

Research Article

MicroRNA-21 and PTEN expression levels are negatively correlated in Doxorubicin resistant MCF-7 Breast Cancer Cell Line

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Abstract:

Background: Breast cancer is the leading cause of cancer mortality and morbidity among Indonesian women. Identification of biological pathways leading into therapeutic resistance through in vitro model is an important step to develop alternative effective therapy in breast cancer. Loss of PTEN expression has been associated with resistance to chemotherapy by involving PI3K/PTEN-dependent apoptosis pathway. We conducted in vitro experiment to investigate the association of hsa-miR-21 and PTEN expression in Doxorubicin-resistant MCF-7 cell line. Methods: Parental MCF-7 cells were periodically incubated with Doxorubicin to obtain specific Dox-resistant variant determined by IC50 using MTT assay. PTEN protein expression was analyzed using immunocytochemistry. Expression of mature has-miR-21 was measured using qRT-PCR. Results: The IC50 of Doxorubicin in parental MCF-7 and Doxorubicin-resistant MCF-7 cells (MCF-7/Dox) was 0.68 and 5.78 µg/ml, respectively. Hsa-miR-21 was significantly overexpressed in MCF-7/Dox cells compared to parental MCF cells (7.94 fold changes). Conclusion: PTEN and hsa-miR-21 expression levels were negatively correlated in Doxorubicin resistant-MCF cells. Further study to confirm the causal relationship of miR-21 overexpression and PTEN downregulation in MCF-7/Dox is required.

Keywords: Resistant; microRNA-21; Doxorubicin; PTEN; MCF-7/Dox Cell Line.

1. Introduction

Breast cancer has emerged as the most common cancer and the leading cause cancer-related mortality among females worldwide including in Indonesia [1, 2]. More than 50,000 patients are diagnosed each year and an estimation of 20,000 patients died due breast cancer in Indonesia [1, 3]. During the past few decades, mortality due to breast cancer has significantly reduced particularly in those who are diagnosed in early stages [3]. New myriads of breast cancer treatment are recently developed with significant impact not only reduced mortality but also improved patient's quality of life [4]. However, a relatively high proportion of breast cancer develops resistance to treatment causing significant clinical problem particularly in metastatic disease in which resistance leads into treatment failure in 90% patients [5]. Efforts to counter drug resistance are important step to improve breast cancer outcome [5]. Therefore, improvement in the knowledge of molecular mechanism leading into drug resistance is very important to develop alternative approach to reverse drug resistance.

Anthracyclines are a spectrum of chemotherapeutic agents that have been used in combination with fluorouracil and cyclophosphamide with considerable efficient in breast cancer treatment [6]. Doxorubicin is among anthracyclines that is commonly used as a combination of adjuvant therapy in breast cancer [6]. However, it is estimated that 20-30% of early stage breast cancer patients develop into progressive disease after treatment [7]. Doxorubicin forms DNA intercalation causing stabilization and local unwinding as well as transcription inhibition [8]. In addition, doxorubicin also inhibits Topoisomerase II and produces free radical to further induce cancer cell death [8]. Resistance to doxorubicin has been previously suggested by inducing enhancement drug efflux, deregulation of Topoisomerase II expression, and alterations of extracellular matrix and microenvironment [9, 10].

Recent studies have shown the essential roles of microRNAs (miRNAs), a class of small non-coding RNAs that are able to negatively regulate gene expression post transcriptional, in breast carcinogenesis [11, 12]. Some miRNAs have been associated with resistance to chemotherapeutic agents in breast cancer [13, 14]. However, systematic screen of miRNAs involved in doxorubicin-resistance in breast cancer cells has not been thoroughly reported. In this study, we performed in vivo study to identify miRNAs specifically dysregulated in doxorubicin-resistant breast cancer cells.

2. Results

Characterization of MCF-7/Dox cells

The Doxorubicin resistant MCF-7 cells were established with 240 nM Doxorubicin. The development of resistant cells phenotype was characterized by changes of morphological features and P-gp expression. Compared to parental MCF-7, the MCF-7/Dox cells were larger with a prominent abnormal shape of the cytoplasm membrane (Figure 1). Additionally, the P-gp was detected in high expression using immunocytochemistry (Figure 2).

