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CRISPR/Cas9-based Editing of *CDK4, p107*, and *TGF*β1 in Human Breast and Lung Cancer Cells

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ABSTRACT

The CRISPR/Cas9 system is considered one of the most controversial yet powerful genome-editing tool with high potentiality and wide-angled array of biomedical applications. Cancer is considered one of the serious health-threat diseases that could be controlled, and even, cured by CRISPR/Cas9. In the present study, three cancer-related genes (*CDK*4, *p107*, and *TGF*β1) were targeted in breast cancer cells (MCF-7) and lung cancer cells (A549) with CRISPR/Cas9 cassettes. Cells were transfected with these crRNAs to knockout *CDK* and *TGF*β1, and to activate *p107* in a dose-optimized manner. Viable cell count was measured using Trypan blue assay. Semi-quantitative PCR was also performed to detect the knockout and activation of the genes under study. Two-dimensional polyacrylamide gel electrophoresis was carried out to ensure the up/downregulation of the studied genes in both MCF-7 and A549. Transfecting MCF-7 and A549 has resulted in a significant reduction in the cell count (p>0.005). Cell viability was also measured using MTT assay, and the results showed a significant decrease in the overall viability of cells after being challenged with CRISPR/Cas9. Semi-quantitative PCR was performed against specific primers to ensure the elimination/activation of the target genes. Data indicated a downregulation of *CDK*4 and *TGF*β1 and upregulation of *p107*. Two-dimensional protein gel electrophoreses was also conducted to indicate the level of up/down regulation of the specific proteins encoded by these genes. Data indicated a partial presence of *CDK*4 and *p107* and a complete absence

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of *TGF*β1 protein. This study might indicate the efficiency of editing tumor-related genes within the genome of malignant cells. However, further confirmative studied are needed to validate the use of CRISPR Knockout (KO)/Activation (AC) in controlling cancer *in vitro*.

KEYWORDS: CRISPR; Knockout; *CDK4*; *p107; TGFβ1*; Lung; Breast; Cancer.

INTRODUCTION

Cancer is a leading cause of death worldwide, accounting for 8.8 million deaths in 2015. The most common causes of cancer death are cancers of breast (571,000 deaths) and lung (1.69 million deaths) [1]. Cancer is caused by a series of alterations in genome and epigenome mostly resulting in activation of oncogenes or inactivation of cancer suppressor genes [2, 3]. This disease is a multistep process in which not one, but several mutations are required to cause functional abnormality that leads to tumorigenesis [4].

Breast cancer, in this context, is emerging by a multistep process, which can be broadly equated to transformation of normal cells *via* the steps of hyperplasia, premalignant change and *in situ* carcinoma [5]. Several genes are involved in the tumorigenesis and progression of breast cancer, but functional validation of candidate cancer genes remains unsolved [6].

Lung cancer, which has a low survival rate, is a leading cause of cancer-associated mortality worldwide. Smoking and air pollution are the major causes of lung cancer; however, numerous studies have demonstrated that genetic factors also contribute to the development of lung cancer [7, 8]. The overall survival rate of lung cancer patients remains poor despite the availability of standard treatments [9].

Clustered regularly interspaced short palindromic repeats (CRISPR) is a powerful genome editing technique [10]. Cancer characterization and modeling have benefitted greatly from the genome editing capabilities of CRISPR/Cas9 [11]. By rapidly introducing genetic modifications in cell lines, organs and animals, CRISPR/Cas9 system extends the gene editing into whole genome screening, both in loss-of-function and gain-of-function manners [12, 13]. This technology, developed based on the discovery that DNA double strand breaks (DSBs), could stimulate endogenous DNA repair machinery, mainly through homology-directed repair (HDR) and non-homologous end-joining (NHEJ) [14]. Recent CRISPR screens detected thousands of essential genes required for cellular survival and key cellular processes; however, discovering novel lineage-specific genetic dependencies from the many hits remains a challenge [15].

Cellular proliferation is controlled by several cell-cycle checkpoint proteins. In malignancy, the genes encoding these proteins are often disrupted and cause unrestrained cancer growth. The proteins are over-expressed in numerous malignancies; thus, they are potential targets for anti-cancer therapies [16]. One of these proteins becomes the master regulators, cyclin-dependent kinases (*CDKs*), which serves as the actual driving forces behind the progression of cell cycle in eukaryotic cells [17]. *CDK*/cyclin complexes regulate each phase of the cell cycle and the breakdown of this regulation in any phase results in uncontrolled growth and thus, tumor formation. Most, if not all of the cancers show direct or indirect deregulation of these kinases, therefore targeting *CDKs* is an important aim or factor to develop new anticancer therapeutics [18].

