RESEARCH ARTICLE

GSTM1 Deletion Compensated in mRNA Expression and 4T1 Viability After Editing Using CRISPR/Cas9 Single and Double gRNA

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Abstract

BACKGROUND: Glutathione S-transferase Mu-1 (GSTM1) is known to undergo polymorphism and plays role in drug metabolism including Paclitaxel (PTX), the first-line chemotherapy for breast cancer. However, the effect of GSTM1 polymorphism against chemotherapy in breast cancer is limited and unexplored. This study was conducted to explore the effects of single and double guide (gRNA) on the GSTM1 knocked out (KO) and its effect on the response of PTX in the 4T1 cell line.

METHODS: The preparatory stage was done by culturing and electroporating 4T1 cells using Ribonucleoprotein of clustered regularly interspaced short palindromic repeats (CRISPR)/Caspase 9 (Cas9). KO validation was examined by quantitative reverse transcription polymerase chain reaction (qRT-PCR), Sanger sequencing, and ICE analysis. The 4T1 viability was examined by MTT Assay.

RESULTS: The number of base pairs of GSTM1 after being engineered by single or double gRNA was 86 bases. The DNA quantity of GSTM1 engineered by gRNA was more than using double gRNAs. The mRNA expression of GSTM1 engineered by single gRNA was lower than using double gRNAs. IC_{50} values of PTX between wildtype and KO were not significantly different, in the range of 30 μM.

CONCLUSION: The base-pair length of GSTM1 exon 4 that is knocked out with single and double gRNA have the same number of base pairs. The quantity of GSTM1 DNA and mRNA expression are contrary between single gRNA and double gRNA, and IC_{50} PTX values in the 4T1 cell line of the control group with single or double gRNA knocked out do not differ markedly. PTX efficiency as chemotherapy is not disturbed in the GSTM1 deletion genetic profile.

KEYWORDS: GSTM1, gRNA, Paclitaxel, CRISPR, breast cancer

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Introduction

Glutathione S-transferase (GSTs) are grouped into three according to their location in the cell, namely cytosolic, mitochondrial, and microsome. Glutathione S-transferase Mu-1 (GSTM1) is the most expressed mu (μ) class GST subfamily.(1) GSTM1 in humans is 4.2 kb consisting of 8 exons located on chromosome 1p13.3. The GSTM1 null variation is widespread in the world with a frequency of occurrence as much as 20-67%.(2) Several studies reported that human GSTM1 has a 78% homology level with *Mus musculus* so it can be used as a model in research.(1) GSTs play a role in the conjugation stage or drug metabolism phase II. Genetic variants of GSTs may cause an alteration in drug pharmacokinetics.(2)

Breast cancer is the type of cancer that causes the number two death in women in the world.(3,4) According to the International Agency for Research on Cancer (IARC), the incidence of breast cancer in the world in 2018 reached 2.1 million.(5) By 2050, the incidence of breast cancer will reach 3.2 million per year and become the most diagnosed cancer among women.(6) Triple Negative Breast Cancer (TNBC) is the most aggressive type of breast cancer. Its incidence rate reaches 15-20%.(7) TNBC does not express the receptor of Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor-2 (HER2) which causes chemotherapy to be the first recommended therapy.(5) A meta-analysis study showed that GSTM1 deletion may increase the risk of breast cancer in Asian, Caucasian, and postmenopausal women. GSTM1 null causes chemotherapy exported by the cells.(8)

In cancer, a patient's response to the therapy may be influenced by inherited factors and external factors. Personalized medicine appeared after the genome project to state that the theory of "one fits all" was not proper to apply. This phenomenon occurred in drug effects among patients, for example, the difference in toxicity.(9) Paclitaxel (PTX) is the first-line chemotherapy to stop the mitosis of cancer cells.(10) PTX toxicity is influenced by metabolism carried out by detoxifying enzymes of the GST group. This process involves CYP3A4, CYP2C8, ABCB1, PXR genes, and P-glycoprotein (P-gp) which work together to regulate, clear, and secrete PTX from cells.(7,11,12)