Effect of Doxorubicin on cell viability

Cell viability assay was performed to quantify the IC₅₀ of parental MCF-7 and MCF-7/Dox cells. As shown on Figure 1, the MCF-7/Dox cells had increased IC₅₀ (5, 78 µg/ml) compared to parental MCF-7 (0, 68 µg/ml). The resistance difference between these two cells variant then presented as resistance index by dividing resistant cells IC₅₀ with parental cells IC₅₀. The MCF-7/Dox cells were 8,5-fold more resistant to Doxorubicin than parental MCF-7 cells.

Expression of miR-21 on breast cancer cell line

qRT-PCR was performed to detect the expression of hsa-miR-21 in parental MCF-7 and MCF-7/Dox cells. Compared to parental MCF-7 cells, the relative level of hsa-miR-21 expression in MCF-7/Dox cells was increased 7,94-fold (Figure 3). This data showed that expression of hsa-miR-21 was higher in MCF-7/Dox cells compared to parental MCF-7 cells.

MicroRNA-21 regulates expression of PTEN

Our results showed the reduction of PTEN in MCF-7/Dox cells compared to parental MCF-7 cells as detected by immunochemistry. The parental MCF/7 cells had the higher PTEN expression whereas the lowest expression was found in MCF-7/Dox cells (Figure 2). Therefore, we found that the up regulation of hsa-miR-21 was parallel to the down regulation of PTEN in MCF-7/Dox cells.

3. Discussion

Some studies have confirmed the important role of miRNAs as biomarker and prognostic indicator of human breast cancer [15] which bring new hope and breakthrough in cancer management. Despite the all known post-transcriptional regulation of miRNAs in cancer[16], the precise molecular mechanism involved in the development of cancer cells resistance to chemotherapy remains not well established. In this report, we provide data showing the differences of miRNAs expression in MCF-7 parental cells (not treated) and in the acquired MCF-7 cell line resistance to Doxorubicin. This was proved by the alteration in expression of microRNA-21 in MCF-7 Doxorubicin resistant cells compared to parental MCF-7 cells. Doxorubicin has widely used as first line chemotherapy agent in the treatment of various cancers, including breast cancer[17, 18]. This drug could inhibit replication by intercalation resulting cleavage on dsDNA[19]. Doxorubicin also targets topoisomerase II affecting inability in relaxing the super coiled structure of chromatin. We demonstrated that the parental MCF-7 cells could develop resistance after serial Doxorubicin intermittent exposure. Results showed that MCF-7/Dox had a phenotype of resistant cells proved by higher IC₅₀ values with 8,5-fold more Dox-resistant compared to parental MCF-7 cells. Many studies have reported PTEN as a target of miR-21 [20, 21]. It was evidenced by in silico analysis that miR-21 has a putative binding site at 3'UTR mRNA sequence of PTEN gene. PTEN, as target of miR-21, has a critical role in apoptosis mediated by PI3K/Akt pathway [22, 23]. Reduced of PTEN expression will eliminate the inhibition of phosphatidylinositol phosphate (PIP) fosforilation in Akt downstream signaling then increasing cell proliferation and apoptosis failure[24, 25].

In this study, we showed high miRNA expression in the MCF-7/Dox cells. Compared to parental MCF-7 cells, hsa-miR-21 expression was up regulated in MCF/Dox cells with significant fold difference of relative expression. This could be considered as important finding related to the expression pattern of miR-21 in breast cancer cells. Some studies reported the high frequently expressed of miR-21 in MCF-7 cells [26, 27] and our results also proved the over expression of miR-21 in Dox-resistant breast cancer cells.

The association of miR-21 deregulation with the establishment of Doxorubicin resistant cells phenotype was showed by the correlation between expression of hsa-miR-21 and corresponding changes in target protein. The up regulation of miRNA could affect PTEN protein[28]. We found that the up regulation of hsa-miR-21 was parallel to the down regulation of PTEN protein in MCF-7/Dox cells as detected by immunochemistry.

This result is consistent with clinical studies reported PTEN under expression on breast cancer patient with the mutation in PI3K pathway[29, 30]. This finding suggests that hsa-miR-21 has negative regulation on PTEN expression resulting aberrant PTEN-dependent apoptosis pathway which ultimately affect in resistance mechanism of breast cancer cells.

