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Overexpression of *hsa-miR-21* Mediates Doxorubicin Resistance in Non-Hodgkin Lymphoma by Targeting PTEN and P-glycoprotein

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Doxorubicin resistance is a problem in lymphoma therapy. The mechanisms for drug resistance include epigenetic regulation by microRNA, drug efflux by transporter protein and lost expression of apoptotic protein. This research aimed to evaluate the potential of *hsa-miR-21* as a predictive biomarker and targeted therapy candidate for cases of doxorubicin resistance in non-Hodgkin lymphoma by investigating the pattern of *hsa-miR-21*, P-glycoprotein (P-gp) and PTEN expression in doxorubicin-resistant Raji cell lines. The interaction between *hsa-miR-21* and *PTEN* mRNA was predicted *in silico*. Epigenetic modifications in the normal and doxorubicin-resistant Raji cells were assessed from their expression levels of P-gp and PTEN by immunocytochemistry, and expression levels of *hsa-miR-21* with RT-qPCR. The doxorubicin-resistant cells showed giant cell morphology and a 2.46-fold resistance to doxorubicin in the MTT assay, along with a 1.74-fold increased expression of P-gp and 2-fold down-regulation of PTEN at protein level, and a 5.06-fold overexpression of *hsa-miR-21*. Such interaction between the *hsa-miR-21* and the proteins involved in the resistance to doxorubicin in Raji cells implied that *hsa-miR-21* could be a potential candidate of biomarker and targeted therapy for identifying and managing non-Hodgkin B cell lymphoma patients who are resistant to therapy with doxorubicin.

Keywords: doxorubicin; *hsa-miR-21*; PTEN; Raji cell line; resistance

I. INTRODUCTION

According to the statistics from the World Cancer Research Fund International in 2013, lymphoma is classified as a haematologic malignancy with 386,000 new cases at that time (Ferlay *et al.*, 2013). In the same year, lymphoma was identified as the fourth most frequent cancer that the patients commonly need intensive care in the hospitals in Indonesia (Ministry of Health, 2013). Chemoresistance to CHOP, a combination chemotherapy including doxorubicin, that serves as the first line treatment was one of the main problems in lymphoma therapy (Cancer Research UK, 2012). Only 30-40% of non-Hodgkin lymphoma patients were cured by doxorubicin in the conventional therapy (Sehn *et al.*, 2007). Doxorubicin stimulates reactive oxygen species production and causes oxidative stress that induces development of resistance to the drug in cancer cells (Steelman *et al.*, 2016). Many factors can influence drug resistance in cancer treatment, including epigenetic regulation by microRNA, drug efflux by transporter protein and lost expression of apoptotic proteins (Housman *et al.*, 2014).

The three major mechanisms of multidrug resistance were decreased uptake of water-soluble drugs, various changes in cells that affect the ability of cytotoxic drugs to kill cells, and increased energy-dependent efflux of hydrophobic drugs. Studies have shown that the major mechanism of multidrug resistance in cultured cancer cells involves P-glycoprotein (P-gp) as drug efflux transporter (Szakács *et al.*, 2006). In 2013, WHO and International

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Agency for Research on Cancer (IARC) developed early detection programme for cancer diagnosis and prognosis, and also the biological therapeutic agents based on individualised therapy to overcome resistance to chemotherapy. One of the early detection and therapeutic agents has been developed based on the epigenetic study of microRNA. The microRNA has been considered as a potential biomarker and targeted therapy agent because of its stability and specificity (George & Mittal, 2010). The microRNA *hsa-miR-21* has been identified as an oncomir in cancer progression, including lymphomagenesis. Raji cell line is known as the *in vitro* Burkitt lymphoma model, a type of B cell non-Hodgkin lymphoma, with the highest aggressivity and recurrence level among other types of lymphoma. Apoptotic signal inhibition in Raji cells is related to the expression of LMP1, an Epstein-Barr virus protein, through the PI3K/AKT pathway that is inhibited by PTEN. PTEN acts as a tumour suppressor (apoptotic protein) that influences the genomic stability in cancer cells, and the expression of PTEN was shown to be regulated by microRNA like *hsa-miR-21* (Wang *et al.*, 2011). As such, doxorubicin-resistant cell line developed from Raji cells could represent a drug resistant model of B cell non-Hodgkin lymphoma for studying the epigenetic modifications of *hsa-miR-21* in mediating doxorubicin resistance.