Clustered regularly interspaced short palindromic repeats (CRISPR) was isolated from microbial and originally known as the cellular immune system. Caspase 9 (Cas9) acts as a nuclease enzyme in the genetic editing tool. Previous research reported that Cas9 conducts to cut various DNA *in vitro* and suggested to adopt in cells or organisms, for example, cancer cell lines. CRISPR/Cas9 system raises the possibility to create a disruption in a targeted gene either deletion or insertion. The modifying gene will contribute an important role in future therapy.(13) CRISPR/Cas9 consists of gRNA and Cas9 endonuclease can be used as a technique for modeling polymorphisms by engineering certain genes.(14) Different cell types provide different results in CRISPR/Cas9 efficiency because this genetic editing tool is highly specific.(15) Exploration of CRISPR/Cas9 for use as polymorphism modeling has not been widely carried out. This study was conducted to create a GSTM1 deletion model in a triple-negative breast cancer cell line, 4T1, and explore the effect on mRNA expression as well as PTX therapy.

Methods

Cell Culture

Dulbecco's modified eagle medium (DMEM) (Gibco, Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Gibco), 1% penicillin-streptomycin (Gibco), and 0.5% fungizone (Gibco) were used for the 4T1 cell lines culture. Cells were incubated at 37°C in 5% CO_2, and counted periodically until 80% confluent. The research was carried out after obtaining an ethical permission from the Ethics Committee of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada (No. KE/FK/0059/EC/2022).

Formation of Ribonucleoprotein (RNP) Complex

The single guide RNA (gRNA) was designed at *https://idtdna.com*. The formation of RNP complex was prepared by mixing 0.3 µL of 62 µM Cas9 enzyme, 2.2 µL of 200 µM crRNA, 2.2 µL of 200 µM tracrRNA, and 0.2 µL buffer. The RNP complex was incubated at room temperature for 20 minutes. Genome editing was performed based on the CRISPR Cas-9 kit manual (Alt-R® CRISPR-Cas9 crRNA; Alt-R® CRISPR-Cas9 trac-RNA; Alt-R® S.p.Cas9 Nuclease V3, Integrated DNA Technologies INC, Coralville, IA, USA). RNPs were made for single and double gRNAs.

Electroporation

The 4T1 cell lines were took in 10 L of medium and 1 L of RNP complex and then added into a 1 mm gap cuvette. Electroporation was carried out at a 125V pulse duration of 1 ms for 1 pulse (following protocol from *www.idtdna.com*). Cells were removed from the cuvette for re-culture on 96-well plates, and the complete medium was replaced after 6 hours. The electroporated cells were then cultured.

Confirmation of Genome Editing

DNA isolation was carried out according to the protocol (FavorPrep DNA Isolation kit, Favorgen, Wien, Austria) and then was continued with polymerase chain reaction (PCR) (Promega Gotaq Green Master Mix, Promega, Madison, WI, USA) using primers that matched to gRNA targets: 5'-ATCGATGGATCACACAAGATCAC -3' (forward), 5'-CCAGGTGGTGCTTTCGGG -3 ' (reverse). Furthermore, electrophoresis and visualization were carried out with Geldoc. The qRT-PCR (Promega) was used to address the possibility of post-engineered exon skipping with CRISPR/Cas9 in DNA level. The PCR product was analyzed by Sanger Sequencing to define the knocked-out score by ICE

analysis (*ice.synthego.com*) (16), whereas the prediction of frameshift mutation was analyzed by Jalview 2.11.2.5 (The Barton Group - University of Dundee, Scotland, UK).

Expression of mRNA GSTM1

RNA isolation was carried out according to the protocol (Favorprep RNA Isolation kit), while synthesis of cDNA was carried out using reverse transcriptase PCR (RT-PCR) (Smobio Reverse Transcription Kit, Smobio, Hsinchu City, Taiwan). The qRT-PCR (Promega) was performed in 40 cycles with a pre-denaturation temperature of 95°C for 2 minutes, denaturation at 95°C for 30 seconds, and annealing at 59°C for 1 minute.