4. Materials and Methods

Cell culture

The MCF-7 human breast cancer cell line originally obtained from Stem Cell and Cancer Institute Jakarta, Indonesia. The cells were maintained as an attached monolayer culture in DMEM high glucose medium (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco), 2% Penicillin-Streptomycin (Gibco) and 0,5% antifungal (Fungizone, Gibco) at 5% CO₂, 37°C.

Establishment of Doxorubicin-resistant cells

To obtain MCF-7 Doxorubicin-resistant variant, the confluent parental MCF-7 cells were exposed to Doxorubicin (Sandobicin) in 5 intermittent treatments to increase Doxorubicin (Dox) concentration starting from 30 nM to 240 nM at final concentration. The resistant cells were characterized by cell morphological features, MTT assay, and P-gp immunocytochemistry.

MTT assay

Cell viability was determined by MTT assay. Both parental MCF-7 and MCF-7 treated Dox (MCF-7/Dox) were seeded into 96-well culture plate (Iwaki) at density 10⁴ viable cells per well. Following 24 h incubation, cells were treated with various concentrations of Doxorubicin. After 24h treatment, cells were washed with Phosphate Buffer Solution (PBS) following an addition of 0,5 mg/ml MTT solution (Sigma). About 4h later, Sodium Dodecyl Sulfate was added. Absorbance was recorded at 550 nm using microplate reader (Bio-Rad).

Immunocytochemistry

Expression of P-gp and PTEN was detected by immunocytochemistry using specific monoclonal antibody. The parental MCF-7 and MCF-7/Dox cells were plated on glass cover slip and cultured in 24-wells plate (Iwaki) at 5x10⁴ cells per well. After 24h incubation, cells were rinsed with PBS and fixed with cold methanol (store at -40°C, 10 min) following the addition of hydrogen peroxide blocking solution. The fixed cells then washed with PBS and pre-diluted blocking serum at room temperature for 10 min. Cells were incubated with primary anti-human P-gp monoclonal antibody (Dako) and primary anti-human PTEN monoclonal antibody (Abcam) for P-gp and PTEN detection, respectively. After 1h at room temperature, antibody binding was performed by incubation with the biotinylated universal secondary antibody (15-30 min). Cells then incubated with streptavidin-biotin complex (15 min) and 3,3-diaminobenzidin solution (3-8 min) followed by counterstained with Mayer-Haematoksilin (3-4 min). After washing, cover slip then immersed with xylol and alcohol. Dark brown color in cells membrane indicating positive expression of P-gp and

PTEN, while negative expression detected as purple.

Quantitative real-time PCR analysis

Total RNA was extracted from confluent cells culture of MCF-7 and MCF-7/Dox using miRCURY RNA Isolation Kit-Cell & Plant (Exiqon) according to the manufacturer's instructions. The total RNA (5 ng/ μ l) was used as the RNA template for cDNA synthesis using miRCURY LNA Universal RT-microRNA PCR, Polydenylation and cDNA Synthesis Kit (Exiqon). The qRT-PCR was performed using miRCURY LNA Universal RT-microRNA PCR Cyber Green Master Mix (Exiqon), hsa-miR-21-5p PCR LNA Primer Set (Exiqon) and U6SnRNA PCR LNA Primer Set (Exiqon) following the manufacturer's instructions. The qPCR reactions contained hsa-miR-21 primer specific for human miR-21 and normalized with U6SnRNA as the reference gene. The qRT-PCR analysis was done on CFX96 Thermocycler (Bio-Rad). The expression level of hsa-miR-21 was measured using Livak method ($2^{-\Delta\Delta Cq}$). The results were presented as fold change of hsa-miR-21 relative expression in the MCF-7/Dox cells compared to parental MCF-7 cells.

In Silico

To predict gene of target and biological process of miRNA and mRNA, we used miRWALK, Diana miR-Path and David KEGG online platform. TCGA dataset is used from cBioportal for Cancer Genomic in September 2018, <http://www.cbioportal.org/> Comprehensive analysis and demonstration role and correlation of miR, target gene, and prognosis of Breast cancer patients.

