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Generating Paclitaxel-Resistant in Cervical Cancer HeLa Cell Line

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Abstract

Cervical cancer is one of the most leading causes of women death. Currently, paclitaxel is still one of the main therapeutic regimens for cervical cancer patients. However, some patients developed to be paclitaxel-resistant. Hence, studies to find out the novel strategies to resolve this problem are important. Generating resistant cancer cell lines can be utilized as the potent tool to evaluate the efficacy of any therapeutic agent toward cancer drug-resistant problems. Current studies describing the methods to establish chemoresistance are lacking. Moreover, study in Indonesia conducting chemoresistance in cell line is limited. This study was aimed to elaborate the characteristics of HeLa cells during generation of paclitaxel-resistant cervical cancer cells. The parental HeLa cells were exposed to an escalating concentration of paclitaxel for a long time period. Subsequently, cells were divided into two groups for the evaluation of resistance characteristics. The values of inhibitory concentration 50 (IC_{50}) and inhibitory concentration 90 (IC_{90}) were analyzed using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. Our data showed that the longer exposing periods of paclitaxel, the higher IC_{50} and IC_{90} values of HeLa cells are. IC_{90} of paclitaxel in HeLa Pac RB was increased from 69 pM, 440 pM, 2,561 pM and 10,337 pM on 0^{th}, 1^{st}, 2^{nd}, 3^{rd} and 4^{th} months, respectively. Interestingly, the resistant cells were recovered to be paclitaxel-sensitive when they were not being continuously exposed to paclitaxel. In addition, the paclitaxel resistant cells become less sensitive against 5-FU but not doxorubicin, cisplatin and etoposide. We were able to generate cervical cancer HeLa paclitaxel-resistant cell line. These cell line could potentially be utilized for further studies in order to understand the molecular mechanisms of drug resistance in cervical cancer and as a tool for cancer drug discovery.

Keywords: *cervical cancer, drug resistant cell line, paclitaxel resistant cells, stepwise escalating concentration*

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INTRODUCTION

Non communicable diseases (NCDs) have become one of the most focused health problems worldwide. Among all of the NCDs, the most common cancer in women is cervical cancer (McGuire, 2016). Due to its high level of prevalence and mortality rate, cervical cancer becomes a cancer that has been discovered intensely through numerous studies. Therefore, in order to support the reliability of the studies to evaluate therapeutic effects toward cervical cancer, a proper tool that reflects a certain condition of cervical cancer patient's is needed.

Currently, multiple cervical cancer treatments available for the patients; surgery, radiotherapy, and chemotherapy, in which could also be done in combination. The standard chemotherapy regimen that has widely given to the patients is a combination of cisplatin with paclitaxel (Koh, *et al*., 2019). As an adjuvant therapy, chemotherapy poses an important role as it prolongs patients' survival rate through preventing the cancer's recurrence and metastasis. However, some patients are-born-to-be or acquired resistant of chemotherapy. The resistance might happen on several chemotherapy drugs, in which one of those is paclitaxel. Studies to evaluate the mechanism of chemotherapy resistance in cervical cancer patients had been conducted. The studies showed the overall response rate is 29.1%–67% and median overall survival is 12.87 months in patients with advanced cervical cancer (Peng, *et al*., 2014; Zhu, *et al*., 2016). There are multitude factors contributed to the conditions that could potentially lead to the development of resistance in which the patient has low response to a given treatment (Mansoori, *et al*., 2017). In order to discover the mechanisms that establish the resistance of chemotherapy and further developing potential drug or substance, a cellular model that reflects human's homeostasis is needed.

Generating resistant cervical cancer cell lines can be utilized as a utility to evaluate the efficacy of any therapeutic agents toward cervical cancer drug-resistant problems. Methods to generate paclitaxel-resistant HeLa cells had been developed for years (Bi, *et al*., 2014; Lee, 2004; McDermott, *et al*., 2014; Peng, *et al*., 2014). To our recent knowledge, there is subtle amount of studies described the process of paclitaxel-resistant HeLa cells formation. Moreover, there is very limited study in Indonesia that utilized the generated cells, making this study might carry more benefits in addition to the complexity of the techniques. Hence, to confirm and establish the techniques is important to create an integral part of further studies in cervical cancer in Indonesia.