MTT Assay

MTT was dissolved in Dulbecco's Phosphate Buffered Saline (DPBS) pH 7.4 in 5 mg/mL. The MTT solution was protected from light and stored at -20°C. Cells were cultured in each well of the 96-well plate to attach. The complete medium was replaced with a complete medium that contain PTX at a concentration of 250 µM; 125 µM; 62.5 µM; 31.25 µM; 15.6 µM; 7.8 µM; 3.9 µM, and 0 µM as control volume 100 µL.(17) Three wells emptied (culture medium only) as blanks. Cells were incubated for 24 hours, then 100 µL of MTT solution was added to each well. After 4 hours, SDS solution was used to stop MTT, and the plate wrapped in aluminium foil overnight. Observations using an enzyme-linked immunosorbent assay (ELISA) microplate reader with a wavelength of 595 nm was then performed.(18)

Statistical Analysis

Results of the Geldoc visualization calculated to determine the standard curve equation and calculate the base length. The Geldoc measurement was done using ImageJ (National Institutes of Health, Bethesda, MD, USA). The results of qRT-PCR DNA and mRNA were calculated using the Livak equation. The absorbance value of the MTT assay calculated using Probit analysis to determine the IC_{50} value. Data were shown in mean±SD. These values were analyzed statistically using SPSS Ver.24 (IBM Corporation, Armonk, NY, USA), while GraphPad Prism 9.0.0 (GraphPad Software, San Diego, CA, USA) was used to design the graphs.

Results

The GSTM1 gene in 4T1 successfully edited using single and double gRNAs. Figure 1 showed the results of the GSTM1 DNA qRT-PCR. The quantity of GSTM1 DNA in cells with single, double, and wild-type gRNA were 0.71±0.07, respectively; 0.01±0.003; and 1.06±0.3. There was a significant difference between the single gRNA group and the double gRNA group ($p<0.05$). A significant difference showed between the double gRNA group and the wild type as well ($p<0.01$). Whereas the single gRNA group was not significantly different from the wild type ($p>0.05$). The result based on the Tukey HSD post-ANOVA test indicated that use of double gRNA produced larger deletion in GSTM1.

The Gel Doc in Figure 2 was a qualitative test to estimate the presence or reduction of GSTM1 in DNA level. The frequency of GSTM1 deletion based on equation described in previous publication was 2.27%.(19) This value was obtained by counting base pairs in the results of Gel Doc Figure 2. The number of base pairs for cells engineered using a single gRNA was 86 base pairs. The number of base pairs for cells engineered with double gRNAs was 86 base pairs. While in wild-type cells the number was 88 base pairs. The number of base pair determined after measuring the distance between each well and band by ImageJ.

Alignment analysis by Jalview showed the prediction of frameshift mutation around the cut site (Figure 3). A full Consensus (black bar) was correspondence to the same nucleotide between edited and wild-type sequences while a half-black bar meant a different nucleotide between edited and wild-type sequences. The number of cut sites same as the number of gRNA that used. Figure 3A had one cut site, Figure 3B had two cut sites. Figure 3B showed more half-black bars than Figure 3A which indicated the use of double gRNAs produced a larger frameshift mutation on GSTM1.

The quantitative result to determine the percentage of GSTM1 deletion showed in Figure 4. The use of single

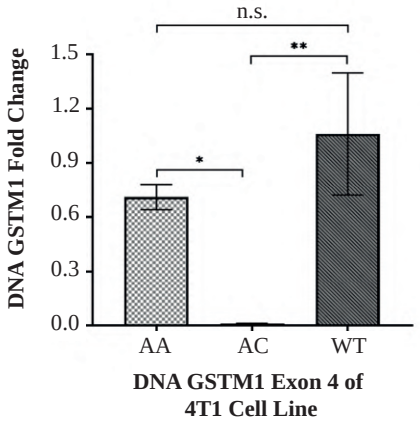

Figure 1. DNA quantity shown by DNA GSTM1 fold change. Value was obtained from ANOVA test followed by Tukey HSD. n.s: not significant ($p>0.05$), *$p<0.05$, **$p<0.01$. AA: single gRNA group; AC: double gRNAs group; WT: wildtype.

