using immunohistochemistry was done to characterize the MCF-7 cell line. The brown colour on the nucleus of the cell was the indicator of the expression of the ER. based on analysis of level expression of targets protein semi-quantitatively through image quantificationby using Image J software, by the total amount of 27-29 cell field of view, the significance level expression of Pgp and ERof 9.191% and 9.557%, respectively from a field of view, which can observe in FIGURE 4.

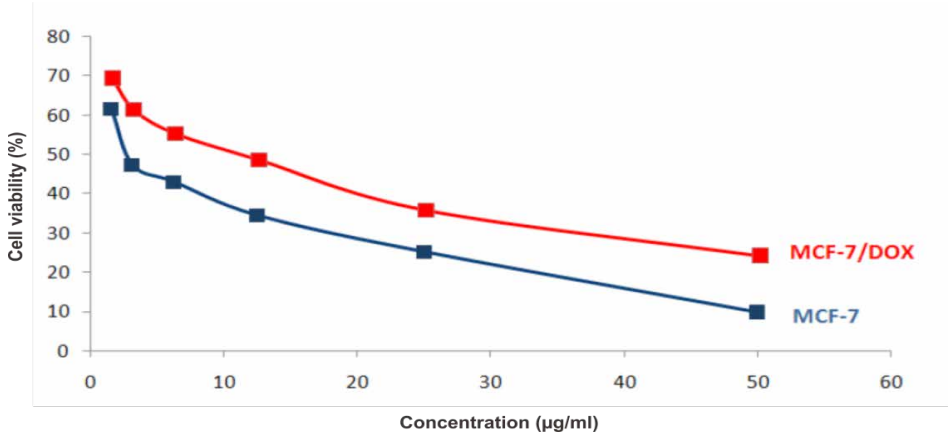

FIGURE 4. MCF-7 and MCF-7/Dox parental cell viability assay using the gradual Dox concentration

Unexpressed miR-145 on MCF-7 Cell line

qPCR was conducted to measure the expression of miR-145. The expression level of miR-145 was undetected. These results were confirmed by normal expression and observed in U6 snRNA as gene reference and UniSp6 as the normalization reference (FIGURE 5). This condition occurs in both cells, MCF-7/Dox and wild-type MCF-7 which shown in FIGURE 6.

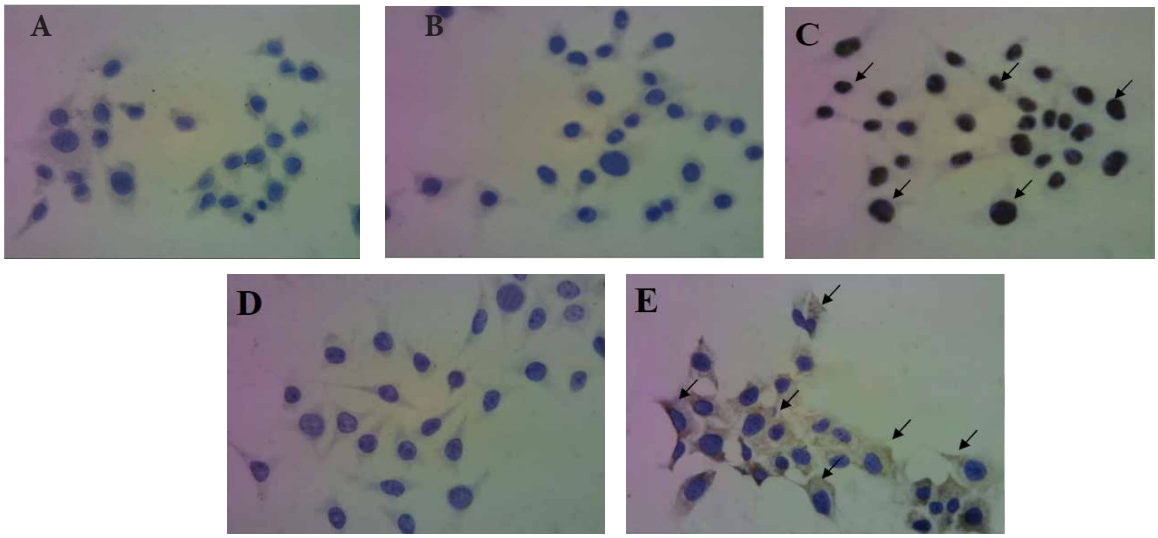

FIGURE 5. Histochemistry results on parental cell line, among others negative control (A), caspase 3 (B), expression ER in nucleus (C), and Pgp in MCF-7 (D), expression of Pgp in cytoplasm and cell membrane of MCF-7/Dox (E), protein expression was detected with brown on the sample showed by arrow (400x).