5. Conclusions

Higher expression of hsa-miR-21 have been shown in MCF-7/Dox positive cell line as Doxorubicin-resistance model compared to MCF7/Dox negative in breast cancer. This finding suggests that over expression of hsa-miR-21 play an important role regulating chemo resistance. Doxorubicine through down regulating mRNA PTEN post-transcriptional.

Figures, Tables and Scheme

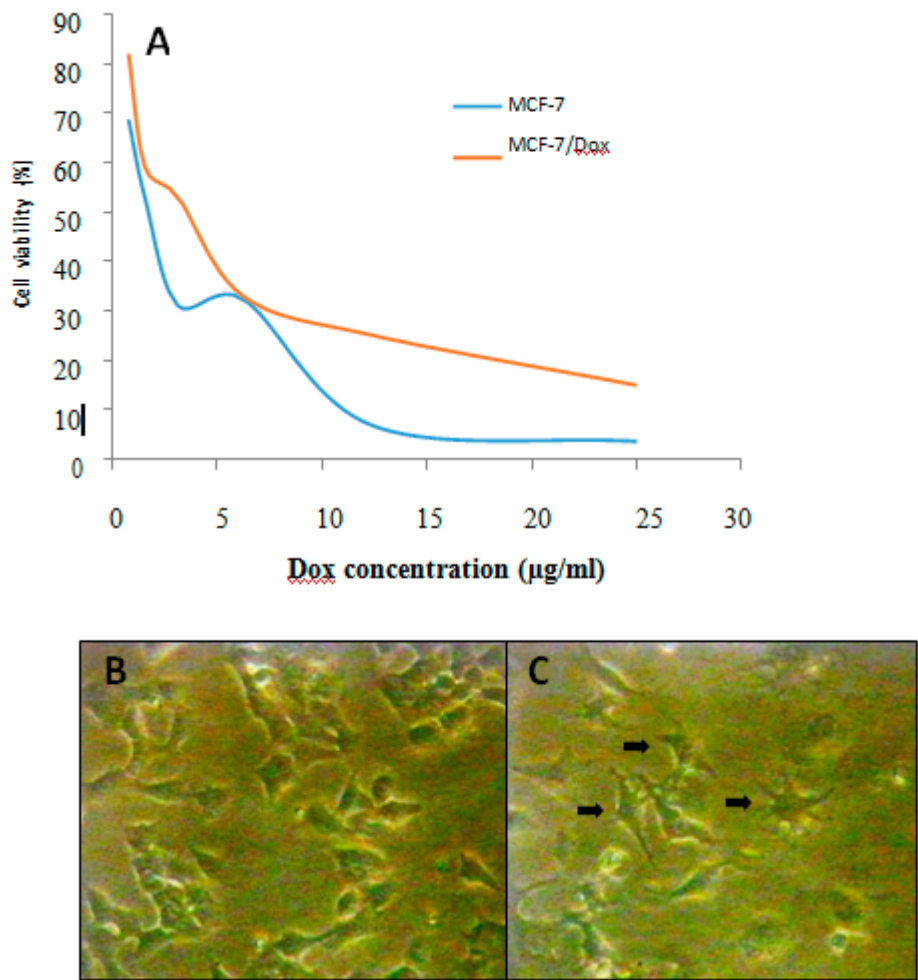


Figure 1. Comparison of cell viability and morphological features of MCF-7/Dox and parental MCF-7 cells, compared to parental MCF-7, the MCF-7/Dox cells have higher IC_{50} corresponding with cell viability (A). The parental MCF-7 appear small, spindle-shaped cells (B). The MCF-7/Dox cells relatively larger appear irregular with abnormal protruded cytoplasm (C). Magnification 100x.