This study investigates how *hsa-miR-21* expression regulates the expression of PTEN and P-gp in doxorubicin-resistant Raji cell line, in an effort to determine the potential of *hsa-miR-21* as a predictive biomarker or target for treating doxorubicin resistance in non-Hodgkin lymphoma. This is important in the development of individualised therapy programme for chemoresistance to doxorubicin in lymphoma patients.

II. MATERIALS AND METHODS

A. In Silico Prediction of Interaction Between hsa-miR-21 and PTEN

Sequences of *hsa-miR-21* and *PTEN* were retrieved from miRBase (http://www.mirbase.org) and Genbank (http://www.ncbi/genbank). Interaction between *hsa-miR-21* and PTEN was searched against miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/), a database that provides comprehensive curated collection of microRNA-target interactions predicted by miRanda based on the minimal free energy of the duplex structure, evolutionary conservation of the whole putative target site, and the microRNA's position within 3'UTR of the target.

B. Raji/WT and Raji/Dox Cell Culture

The Raji cell line used in this study was provided by the Molecular Biology Laboratory at Faculty of Medicine, Gadjah Mada University. The wild type Raji (Raji/*WT*) cells used as the Burkitt lymphoma model were grown in the RPMI complete medium [RPMI 1640 (Gibco, 31800-014) supplemented with 2% penicillin-streptomycin (Gibco), 0.5% fungizone (Gibco) and 10% foetal bovine serum (Gibco)]. The doxorubicin-resistant Raji cell line (Raji/*Dox*) was developed from the wild type according to the resistance induction method in Kartika *et al.* (2013) with modifications as summarised in Figure 1. Resistance was induced by intermittently exposing the Raji/*WT* cells to increasing doxorubicin concentrations in three cycles. The first two treatment cycles involved growing the cells in RPMI 1640 complete medium added with 151.78 nM doxorubicin (Novell) for 24 h with a 3-day recovery period after treatment. The cells were then grown in medium supplemented with doxorubicin at a final concentration of 303.5 nM for 24 h with a 5-day recovery period in the third treatment cycle. Doxorubicin was not applied in the medium during the recovery period which depends on the time needed for the cells to grow to confluence.

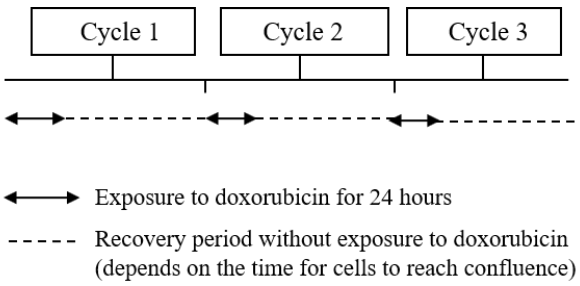

Figure 1. Doxorubicin re-treatment schedule to develop doxorubicin-resistant Raji cell line (modified after Kartika *et al.*, 2013).