The retinoblastoma (*RB*) tumor suppressor and its family members, *p107* and *p130*, function by repressing E2F transcription factor activity to attenuate the expression of genes essential for cell cycle progression through modulation of the E2F family of transcription factors [19]. Transforming growth factor-beta1 (*TGF*β1) is a cytokine, which intricately controls a plethora of physiological and pathological processes during development and carcinogenesis. *TGF*β1exerts anti-proliferative effects and functions as a tumor suppressor during early stages of tumorigenesis, whereas at later stages it functions as a tumor promoter aiding in metastatic progression through an autocrine *TGF*β1 loop. For that, targeting *TGF*β1is a valid approach to control cancer, at least *in vitro* [20].

Here, in the present study we are aiming to assess the efficiency of using CRISPR/Cas9-based approach to control breast and lung cancers via knockout of both *CDK4* and *TGF*β1, and activation of *p107* genes.

MATERIALS AND METHODS

Cell line maintenance

Lung cancer cell line (A549) and breast cancer cell line (MCF-7) were obtained from the Holding Company for Biological Products and Vaccines (VACSERA, Giza, Egypt). All cells were seeded at a density of 104 cells/cm2 in 12-well plate, on RPMI 1640 media (GIBCO/Invitrogen Life Technologies, Carlsbad, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA) and 1% antibiotic mixture. Cells were cultured under standard

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laboratory conditions; at 37 °C and 5% CO2. MCF-7 and A549 cells were limited to use within the first 10 passages from the original purchased flask to control genomic drift due to instability. RPMI-1640 was changed every 3 days and cells were passaged whenever it reached 65–80% confluence or confluent?

CRISPR/Cas9 transfection

All CRISPR/Cas9 kits (*p107* activation, *CDK4* knockout, and *TGF*β1 knockout) were purchased from Santa Cruz Biotechnology (USA) **(Figure 1)**. The protocol of transfection was as follows: approximately 2 x 10^5 cells were seeded in a 6-well round U-bottom tissue culture plate on 3 mL of antibiotic-free standard growth medium per well 24 hours prior to transfection. Cells were grown to about 60-80% confluence, which was obtained normally after 24 hours. The following solutions were prepared: *Solution A:* For each transfection (and for each gene), 1µg (dissolved in 10 µL of deionized water) of plasmid solution was added to 140 µL of plasmid transfection medium to bring the final volume to 150 µL. The mix was pipetted up and down and incubated for five minutes at room temperature. Solution B: For each transfection (and for each gene), 10 µL of UltraCruz transfection reagent was diluted in 140 µL of plasmid transfection medium to bring the final volume to 150 µL. The mixture was pipetted up and down and incubated for 5 minutes at room temperature. The Plasmid DNA solution (Solution A) was added dropwise directly to the dilute UltraCruz transfection reagent (Solution B), vortexed immediately and incubated for 20 minutes at room temperature.

Prior to transfection, the old antibiotic-free medium was replaced with fresh media and the 300 µL of Plasmid DNA/ UltraCruz transfection reagent mix (Solution A + Solution B) was added dropwise to the each well, and then gently mixed by swirling the plate. After that, the cells were incubated for 48 hours under normal laboratory conditions. Finally, the cells were harvested and resuspended in a 1 mL of fresh serum-containing medium to inactivate trypsin and cultured normally or stored at -20 °C for further analysis. The same cell lines were kept as control, where it was treated with transfection media and transfection reagents only.

Trypan Blue exclusion viable cell assay

After treatments, cells were trypsinized and resuspended in equal volumes of medium and Trypan blue (0.05% solution) and counted using a haemocytometer. Trypan blue dye (Invitrogen) exclusion was used to assess cell viability. Blue cells were considered non-viable, and unstained cells were considered viable.

RNA extraction

Total RNA was extracted from CRISPRized and unCRISPRized cells with the RNeasy kit (Qiagen, Hilden, Germany). RNA was treated with DNase I (Boehringer-Mannheim, Mannheim, Germany) for 50 min and purified according to the kit's protocol. The quality and integrity of RNA were checked by spectrophotometry and ethidium bromide agarose gel electrophoresis.