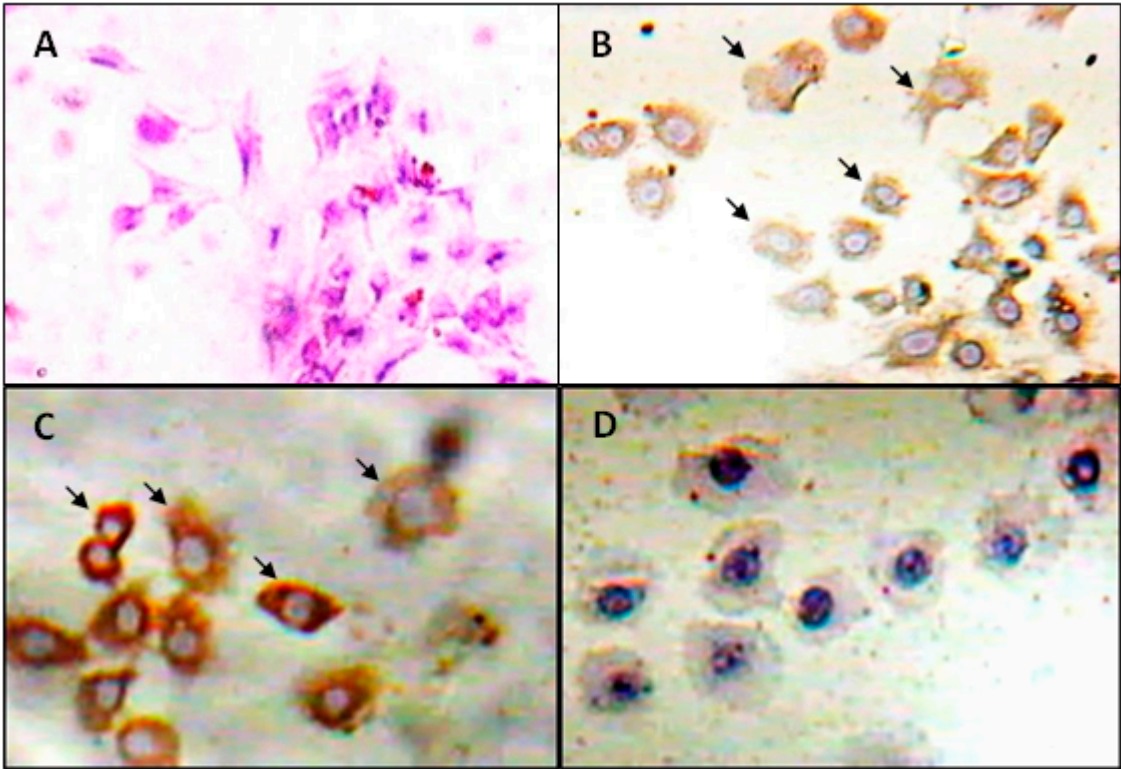


Figure 2. Immunocytochemistry of P-gp and PTEN. Compared to parental MCF-7 cells (A), the MCF-7/Dox cells with primary anti P-gp showed brown color on the cell membrane demonstrating P-gp expression (B). In contrast, the brown color was detected on the parental MCF-7 cell membrane (C) as no seen on the MCF-7/Dox cells stained with primary anti-PTEN showing the reduction of PTEN expression in MCF-7/Dox cells (D). Magnification 400x.