C. MTT Assay

MTT assay was used to evaluate the development of resistance to doxorubicin in the Raji cells for further use in this study. Suspension of each Raji/*WT* and Raji/*Dox* (induced with 151.78 nM and 303.5 nM doxorubicin) cell

line was incubated in microplate at a density of 1×10^4 cells in 100 µL per well at 37°C and 5% CO_2 for 24 h, and treated with doxorubicin in a range of concentrations prepared from two-fold serial dilution (25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL, 1.56 µg/mL, 0.78 µg/mL and 0.39 µg/mL) in three replicates. The cells were washed after 24-h treatment with doxorubicin, and yellow MTT (Bio Basic Inc.) was added to each well at a working concentration of 0.5 mg/mL. The microplate was incubated for 2-4 h until formazan crystal formed. 100 µL of 10% SDS (Merck) in 0.1 N HCl (Merck) was added to each well and mixed for 30 s to stop the reaction. The absorbance of solubilised formazan crystal was measured in an ELISA reader at 550 nm. The viability of cells was expressed as a percentage relative to the control (untreated cells) using the following equation:

$$\textit{Relative cell viability (\%)} = \frac{A_T - A_{B\ with\ doxorubicin}}{A_C - A_{B\ without\ doxorubicin}} \times 100\% \quad (1)$$

where, A_T = Absorbance of treatment

A_B = Absorbance of blank (medium)

A_C = Absorbance of control

A dose-response curve was plotted with relative cell viability against the range of doxorubicin concentrations for each cell line (Raji/*WT*, and Raji/*Dox* induced with 151.78 and 303.5 nM doxorubicin) in Excel. The IC_{50} value which represents the concentration of doxorubicin needed to cause 50% growth inhibition was determined from the value of *x* by substituting *y* with 50% in the linear regression equation fitted to the curve. Increased IC_{50} value in Raji/*Dox* exposed to higher dose of doxorubicin indicated the developed resistance in cells.

D. Immunocytochemistry of P-gp and PTEN

Immunocytochemical staining of the P-gp and PTEN in the Raji cells (both Raji/*WT* and Raji/*Dox*) was performed using the Starr Trek Universal HRP Detection system (Biocare Medical, STUHRP700, L10). A drop of Raji cell suspension was placed on a poly-L-lysine-coated microscope slide (Polyscience, 22247-1), fixed by cooled methanol (4°C) and rinsed with PBS (Invitrogen). Following the blocking of endogenous peroxidase activity with H_2O_2 for 20 min, the cells were incubated in pre-diluted blocking serum (Background Sniper) for 10 min to avoid non-specific background staining. The primary monoclonal Anti-P-Glycoprotein (MDR) antibody produced in mouse (Sigma Aldrich, P7965) and anti-PTEN antibody (Y184) (Abcam, ab32199) were added at a dilution of 1:1000 and 1:100 each, and incubated for 2 h at room temperature. The cells were rinsed with PBS for 5 min. Biotinylated secondary antibody was added and incubated for 10 min. The slide was then processed with streptavidin-peroxidase, DAB chromogen and CAT haematoxylin (BioCare Medical, CATHE-H) according to the manufacturer's instruction, followed by washing in alcohol and xylol for dehydration and clearing, mounted and covered with coverslip before examination under microscope. Semi quantitative analysis was carried out using ImageJ to determine the percentage of positive expression and cellular localisation of the P-gp and PTEN proteins (Gutiérrez *et. al.*, 2012; Toprak *et. al.*, 2006; Vrekoussis *et al.*, 2009).

E. Total RNA Isolation and RT-qPCR

The total RNA isolation was carried out using a miRCURY™ RNA Isolation Kit for Cell & Plant (Exiqon, 300110) according to the manufacturer's instructions. The levels of *hsa-miR-21* in Raji/*WT* and Raji/*Dox* cells were detected by RT-qPCR using the miRCURY LNA Universal RT microRNA PCR system (Exiqon, now miRCURY LNA microRNA PCR system). A total of 50 ng (5 ng/µL) RNA was used to synthesise cDNA using the Universal cDNA synthesis kit II (Exiqon, 203301, now miRCURY LNA miRNA RT kit) by incubating the reaction at 42°C for 1 h, followed by inactivation at 95°C for 5 min and immediate cooling at 4°C. The qPCR was performed using the ExiLENT SYBR Green Master Mix Kit (Exiqon, 203402, now miRCURY LNA SYBR Green PCR Kit), *hsa-miR-21-5p*-specific primers (Exiqon, 204230), and primers for the candidate reference genes U6 snRNA (Exiqon, 203907) and UniSp6 (Exiqon, 203956). PCR amplification was performed at 95°C for 10 min for one cycle, followed by 39 cycles of amplification at 95°C for 10 s and 60°C for 1 min. The specificity of PCR products and expression levels of *hsa-miR-21* were analysed in triplicates using the Bio-Rad CFX Manager Software. The expression stability of candidate reference genes in both Raji/*WT* and Raji/*Dox* cell lines were analysed by comparing the standard error calculated with the statistical software CoStat. Assuming optimal amplification efficiency, the fold change of *hsa-*