MATERIALS AND METHODS

Cell Culture

This study was subjected parental HeLa cells from Dr. Hasan Sadikin General Hospital, Bandung, Indonesia. HeLa cells were cultured in RPMI medium (cat No. 11875093, Gibco, New York, USA) supplemented with 10% fetal bovine serum (FBS) (cat No. 10270106, Gibco, USA) and 1% penicillin-streptomycin, incubated with a controlled temperature at 37^{o}C containing 5% CO_2.

Generating Paclitaxel-Resistant HeLa Cells

The parental HeLa cells were exposed with modified escalating concentration in a long period of time according to previous study (McDermott, *et al*., 2014). Briefly, after having data of inhibitory concentration 50 (IC_{50}) of paclitaxel in the parental cells, the cells were treated with IC_{50} of paclitaxel (from Pharmacy Dr. Hasan Sadikin General Hospital, Bandung, Indonesia) for three days followed by replacing with fresh medium to let the cells recovered. The HeLa cells were divided into 4 groups of exposure; the control (HeLa Parent), IC_{50} exposure (HeLa Pac RA), inhibitory concentration 90 (IC_{90}) exposure (HeLa Pac RB) and two-fold of IC_{90} exposure (HeLa Pac RC). These groups of exposure were done in two until three days depends on cells characteristics. If the cells had

seen dying microscopically, the culture medium was replaced with fresh medium until the cells are recovered. The experiment was repeated until reaching four months. At the end of each month, the cells were being evaluated for its resistance towards paclitaxel. The evaluation of the resistance characteristic was done through determination of its IC_{50} and IC_{90} values using (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay, (cat No. M2128, Sigma-Aldrich, Missouri, USA). Additionally, after the cells were confirmed to become paclitaxel-resistant, some of them were cultured without any exposure to paclitaxel for two months to understand further characteristic of HeLa cervical cancer resistant cells.

Cytotoxic Assay

In order to determine the IC_{50} and IC_{90} values as well as cytotoxic effects of tested drugs, the cells were exposed to MTT (Meerloo, *et al*., 2011). Briefly, the cells were seeded on 96-well plate then treated with serial concentrations on the next day. After that, the cells were placed in incubator for 72 h with 5% CO_2 at 37^0C. Cells contains medium with 1% Dimethyl Sulfoxide (DMSO) were used as control. The MTT solution was added and the mixture was incubated for 4 h. Next, DMSO was added to dissolve the formazan crystal. Absorbance was measured at the wavelength 550 nm for percentages of viable cells on treated cells, compared to the control cells.

In addition to evaluate the characteristic of paclitaxel resistance cells, both parental and paclitaxel resistance cells were exposed to different chemotherapy agents including 5-FU, doxorubicin, cisplatin and etoposide. This evaluation was aimed to know whether any cross resistant with other chemotherapy drugs. All procedures were conducted in the laboratory of cell culture and cytogenetic, Faculty of Medicine, Universitas Padjadjaran, Indonesia.

Data obtained from MTT assay was then analyzed for drug curves, the IC_{50} and IC_{90} value using four-parametric logistic regression by SigmaPlot for windows version 12.0 software (Systat Software Inc., San Jose, California, USA).

RESULTS

To evaluate the ability of HeLa cells to resist against chemotherapy, we used MTT assay to assess cell death after given in serial concentrations. Each condition of both parental and paclitaxel exposing cells were compared. The result showed that the higher of resistance level of the cancer cells the higher of their threshold to be able to reach the maximum number of cell death (Figure 1). Moreover, as the level of HeLa cell paclitaxel-resistant progressed increasingly in each month, the results showed an increased value of the IC_{50} and IC_{90} of the HeLa Pac RB. Upon exposing impulse concentration of paclitaxel, the IC_{90} of paclitaxel in HeLa Pac RB was increased from 69 pM, 440 pM, 2,561 pM and 10,337 pM on 0th, 1st, 2nd, 3rd and 4th months, respectively. (Figure 2).

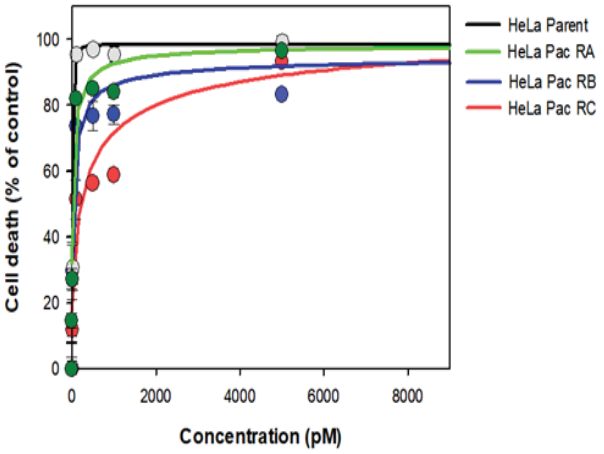

Figure 1. Drug curves of paclitaxel in HeLa cells after 3 months exposing different conditions of paclitaxel for generating HeLa paclitaxel resistant cells. HeLa Parent=parental HeLa cells. HeLa Pac RA=impulse exposing of IC_{50} concentration. HeLa Pac RB=impulse exposing of IC_{90} concentration. HeLa Pac RC=impulse exposing of two-fold of IC_{90} concentration.