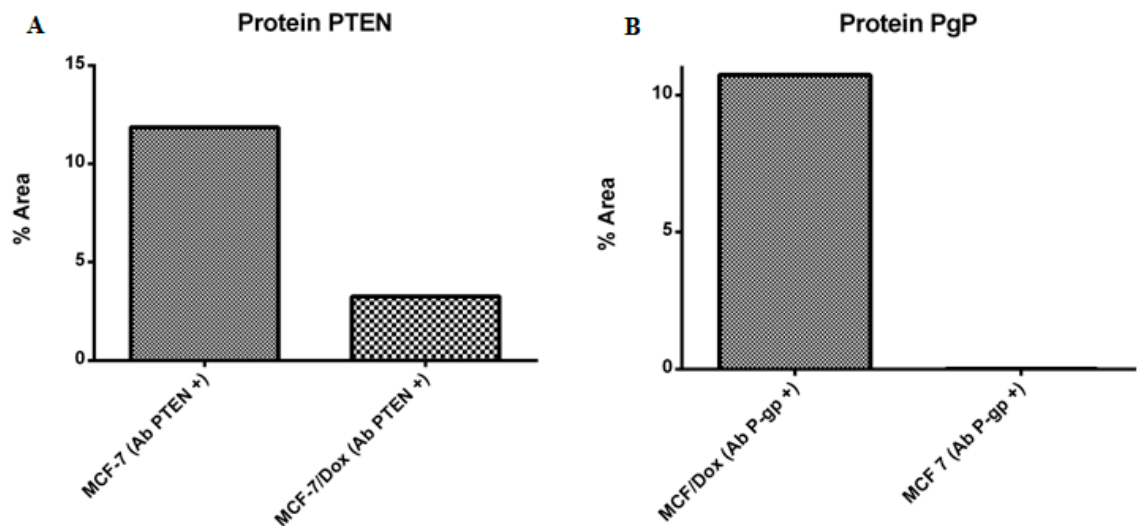


Figure 3. Profile expression protein parental MCF-7 cells compared with MCH-7/Dox cells, quantification from Immunochemistry assay using Image J (A), PTEN down expression 3,258 MCF-7/Dox compared with MCF-7 11,854, (B). down expression of PgP protein in MCF-7/dox 0.008 compared with MCF-7 10.725.

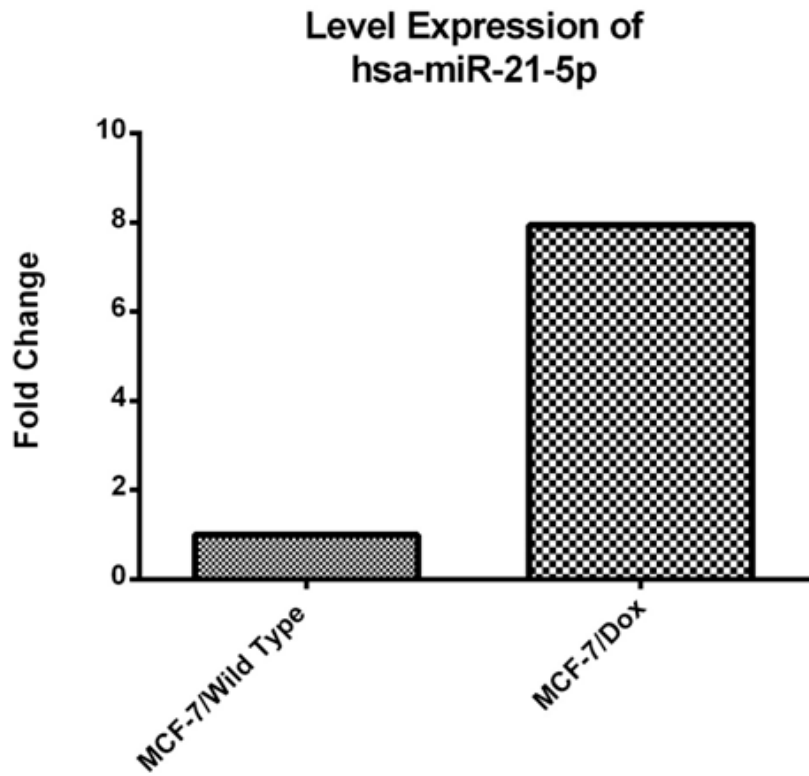


Figure 4. The overexpression of *hsa-miR-21* in breast cancer cell line. The *hsa-miR-21* was upregulated in MCF-7/Dox cells with 7, 94 fold change expression relative to parental MCF-7 cells.