miR-21 expression in doxorubicin-resistant Raji cells relative to that in normal cells was quantified using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), where $\Delta\Delta Ct = \Delta Ct_{(Raji/Dox)} - \Delta Ct_{(Raji/WT)}$, while $\Delta Ct_{(Raji/Dox)}$ and $\Delta Ct_{(Raji/WT)}$ were obtained by subtracting the Ct of *hsa-miR-21* from Ct of the reference gene for each cell line. The expression level of *hsa-miR-21* normalised to the reference gene in each Raji/*WT* and Raji/*Dox* cell line was quantified using the ΔCt method, which is a variation of the $2^{-\Delta\Delta Ct}$ method, with the expression ratio = $2^{Ct(reference\ gene) - Ct(hsa\text{-}miR\text{-}21)}$.

III. RESULTS

A. In Silico *Prediction of Interaction Between* hsa-miR-21 *and* PTEN

The *PTEN* located on the chromosome 10q23.3 (positions 87863438-87970345 in assembly GRCh38.p13) is a target of *hsa-miR-21*. *PTEN* mRNA has complementary sites that can bind with *hsa-miR-21-5p*. *In silico* prediction by miRanda in miRTarBase suggested that *hsa-miR-21* can inhibit the expression of *PTEN* instead of degrading the mRNA, based on their partial complementation that resulted in a duplex structure with stability indicated as the lowest minimum free energy of -6.90 kcal/mol (Figure 2).

Duplex structure	Position	Score	MFE
miRNA 3' aguugUAGUCA--GACUAUUCGAu 5' ||:: | || ||||:| Target 5' tattgATTGATTGCTCATAAGTTa 3'	1993 - 2016	135.00	-6.90

Figure 2. Screenshot showing the interaction between *hsa-miR-21* and *PTEN* predicted by miRanda in miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/detail.php?mirtid=MIRT001190#target).

B. Raji/WT and Raji/Dox Cell Culture

The development of doxorubicin resistance in wild type Raji cells involved several cycles of doxorubicin treatment with increasing concentrations and recovery periods in between. The time required for cells to recover depends on the concentration of the drug. Raji/*WT* cells showed homogenous morphology while the morphology of Raji/*Dox* cells was heterogenous with the presence of giant cells (Figure 3). Giant cells were observed in the culture exposed to 151.78 nM doxorubicin after the first cycle of resistance induction, and their presence became more abundant during the following cycles of exposure to doxorubicin. In addition, the Raji/*Dox* cells treated with 303.5 nM doxorubicin needed a longer time to grow to confluence (Figure 3B) while those treated with doxorubicin at lower concentration grew to confluence within a shorter recovery period (Figure 3D).

The resistance of Raji cells to doxorubicin was tested using the MTT assay. The Raji/*Dox* cells were more resistant to doxorubicin than Raji/*WT* cells, as indicated by the IC_{50} values which were higher than that of the Raji/*WT* cells at 9.86 μg/mL (Figure 4). The IC_{50} for Raji cells increased over the period of doxorubicin resistance induction until an IC_{50} of 24.3 μg/mL (41.79 μM) was achieved in cells treated with 303.5 nM doxorubicin. The increase in the IC_{50} value for Raji/*Dox* cells induced with heavier dose of doxorubicin indicated the ability of the cells to tolerate the increased doxorubicin doses. The Raji/*Dox* cells were 2.46-fold more resistant to doxorubicin than the Raji/*WT* cells.