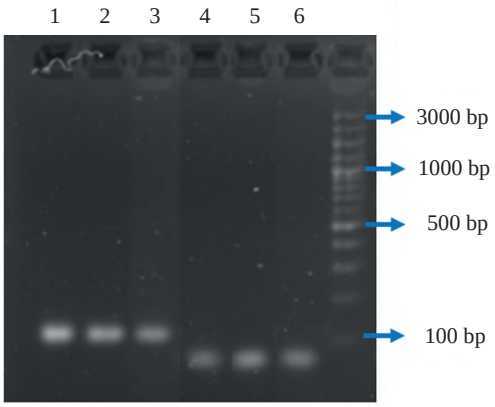

Figure 2. Band of DNA GSTM1 Exon 4. Lane 1: edited GSTM1 by single gRNA; Lane 2: edited GSTM1 by double gRNAs; Lane 3: GSTM1 of wild type; Lane 4: GAPDH of single gRNA group; Lane 5: GAPDH of double gRNAs group; Lane 6: GAPDH of wild type.

gRNA produced a 5% deletion of GSTM1 compared to the wild type. While the use of double gRNAs produced higher deletion, 14% compared to the wild type. The base deletion occurred at the cut site which separated as far as 2 or 3 bases from the PAM. These ICE analysis results had a similar trend to the DNA quantity in Figure 1. We predicted multiple gRNAs will cause a higher deletion at the targeted gene.

GSTM1 in RNA level determined by qRT-PCR. The mRNA expression showed in Figure 4. Expression values of GSTM1 mRNA engineered with single, double, and wild-type gRNAs were 0.14±0.004, 0.94±0.1, and 1.02±0.2. There was a significant difference between the single gRNA group and the double gRNAs group (p<0.01). A significant difference showed between the single gRNA group and the wild type as well (p<0.01). Whereas the double gRNAs and wild type were not different significantly (p>0.05). The result based on the Tukey HSD post-ANOVA test. Figure 5 showed the opposite result from Figure 1 which may indicated the phenomenon of genetic compensation or exon skipping.

The viability test results indicated by the IC_{50} values in Figure 6. The IC_{50} value of each group of single gRNA, double gRNAs, and wild-type cells respectively was 33.5±1.4 µM; 35.3±2.2 µM; and 33.2±0.6 µM. After the statistical tested by the Kruskal-Wallis , we found there were no significant differences between each group (p>0.05). The IC_{50} value of all groups was around 30 µM. The similarity of cytotoxicity could be assumed that GSTM1 presented in both edited groups.

Discussion

Data on the gene bank (NCBI) showed *M. musculus* GSTM1 has 8 exons. Two types of independent gRNA target exon 4 which has a base pair number ranging from 80-90 base pairs. Figure 2 shows the location of the GSTM1 band below the 100 bp ladder line which indicates that GSTM1 has a molecular weight of less than 100 bp. Gel Doc visualization shows the similarity in density and molecular weight between AA and AC cells with wild type. Factors that can affect indel frequency in genetic engineering using CRISPR, include protospacer adjacent motif (PAM) direction, formation of DNA double strand breaks (DSB) and non-homologous end joining (NHEJ), local chromatin context, and electroporation technique used according to specific cell types.(20)

In this study, both single and double gRNAs had PAM-in and PAM-out directions with a precision level of more than 50%.(21) However, double gRNAs have overlapping