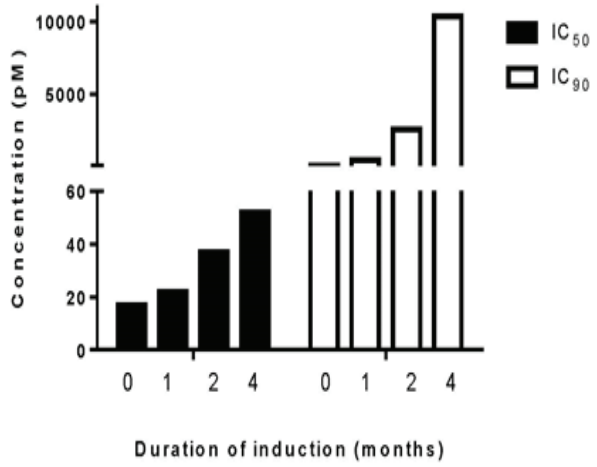

Figure 2. Both IC_{50} and IC_{90} increase following the exposing paclitaxel in HeLa Pac RB cells.

Subsequently, to observe whether the resistance occur permanently or temporarily, we stopped paclitaxel treatment for some resistant cells. Intriguingly, the experiment showed that the resistant cells become more sensitive to paclitaxel if the cells were not being treated (Figure 3).

Finally, we evaluate sensitivity of paclitaxel-resistant HeLa cells to other chemotherapeutic agents. We treated both parental HeLa cells and the paclitaxel-resistant HeLa cells with other conventional chemotherapeutic agents (5-FU, doxorubicin, cisplatin and etoposide). The result showed the paclitaxel-resistant HeLa cells become less sensitive against 5-FU but not doxorubicin, cisplatin and etoposide (Figure 4).

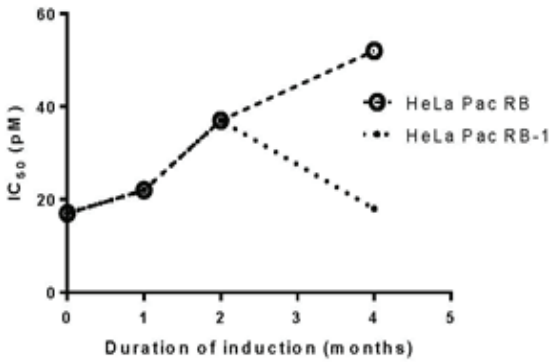

Figure 3. De-escalation of induction drug sensitizes the resistant HeLa Pac RB cells. After 2 months exposed with paclitaxel, the HeLa Pac RB cells and HeLa Pac RB-1 cells were exposed and not exposed, respectively, with paclitaxel for another 2 months.

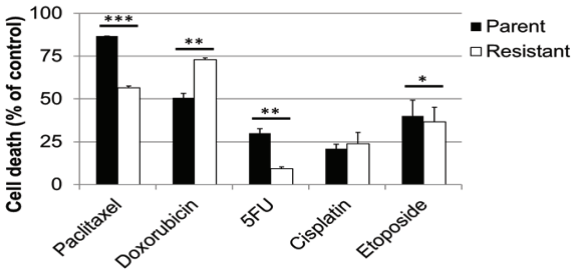

Figure 4. Different cytotoxic response of chemotherapeutics in parental HeLa cells and paclitaxel resistan ce HeLa cells. *)p<0.05; **)p<0.01, ***)p<0.001.