First-strand cDNA synthesis

In a fresh tube, 200 ng of RNA and 1 µL of 25 µM random hexamer primer are mixed together and completed with RNase-free water to a final volume of 5 µl. The mixture was incubated at 72 °C for 3 min, and immediately placed on ice. Then, 2 µL of 5× SMARTScribe buffer, 1 µL of 10 mM deoxynucleotide triphosphate (dNTP) mix, 1 µL of 20 mM DTT and 1 µL of SMARTScribe Reverse Transcriptase (100 U/ µl) (Clontech) were added to the mixture and mixed gently by up and down pipetting. The first-strand cDNA reaction mixture was incubated at 42 °C for 60 min and the reaction was terminated by heating at 70 °C for 15 min in a thermal block.

Table 1: The primers sequences used in the present study.

Primer name	Sequence
CDK4-F	5′-GCGCCAGTTTCTAAGAGGCCTAGAT-3′
CDK4-R	5′-CGGGTGTAAGTGCCATCTGGTAGCT-3′
p107-F	5′-CAATGCTATAATGTGCCCAA-3′
p107-R	5′-TAGGATTCCGCATACAAGAT-3′
*TGF*β1-F	5′-CATCCATGACATGAACCGACCCTT-3′
*TGF*β1-R	5′-ACGAAGTTGGCATGGTAGCCCTT-3′

Semi-quantification of gene expression

PCR was performed on all treated and untreated cells against the primers under study **(Table 1)**. The following set-up was used and measured in triplicate: PCR mix 12.5 µL Master mix (Invitrogen), 0.5 µL forward primer (10 µM), 0.5 µL reverse primer (10 µM), and 1 µL cDNA (~ 30 ng) and dH_2O up to 25 µL. The thermal profile set-up was: initial denaturation at 95 °C for 10 min; 35 cycles of 45 sec at 95 °C, 30 sec at (58 °C for *CDK4*, 57 °C for *p107*, and 60 °C for *TGF*β1), and 75 sec at 72 °C; and then 5 min final extension at 72 °C. The PCR products were electrophoresed for 30 min on 1.6% agarose gel after being stained with ethidium bromide. The PCR assays were

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performed on the Uno II, Biometra. The relative expression of a specific mRNA compared to a control was calculated by measuring the bands intensity using Gel Pro analyzer, UK.

Protein extraction

Total protein extraction was performed using phenol extraction method; briefly, cells were centrifuged at high speed and resuspended in 3 mL of extraction buffer and stored at 4 °C. Just prior to use, 2-ME and 1 mM PMSF were added to pre-chilled buffer. The suspension was incubated for 10 min with continuous shaking at 4 °C. An equal volume of Tris-buffered phenol was then added, and the solution was again incubated on a shaker for 10 min at 4 °C. The aqueous and organic phases were separated by centrifugation at 13,000 rpm for 15 min at 4 °C. The phenolic phase was carefully recovered and re-extracted with equal volume of extraction buffer. The samples were vigorously vortexed and centrifuged for phase separation at 13,000 rpm for 15 min at 4 °C. The phenolic layer was transferred to a fresh tube for precipitation of proteins by addition of ammonium acetate (0.1 M) in cold methanol with subsequent overnight incubation at -20 °C. The precipitate/protein pellet was washed thrice with precipitation solution (stored at -20 °C), and final washing was performed with chilled acetone. The pellet was air-dried and then dissolved in rehydration buffer (7 M urea, 2 M thiourea and 2% (w/v) CHAPS).

Two-dimensional gel electrophoresis

The cellular proteins were solubilized in urea lysis buffer (7 M urea, 2% wt/vol CHAPS, and 50 mM DTT). The protein concentration was estimated by reagent compatible and detergent compatible (RC-DC) protein assay kit (Bio-Rad), and the readings were taken at 750 nm. To remove the contamination from the solubilized protein samples, urea/thiourea lysis buffer with ice-cold acetone was used. The precipitated proteins were suspended in rehydration buffer mentioned above.

Statistical analysis

Statistical significance was assayed by Student's t-test. The mean and standard error of the mean of the results of each experiment are shown in the figures. An asterisk indicates that $p \leq 0.05$.