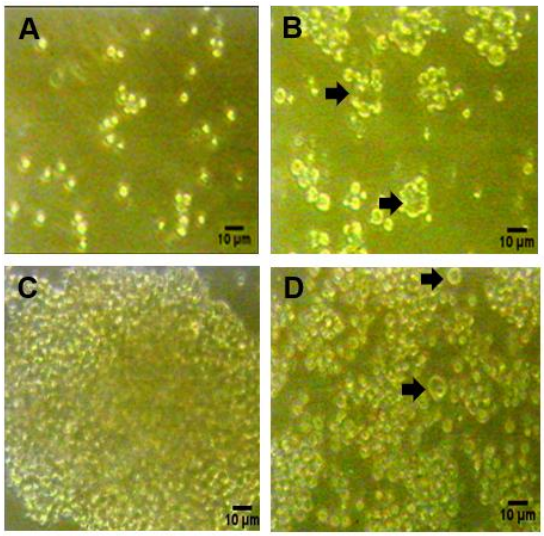

Figure 3. Morphological comparison of Raji cells before (A & B) and after (C & D) growth to confluence. (A) Raji/*WT* cells after thawing, (B) Raji/*Dox* cells exposed to 303.5 nM doxorubicin did not grow into confluence after 3 d of recovery, (C) Raji/*WT* cells grown to confluence after 3 d, and (D) Raji/*Dox* cells treated with 151.78 nM doxorubicin almost grew into confluence after 3 d of recovery at the first cycle of resistance induction. Arrows indicate giant cells.

C. Immunocytochemistry of P-gp and PTEN

Immunocytochemical staining analysis was performed to determine the expression level and specific localisation of two protein markers, P-gp and PTEN, which are implicated in drug resistance in cancer cells. The expression of P-gp at protein level in Raji/*Dox* was higher than that in Raji/*WT*, while that of PTEN was lower in the doxorubicin-resistant cells (Figure 5). The up-regulation of P-gp by 1.74-fold in Raji/*Dox* cells was accompanied by a 2-fold repression of PTEN. The P-gp protein was found to be distributed in plasma membrane of Raji/*Dox* cells (Figure 6A) while PTEN was localised in the nucleus and cytoplasm (Figure 6B).

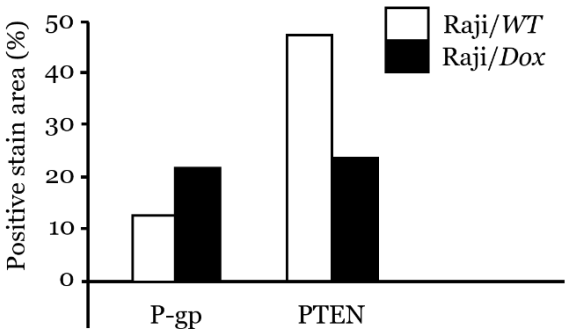

Figure 5. Comparison of the expression levels of P-gp and PTEN in the normal and doxorubicin-resistant Raji cells, indicated by the DAB chromogen positive stain percentage from the immunocytochemical staining analysis.