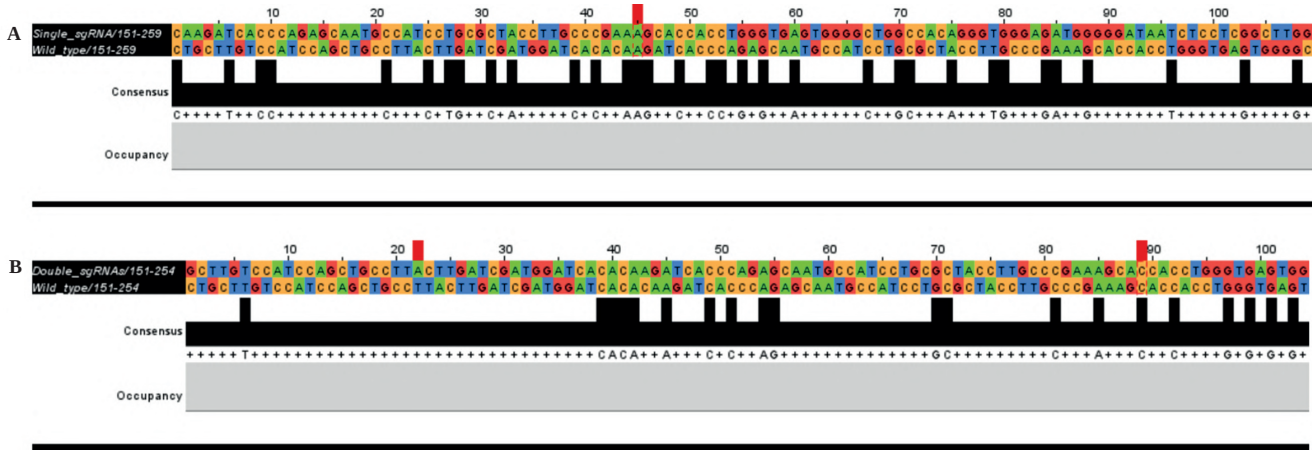

Figure 3. The result of sequence alignment by Jalview. A: Alignment between single gRNA sequence and wild type. B: Alignment between double gRNAs sequence and wild type. The red box above the edited sequence represents the cut site.

Figure 4. The result of ICE Analysis. A1: The comparison between wild type (above) and knocked out by single gRNA (below). A2: Indels percentage (blue bar) and discordance plots between wild type (orange line) and knocked out by single gRNA (green line). B1: The comparison between wild type (above) and knocked out by double gRNAs (below). The vertical black dots line indicates the cutting site while the horizontal red dots line is the PAM site. B2: Indels percentage (blue bar) and discordance plots between wild type and knocked out by double gRNAs.

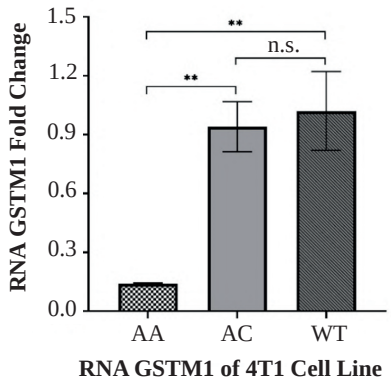

Figure 5. The expression level of GSTM1 mRNA. Value was obtained from ANOVA test followed by Tukey HSD. n.s.: not significant (p>0.05), **p<0.01. AA: single gRNA group; AC: double gRNAs group; WT: wildtype.

regions which cause the deletion target location to be very narrow. The distance of two gRNAs that are very close (less than 150 bp) can trigger DSB to form point mutations and frameshift mutations. Furthermore, the cells will perform a DNA repair mechanism, namely non-homologous end joining-precision deletion (NHEJ-PD).(22)

The DNA quantity in Figure 1 shows that the GSTM1 exon was successfully edited by both single and multiple gRNAs. The quantity of exon 4 GSTM1 DNA engineered with single gRNA resulted in more deletions than double gRNA. This happened in several studies, which was thought because of the close distance between the two gRNAs resulting in fewer cut sites. The use of two types of primers, which are near and far from the cut site, can be a solution to detect the narrow cut site area.(20)

The phenomena in Figure 1 and Figure 5 can be explained by two hypotheses, engineering techniques,

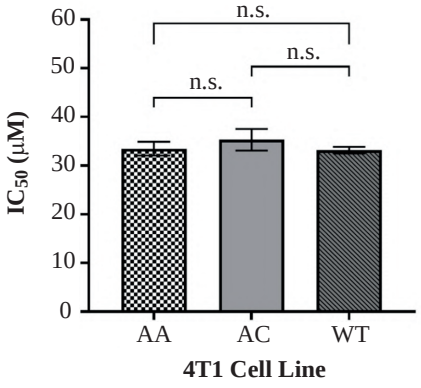

Figure 6. IC_{50} value. Data werre shown in mean±SD, value was obtained from Kruskal-Wallis test. n.s.: not significant (p>0.05). AA: single gRNA group; AC: double gRNAs group; WT: wildtype.