RESULTS

Knockout/activation by CRISPR/Cas9

The present investigation aimed to control the proliferation of cancer cells that were resistant to traditional therapies by activation of *p107* and knocking out of both *CDK*4 and *TGF*β1. In the present study, two malignant cell lines (lung and breast) were used to study the impact of activation/knocking out of the target genes. Cell count was measured by trypan blue assay. Results obtained indicated that cell count was significantly reduced due to transfecting the two cell lines (p= .003). Breast cancer cells (MCF-7) were challenged with three plasmid transfections aiming to activate *p107* and knockout both *CDK*4 and *TGF*β1 **(Figure 1-3)**. Transfecting MCF-7 cells with *p107*, *CDK*4, and *TGF*β1 resulted in 63%, 35%, and 58.3% reduction in the cell count compared to non-treated ones, respectively.

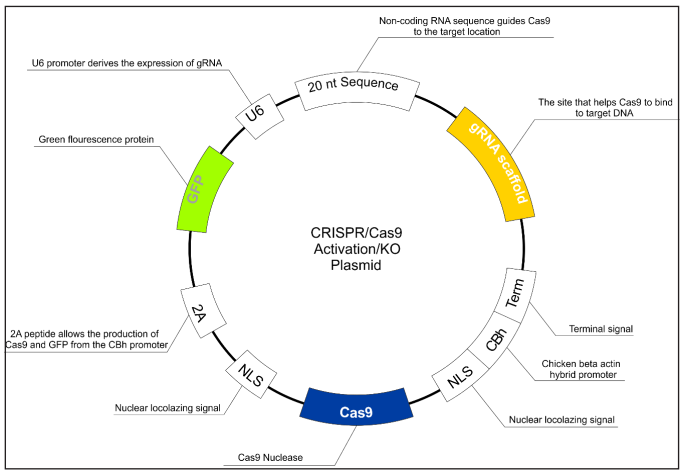

Figure 1: The CRISPR/Cas9 activation/KO plasmid structure.

On the other hand, transfecting A549 lung cancer cells with *p107*, *CDK*4 and *TGF*β1 resulted in 26.9, 41.6, and 45.1% reduction in the cell count, respectively **(Figure 4 and 5)**.

Detection of the edited genes

In the present study, MCF-7 and A549 cells were CRISPRized to edit *CDK*4, *p107*, and *TGF*β1 in order to assess their role in the progression of these types of cancer. After activation of *p107* and knocking out of both *TGF*β1and *CDK*4, the presence/absence of these genes was detected using semi-quantitative PCR. Results obtained indicated that for breast cancer cells **(Figure 6 and 8)**, knocking out both *CDK*4 and *TGFβ1* affected the overall cell viability. Meanwhile, the activation of *p107* caused the cells to commit suicide.

Lung cancer cells exhibited the same profile after being challenged with CRISPR/Cas9 to knockout *TGF*β1and *CDK*4 and to activate *p107* **(Figure 7 and 8)**.

Our results showed that CRISPRising lung cancer cells to knockout *CDK*4 was not efficient in completely removing

this gene. Although, a significant decrease (P= 0.0035) in the cells count was observed. Activation of *p107* also resulted in decreasing the cell count compared to control.

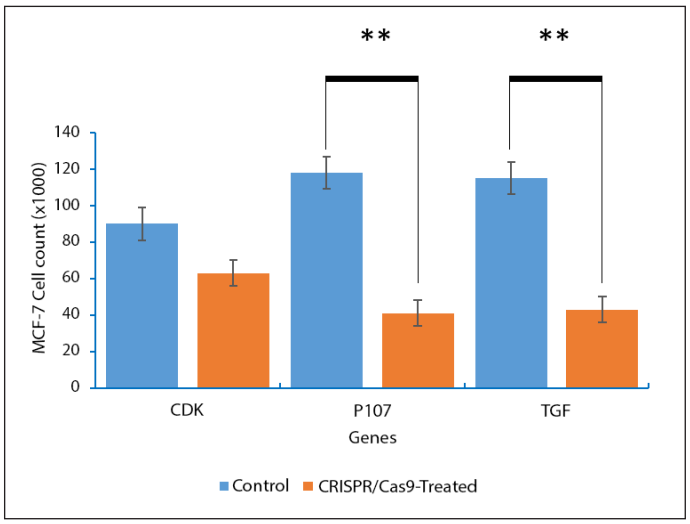

Figure 2: The MCF-7 breast cancer cells count after being knocking out of with CDK4 and TGFβ1, and activation of p107.