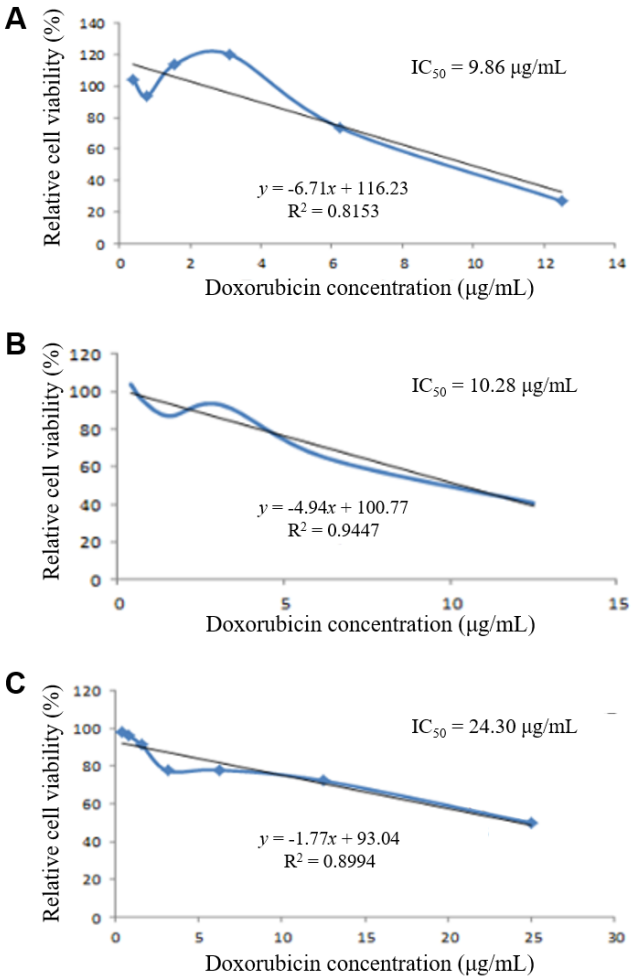

Figure 4. Assessment of cell viability after treatment with doxorubicin of various concentrations based on the MTT assay. Relative cell viability plotted against a range of doxorubicin concentrations applied to Raji/*WT* (A), Raji/*Dox* cells induced with 151.75 nM doxorubicin (B), and Raji/*Dox* cells induced with 303.5 nM doxorubicin (C).

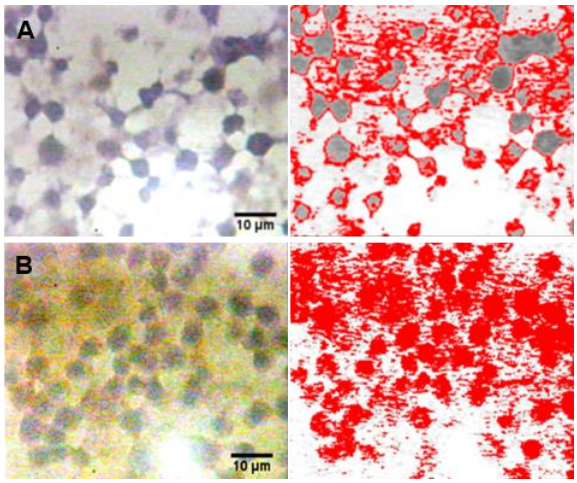

Figure 6. Immunocytochemical staining for P-gp and PTEN expression in Raji/*Dox* cells showing localisation of P-gp protein in the cell membrane (A), and PTEN protein in the nucleus and cytoplasm (B). Brown DAB staining corresponding to target proteins (left) was processed for better contrast (right) using ImageJ software.

D. Total RNA Isolation and RT-qPCR

Pure RNA with a ratio of A_{260}/A_{280} at 2.371 and 2.026 was isolated from the Raji/*WT* and Raji/*Dox* cell lines. The RNA was used for assessing the expression of *hsa-miR-21* by RT-qPCR. The melting temperatures for *hsa-miR-21*, U6 snRNA and UniSp6 were 68°C, 77°C and 68.6°C. There was no significant deviation across the replicates in the melting temperatures for all genes (Figure 7). The amplification specificity of *hsa-miR-21* and reference genes was confirmed by the single peak in the melt curve generated during RT-qPCR.