and genetic compensation. The engineering techniques considered are PAM direction, the ability of chromatin to perform breathing and remodeling, and the electroporation technique performed with more than one pulse for groups of eukaryotic cells.(21-25) The quantity of DNA as a result of the knockout with CRISPR is influenced by several factors, such as the value of the GC content of the target sequence and the specificity of the Cas9-gRNA complex by knowing which spacer sequence will correspond to the target DNA. (26-28)

GSTM1 is a member of the GSTs subfamily. Other members of the GSTs family are GSTM2, GSTM3, GSTM4, and GSTM5. Each subfamily has a specific substrate but shares the level of homology. This indicates an opportunity for other GSTM subfamilies to overcome the absence of other subfamilies.(2,29) Previous study reported a result of genome analysis using ClustalW to determine homology between GSTM subfamilies.(29) Based on the research, it is known that the homology between GSTM subfamilies is 75-99%. Observation of the 3D structure shows that GSTM2 has identical levels that are most similar to GSTM1. Experiments on HeLa cells that have experienced knocked out GSTM1 and it is known that there is compensation in the form of overexpression of GSTM2. Formation of GSH-SF conjugation in dead GSTM1 null cells (30), which showed the contribution of GSTM3 as a risk factor for breast cancer in the state of GSTM1 null pepper isoenzyme substrate overlap between GSTM1 and GSTM3. the absence of GSTM1 can be compensated by GSTM3 to maintain cell biological function. Compensation of GSTM1 by its isoform also occurred in this study.

Meanwhile, the cell has the ability to maintain its integrity which is done by the nucleosome. This mechanism is known as local chromatin context which can occur by two mechanisms, namely nucleosome breathing, and remodeling. This mechanism can increase the efficiency of Cas9 activity because for a while the chromatin will have a weak bond with histone proteins. The use of remodeling, such as DNA methyltransferase can be a strategic choice to reduce the strong binding to nucleosomes and increase the efficiency of Cas9 in genetic engineering.(25)

IC_{50} values in AA and AC cells were in the same range as the wild type and were not significantly different between groups. From this result, we stated electroporation is a tricky editing method because the voltage, number of pulses, pulse duration, and enzyme concentration must be specific for every cell line. This limitation is covered by the easy, cheap, and fast method of electroporation. Meanwhile,

IC_{50} is limited to measuring the cell viability and cannot fully explain the further effect of the edited gene. Our result shows the predictive implication of exon skipping, compensation events, and the need of electroporation optimization techniques for the 4T1 cell line. GSTM1 acts as a modulating signal factor and responds to toxicity conditions. *In vitro* studies have shown that GSTM1 is associated with the JNK signaling pathway that regulates apoptosis.(1) In this signaling kinase pathway, the presence of GSTM1 binds to apoptotic signal-regulating kinase 1 (ASK1) so that it becomes an inhibitor of JNK signaling. JNK is pro-apoptotic signaling.(31) Meanwhile, dephosphorylation of GSTM1 from ASK1 will cause the ASK1 and GSTM1 complexes to separate so that ASK1 can phosphorylate with JNK to activate the apoptotic pathway. (32) Further studies to explore engineering on the 4T1 cell line are needed for modeling and other functional assays.

Conclusion

Engineering using single and multiple gRNAs can specifically edit GSTM1. The quantity of DNA in single gRNA engineering was higher than in double gRNA. This trend was reversed with GSTM1 RNA expression. The IC_{50} value of PTX showed GSTM1 may be compensated by another GSTs subfamily or cellular mechanism to defend its integrity. Compensation should be considered for further research. The exploration of genetically engineered CRISPR/Cas9 in the 4T1 cell line as a model has the potential for precision medicine or pharmacogenetics research in the future.

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Authors Contribution

EVA, DAAN, AHS planned the idea of the study; EVA performed experiment and wrote the manuscript; EVA and DAAN analyzed and interpreted the result; meanwhile DAAN and AHS supervised the study.

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