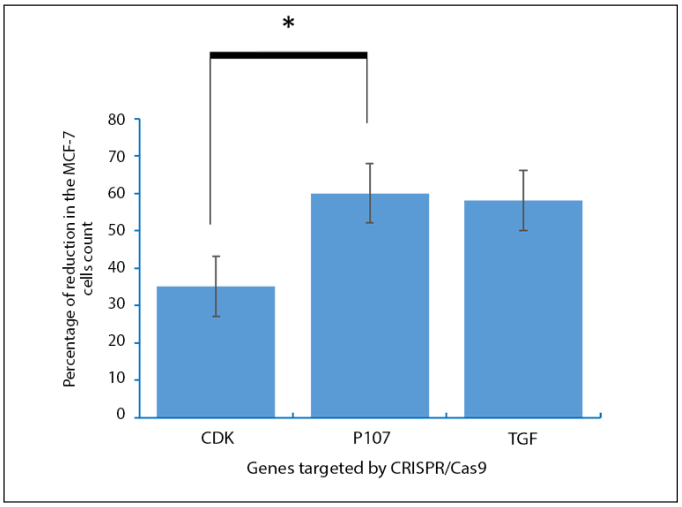

Figure 3: The percentages of the reduction in the MCF-7 breast cancer cells count caused by editing *CDK4*, *p107*, and *TGFβ1* using CRISPR/Cas9.

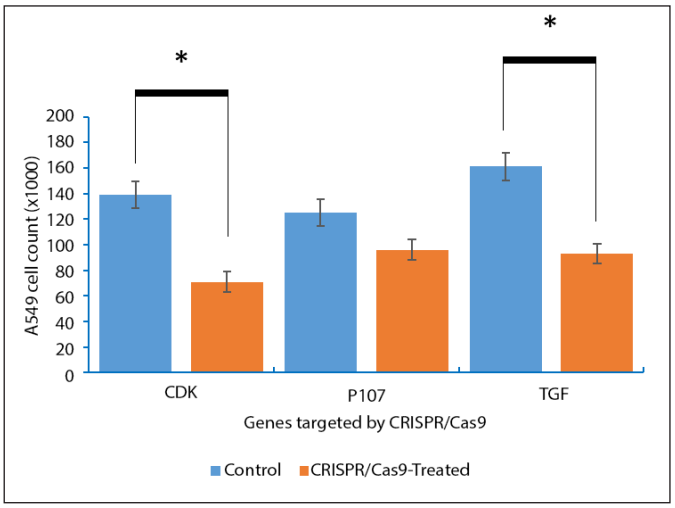

Figure 4: The A549 lung cancer cells count after being knocking out of with *CDK4* and *TGFβ1*, and activation of *p107*.

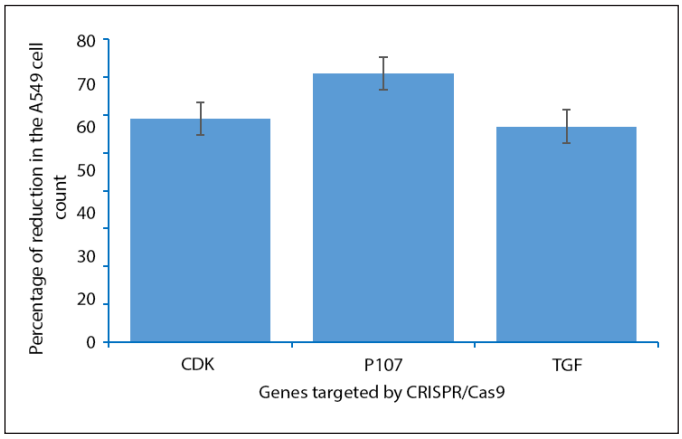

Figure 5: The percentages of the reduction in the A549 lung cancer cells count caused by editing *CDK4*, *p107*, and *TGFβ1* using CRISPR/Cas9.

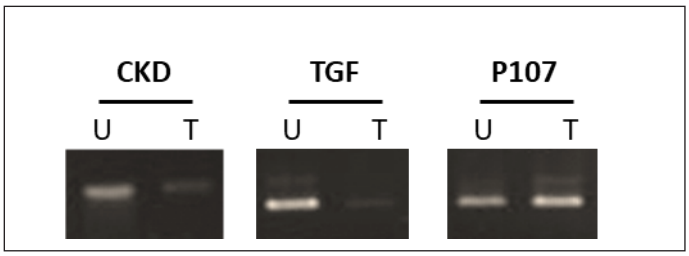

Figure 6: Detection of *CDK4*, *p107*, and *TGFβ1* in treated and untreated MCF-7 breast cancer cells by semi-quantitative PCR. T: treated with CRISPR/Cas9 and U: untreated.