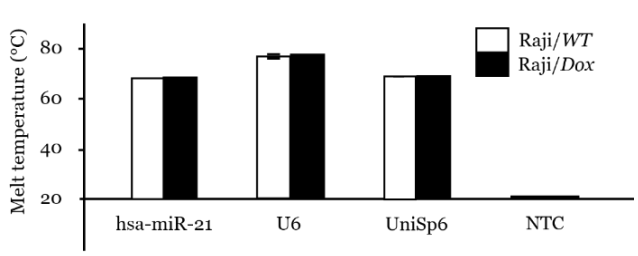

Figure 7. Melting temperature for *hsa-miR-21*, candidate reference genes (U6 snRNA and UniSp6) and no template control (NTC) in both normal and doxorubicin-resistant Raji cell lines. Error bars represent standard error of mean of three replicates used for each sample.

Quantification cycles (Cq), or the fractional cycle number at which the amount of amplified target reaches a fixed threshold (Ct), reflects the abundance of transcripts in the samples. The non-overlapping standard error bars for UniSp6 in both Raji/*WT* and Raji/*Dox* indicated there was no significant difference in the transcript abundance for this gene across both cell lines (Figure 8). As UniSp6 showed a stable expression in both normal and doxorubicin-resistant Raji cell lines (Figure 8), it was selected as the reference gene. Therefore, the expression of *hsa-miR-21* in both Raji cell lines was quantified relative to UniSp6.

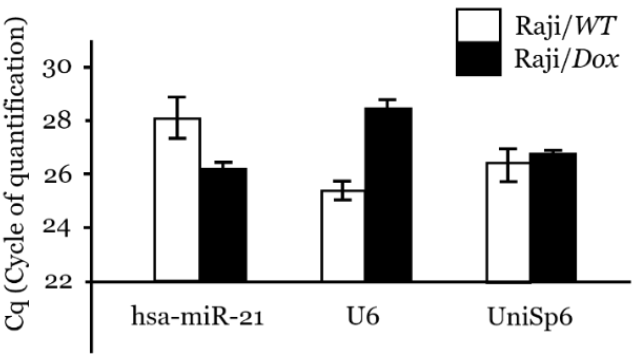

Figure 8. Raw Cq (or Ct) values for *hsa-miR-21* and the candidate reference genes (U6 snRNA and UniSp6) in both normal and doxorubicin-resistant Raji cell lines. Error bars represent standard error of mean of three replicates used for each sample.

Figure 9 showed the normalised expression of *hsa-miR-21* which was higher in Raji/*Dox* cells compared to the Raji/*WT* cells. The expression of *hsa-miR-21* in doxorubicin-resistant Raji cells was up-regulated 5.06-fold relative to the normal sensitive Raji cells.

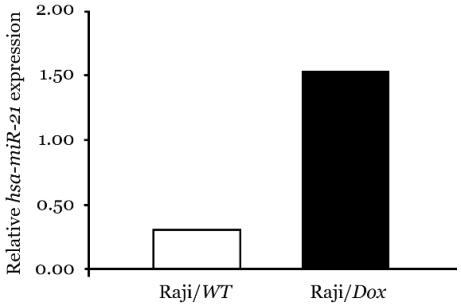

Figure 9. Relative expression of *hsa-miR-21* normalised to UniSp6 in Raji/*WT* and Raji/*Dox* cells.

IV. DISCUSSION

A doxorubicin-resistant cell line was developed from Raji cells to represent a drug resistant model of B cell non-Hodgkin lymphoma for investigating the epigenetic modifications of *hsa-miR-21* expression in mediating doxorubicin resistance. The intermittent exposure of Raji cells to doxorubicin resulted in a change in cellular morphology which is associated with the development of resistance to the drug. According to Liang and Huang (2001), alterations of membrane lipid structures and functions, including the tight lipid packing density and reduced membrane fluidity, may have contributed to drug resistance associated with the higher drug efflux rate in the human lung adenocarcinoma A549 cell line.