DISCUSSION

Paclitaxel is one of the chemotherapeutic agent that belongs to class of *taxane* and was produced from pacific pine trees (*Taxus brevifolia*) and European pine trees (*Taxus baccata*) (Soca-Chafre, *et al*., 2011). Paclitaxel has also known for its anticancer effects against various malignancies such as cervical cancer, ovarian cancer, breast cancer, lung cancer and other cancer types. The drug has a high affinity to bind with β-tubulin, thereby allowing polymerization of tubulin cells leading to disruption of decomposition of microtubules (Mariani, *et al*., 2015; Zhang, *et al*., 2012). This process leads to breakdown of mitosis processing, causing cancer cells apoptosis or cellular death. Moreover, paclitaxel has other mechanism by inactivating bcl-2 protein which also leads to cellular apoptosis (Demidenko, *et al*., 2008).

Paclitaxel has been used as chemotherapy regiments for cervical cancer patients. Unfortunately, during treatment some of the patients are developing resistance to the drug. Therefore, research to explore mechanisms of cancer cells' chemoresistance had also widely conducted to resolve the problems. There are numerous mechanisms of

cancer chemoresistance that have been discovered. The main mechanism of paclitaxel-resistant cancer cells is the overexpression of P-glycoprotein (P-gp) that works as a drug efflux pump. The P-gp will pump out the ingested drug throughout the cell. However, the use of P-gp inhibitors are sometimes ineffective or even toxic at the optimum dose to interfere P-gp functions (de Figueiredo-Pontes, *et al*., 2008). Other possible mechanisms of cancer cells' resistance to paclitaxel are: alteration of the drug affinity to bind with microtubules, alteration of tubulin structure and deregulation of cancer cell cycle (Dumontet and Jordan, 2010; Kavallaris, 2010). Therefore, the mechanisms of cancer cells' resistance to paclitaxel, as tubulin binding agent, are complex and remain unclear.

The parental HeLa cervical cancer cells were being exposed to high concentration of paclitaxel continuously for a long time period. The cells which successfully recovered after being exposed with higher IC concentration were considered as paclitaxel-resistant cells. Figure 1 showed the HeLa Pac RB and HeLa Pac RC have level of resistance on IC_{90} and two-fold IC_{90} respectively, and they never reach the rate of the cell death as HeLa parental cells. Furthermore, data from Figure 2 showed the longer cells were being exposed, the higher their IC_{50} and IC_{90} values. The most likely explanation to this finding is the cells were successfully adapted by overexpressing P-gp to pump out the ingested drug throughout the cell (Zhou, *et al*., 2019). Another possible explanation is the cells might specifically alter its tubulin structure, thus disturbing paclitaxel's affinity in tubulin binding. Therefore, the concentration of the drug inside resistant cell could not reach its maximum therapeutic effect.

Another interesting finding from our study is the resistant HeLa cells which not exposed to paclitaxel after two months (HeLa Pac RB-1), were regain their sensitivity to paclitaxel as their prior condition (Figure 3). Although the exact mechanisms of this finding has not been fully understood, we hypothesized a mechanisms based on study by Peng, *et al.* which proposed a novel paclitaxel-resistant pathway involving Warburg effect activated Hypoxia-inducible factor 1-alpha-mediated (HIF1-α-mediated) signaling induced autophagy (Peng, *et al*., 2014). It is well known that Warburg effect on cancer cell explain the cells' preference to solely use glycolysis as their energy source. Glycolysis end product, lactate and pyruvate, will up-regulate HIF1-α expression which then benefit cancer cell to undergo malignant transformation, metastasis, and develop chemo-resistance. (Lu, *et al*., 2002) Overexpression of HIF1-α will increase cancer cells' level of autophagy and creating paclitaxel-resistant cancer cell. Contrarily, if autophagy is inhibited, sensitivity to paclitaxel will be recovered (Peng, *et al*., 2014). Other study also confirmed that induction of autophagy correlate with paclitaxel resistance in cervical cancer. (Park, *et al*., 2018; Zou, *et al*., 2018) Another mechanism found to be correlated with acquired paclitaxel resistance is overexpression of microRNA miR-375. Paclitaxel up-regulates miR-375 expression during continuous exposure with the drugs, miR-375 will over-expressed and consequently, the chemo-resistance is developed. After discontinuation of paclitaxel in chemo-resistant cell, there was a gradually decreasing expression of miR-375 and its level of expression was restored to the level before paclitaxel administration. (Shen, *et al*., 2014) This finding may partially explain our finding, further study with several molecular markers are needed to explain mechanisms of paclitaxel resistance in cervical cancer.

CONCLUSION

In conclusion, HeLa parental cells that were being exposed in escalated concentration for a long period developed resistance to the chemotherapy. The duration of the constant exposure is positively correlated with the level of resistance. Also, resistant cancer cells will back to be more sensitive if the exposures were not being continued.

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