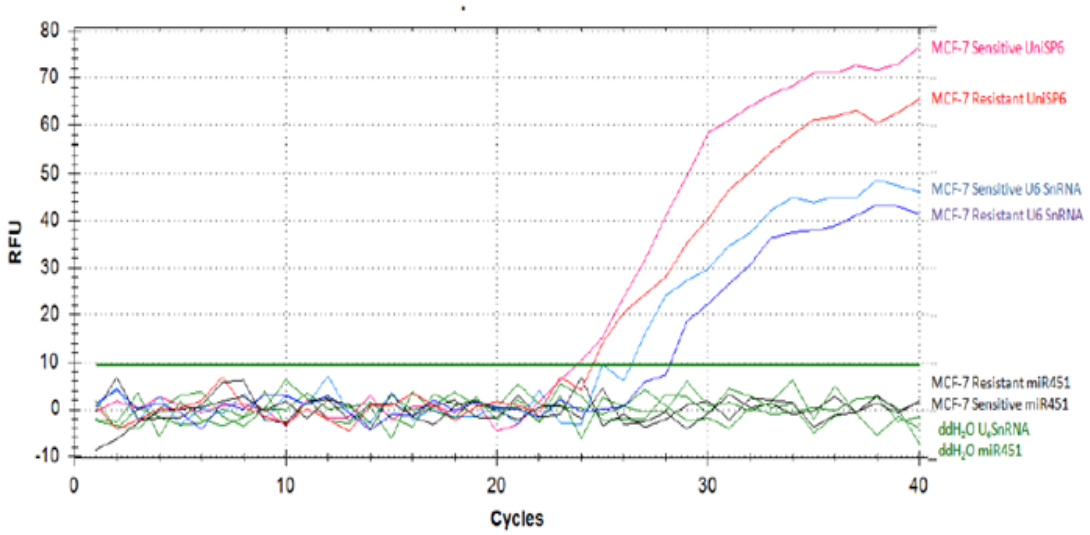

FIGURE 6. Quantification curve/ amplification curve on expression of miR-451 byBiorad CFX Manager™ software

The results showed of level expression of U6 snRNA with the length 107 bp and Unisp6 have a length less of 100 bp. Three-time replicates experiments resulted in the similar phenomenon unexpressed miR-451 on qPCR.

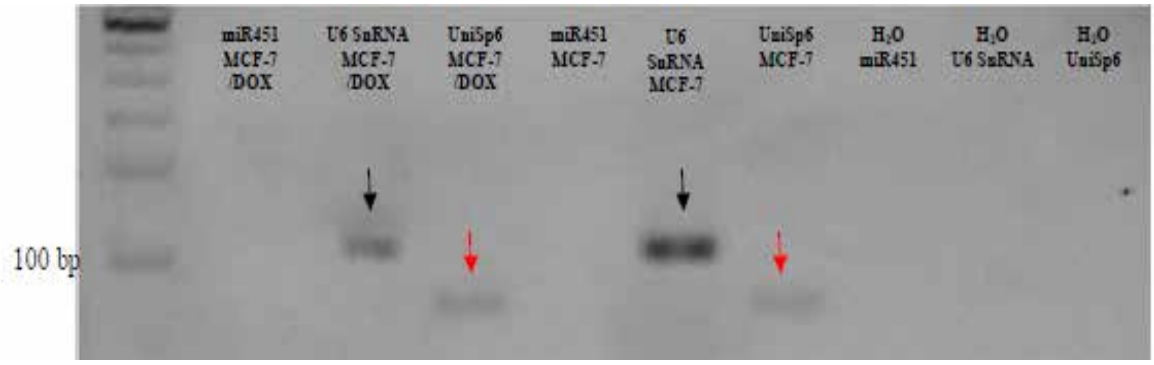

FIGURE 7. Confirmation of amplification of qRT PCR products through electrophoresis. The black arrow shows the ribbon of the U6 SnRNA product with a length of 107bp and the red arrow shows the ribbon of UniSp6 products with a length of <100bp

DISCUSSION

Prediction analysis of miR451 toward mRNA MDR was a validation of prediction *insilico* analysis used hybrid RNA. According to Brennek *at al.*[14] the principle of complementation/base pairing and thermodynamic binding between miRNA and mRNA target. The binding position miR451 was on 3'UTR sequence with 5 base nucleotide that complimented on 5'UTR position. Thermodynamically all interaction resulted from a value of minimum free energy of -6.10 kJ/mol. According to Leitner,[15] the binding of miRNA and mRNA was inversely proportional to ΔG value. Based on principal thermodynamic by Gibbs there were chemical bonds, the value of ΔG negative resulted by *insilico* analysis showed spontaneity reaction between miR451 and MDR1.