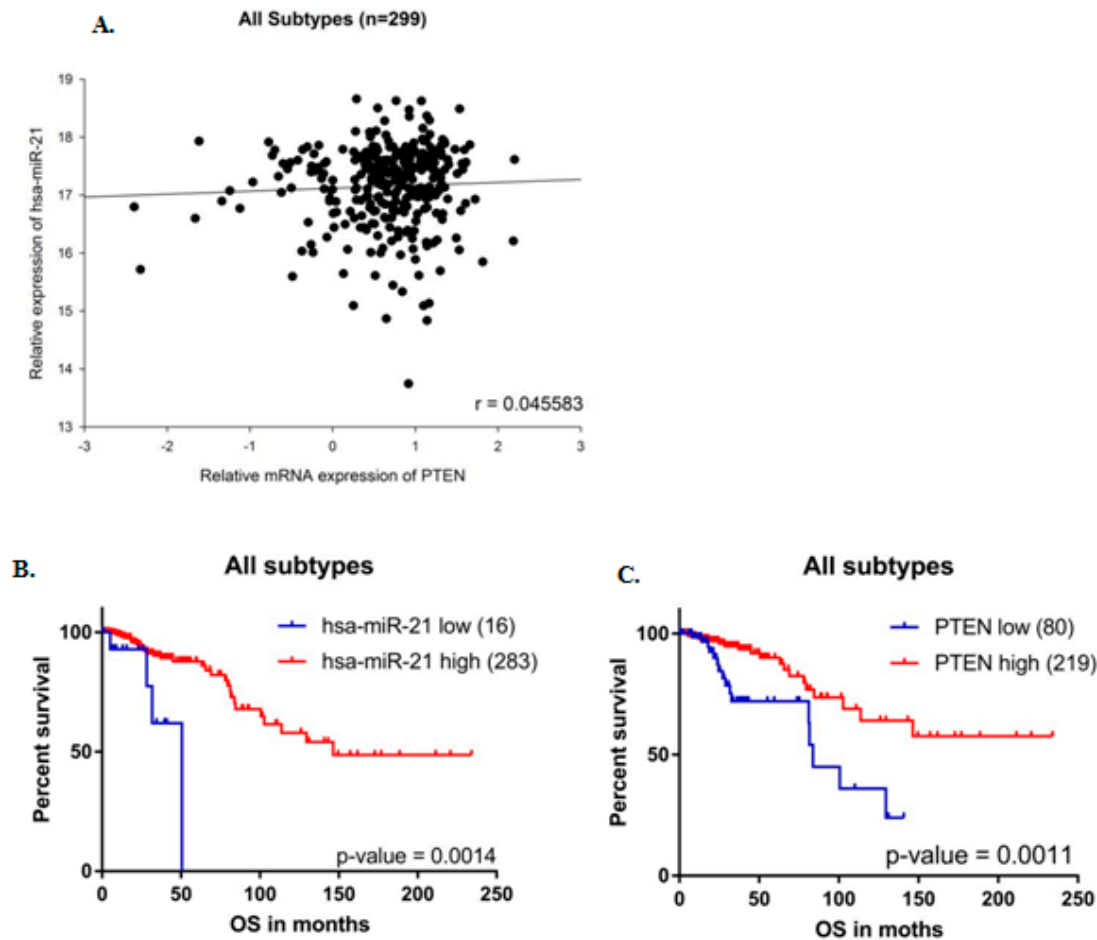


Figure 5. miRNA and mRNA PTEN expression, data set from TCGA cBioportal Correlation expression and Kaplan-Meier overall survival curves by log rank test. (A) Correlation between of miR-21 and mRNA PTEN expression; (B) miR-21; (C) mRNA PTEN

Author Contributions: GMS and RO performed the experiment. IA analyzed the MCF-7 Doxorubicin-resistant cells. TA, TW and SM contributed to study design, MCF-7 cells collection and analyzed the miRNA study. GMS, RO, TW and SM prepared the manuscript. All authors read and approved the final manuscript.

Ethics Statement: This study was approved by The Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine Gadjah Mada University-Dr. Sardjito Hospital, Yogyakarta (Reference number: KE/FK/735/EC).

Funding: the financial support by Hibah Pascasarjana 2014 and 2015 (The Graduate School Research Grant) from The Ministry of Research, Technology and Higher Education of Republic of Indonesia.

Acknowledgments: We would like to thank Stem Cell and Cancer Institute Jakarta Indonesia for providing the MCF-7 cell line, Istini for her technical assistance in culturing MCF-7 cells, Agustin for immunocytochemistry.

Conflicts of Interest: The authors state that there are no conflicts of interest in this study.

Abbreviations

PTEN	Phosphatase Tensin Homolog PI3K Pathway
MCF-7	Human Breast Cancer Cell Line
MCF-7/Dox	Human Breast Cancer Cell Line Doxorubicin resistant cell

Hsa-miR-21-5p

DMEM

P-gP

Homo Sapiens microRNA 21 5p

Dulbecco’s modified Eagle’s medium

P-Glycoprotein

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