We found that the overexpression of *hsa-miR-21* was related to the up-regulation of P-gp and PTEN repression in doxorubicin-resistant Raji cells. Regulatory changes in the expression of P-gp and PTEN, proteins that are involved in drug transport and apoptotic pathway, are the mechanisms for the development of doxorubicin resistance. Cancer cells including lymphoma could become resistant to doxorubicin due to inactivated transporter or substrate protein. The alteration of transporter protein affects the capability of drug influx into cells. P-gp is a transport protein that protects the cells from toxicity by pumping the drugs out. Its expression is regulated by the MAPK (Ras/Raf/MEK/ERK) pathway that controls cell proliferation, survival and differentiation of cancer cells (Katayama *et. al.*, 2007; 2014; Shen *et al.*, 2011). The distribution of P-gp on the plasma membrane of the Raji/*Dox* cells was related to its function as transporter protein which has a role in toxin efflux mechanism in

normal cells. An increased expression of P-gp might confer resistance to doxorubicin in Raji cells and allow cell survival. Overexpression of P-gp as transporter protein in doxorubicin metabolism may have stimulated cancer resistance through the drug efflux mechanism (Brunton *et al.*, 2005).

PTEN can be localised to both the cytoplasm and nucleus, and its function is determined by its subcellular localisation. PTEN has a role in repressing the PI3K/AKT pathway when localised in the cytoplasm. It acts as a lipid phosphatase that down-regulates AKT that inactivates p27, leading to cell apoptosis. If there was a mutation or repression of PTEN, the PI3K/AKT pathway would be activated with an up-regulation of AKT to control the cell survival and inhibit apoptosis. Nuclear PTEN has more functions: it down-regulates MAPK (ERK) and cyclin D1 expression to mediate G0-G1 arrest in cycle cell; increases the activity of RAD51 and DNA damage repair; and maintains chromosomal stability by specifically enhancing centromere stability through interaction with CENP-C (Planchon *et al.*, 2008). Nuclear PTEN also regulates p53 and promotes a reduction in p53-mediated cellular reactive oxygen species production, which in turn reduces the oxidative stress-induced DNA damage (Chang *et al.*, 2008). PTEN activity has been shown to be repressed by *hsa-miR-21* that affected the apoptotic inhibition (Zhang *et al.*, 2010). In line with the down-regulated expression of PTEN in doxorubicin-resistant Raji cells, the decline in PTEN would inhibit apoptosis through the activation of AKT pathway that promotes cell growth, and allow the progression of lymphoma.

Based on the immunocytochemical analysis and RT-qPCR assay, a two-fold down-regulation of PTEN and 1.74-fold up-regulation of P-gp expression, along with a 5.06-fold overexpression of *has-miR-21* were observed in the Raji/*Dox* cells compared with the Raji/*WT* cells. Concomitant overexpression of *has-miR-21* and PTEN down-regulation in the Raji/*Dox* cells indicated that *has-miR-21* could be a potential biomarker that predicts doxorubicin resistance in chemotherapy treatment for non-Hodgkin B cell lymphoma. The microRNA can be considered as an alternative diagnostic marker for chemotherapy resistance, in addition to the protein-based biomarkers such as P-gp and PTEN antibodies. It can also serve as the target for managing doxorubicin resistance in the non-Hodgkin B cell lymphoma chemotherapy. The application of microRNA-based targeted therapies such as microRNA masks or target protectors, small molecule inhibitors, anti-microRNA oligos or microRNA sponge, to stabilise the expression of *has-miR-21* in cases of doxorubicin resistance of lymphoma may up-regulate the expression of PTEN so as to increase the sensitivity of lymphoma to doxorubicin chemotherapy.

V. CONCLUSION

This study confirms that *has-miR-21* has a role in mediating the resistance to doxorubicin based on the concomitant overexpression of *has-miR-21* and down-regulation of PTEN, a tumour suppressor, in the doxorubicin-resistant Raji cells. The microRNA could be used as a potential biomarker and serve as the target for doxorubicin resistance in chemotherapy treatment for non-Hodgkin B cell lymphoma.

VI. ACKNOWLEDGEMENTS

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