The development prognosis biomarker research on the cell line of cancer resistance is the pharmacological and chemical drugs. The multilevel drugs treatment iso ne way that used to induce cell line toward cell cancer resistance.[9] Overexpression and unexpressed caspase 3 on cell line MCF-7 in accordance with previous studies. ER is a protein that playsroles in proliferation and metastasis signalling pathways whereas caspase 3 roles in apoptosis regulation.

Adaptation of induction regulation by Doxaccordance with McCubrey *et al.*[16] showed reactive oxygen species (ROS) accumulation that caused by Dox. This condition can increasing excessive phosphorylation Raf/MEK/ERK pathway that played important role as activation inducer of the transcription factor on Gene MDR1. Overexpression of Pgp on MCF-7/Dox was found with significant value in cytoplasm and membrane cell. Mechanism expression of Pgp was adaptive mechanism cellular accumulated in the cytoplasm through efflux pump mechanism. The results of analysis assay IC_{50} on cell line MCF-7 were overexpression more 7.5 fold times compared with cell line parental. It was resulted by physiological adaptation of increasing dose of Dox that previously administrated, which also affect the upregulation of Pgp expression level.

According to Davis *et al.*[17] there was resistance mechanism through increased expression of BCL-2, this might have been expected of Bcl-2 produced by Dox. Free radical is able to activate Raf/MEK/ERK signalling pathway via phosphorylation cascade. Protein Bcl-2 is a protein that playsrole in apoptosis to increase proliferation and able to increase viability cell resistance. The morphologically on MCF-7/Doxisfound lamellopodia structure. The study result of Lukyanova *et al.*[6] showed the activation of Rac-1 as extracellular response activated Pak1 so that induced phosphorylation myosin and F-actin on growing cytoskeleton and lamellopodia.

MiRNA is one type of non-coding RNA that expressed endogenously and plays role in regulating gene expression via post-transcriptional inhibition mechanisms. qPCR isone type of methods that used as detection methods to quantification miRNA.[18] The results analysis of miR-451 expression was unexpressed while U6 snRNA expressed significantly. It was role as small nucleolar RNA housekeeping gene, which act as catalyst splicing on the mammalian cell.[19,20] The Previous study performed inconsistent from several types of research about miRNA expression, it was suspected because of the change of physiology factor on cell line material. Previous observation has been conducte by Osborne *et al.*[21] on MCF-7cell line in several different laboratories. It was known to form the same morphology, but it occurred differences in growth rates, karyotypes, and activated significantly protein.[21] It was expected to occur because the using of trypsin upon subculture to remove the adherent cell from *substratum* repeatedly on MCF-7 cell line which could led to conformational change of proteins in the cells via proteolytic activity.[22] The others mechanism enabled to occur disruption expression on miR-451 because due the mutations on the gene encode miR451, but it is rare.[23]

Based on the results of research had been done, it suspected abnormalities on protein activated which played role in biogenesis mechanism of miRNA. Although visually, the parental MCF-7 cell line used in this study possess similar morphological appearance compared to MCF-7 cell line, the alteration of gene expression or protein activity had possibly occurred in this cell line.

The changes of profile protein that contribute to miR451 biogenesis were one of the things that may be causing interference on the pri-miRNA into mature miRNA.[24] Currently, the main protein was known as a regulator of miRNA biogenesis among others Drosha, Argonaute (Ago), Dicer, DGCR, TRBP, and RISC protein complex, while the protein which have role in this mechanism unclear. The changes on proteins that play role in biogenesis miRNA were presumed to disruption to cutting process pri-miRNA to mature miRNA, it resulted in changes in the sequence of bases, either point mutations or frameshift mutation on mature miRNA. In the laboratory, this study has been shown unexpressed of miR451 on MCF-7 cell line and MCF7/Dox because of changes characterization. It was confirmed through literary and in-silico analysis that performed expression and strong chemical bonds of miR451 and mRNA MDR1on MCF-7cell line. The research also had been shown increasingly level expression of Pgp independently on MCF-7/Dox which was not affected by expression of miR451.

CONCLUSIONS

The miR451 expression on both MCF-7 and MCF-7/Dox cell lines under go down regulation. On MCF-7/Dox cell line, the downregulation is associated withover expression of Pgpas indicated its higher expression compare to that on MCF-7 *wild-type*. It is concludet that

the Pgp over expression is associated with the MCF-7 cell line resistance to doxorubicin.

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