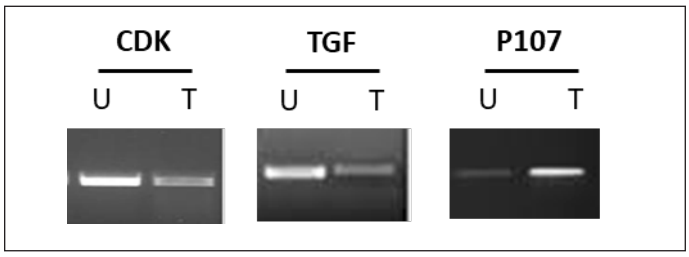

Figure 7: Detection of *CDK4*, *p107*, and *TGFβ1* in treated and untreated A549 lung cancer cells by qualitative PCR. T: treated with CRISPR/Cas9 and U: untreated.

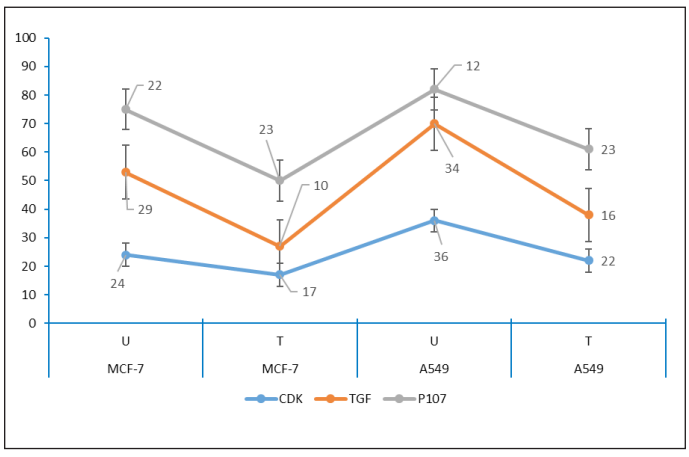

Figure 8: The amount of DNA in control and each treatment MCF-7 and A549 cells.

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2-D PAGE

The two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) technique is a powerful proteomics technique aiming at separation of the complex protein mixtures; it can be used to discover proteins that are differentially expressed, and thus obtaining a set of potential biomarkers. The map was analyzed by the image created by Master 2D Elite Software. Two-dimensional protein electrophoresis experiments revealed some differences on the level of expressed proteins (Figure 9). There were some missing protein spots and, in the meantime, there were some *de novo* expressed protein spots.

*TGF*β1 was also knocked out from MCF-7 and A549 cells *via* CRISPR/Cas9. Treated cells exhibited small amounts of the resemble protein. *TGF*β1 is known for its multifaceted roles in breast cancer progression.

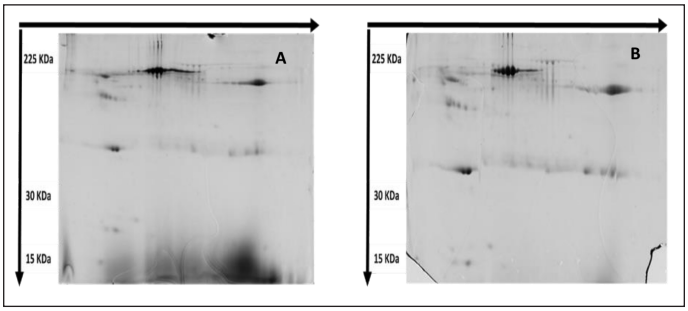

Figure 9: Protein expression map of (A) Lung cancer cell line (A549) and (B) Breast cancer cell line (MCF-7). One hundred-microgram proteins were separated by 2-DE using 13 cm pH 3-10 IPG strips and 12.5% homogeneous SDS-PAGE.

DISCUSSION

Knockout/activation of the target genes

P107 interacts with Retinoblastoma protein (RB) whose functions include DNA repair, telomere maintenance, chromosome condensation and cohesion, and silencing of repetitive regions [21], and thus, by editing (activating) *p107*, E2F-dependent transcription is suppressed, which might lead to cell death [21, 22]. This might explain the high percentage of reduction in the MCF-7 cell count reported in this study. Meanwhile, CDK family (such as CDK4) is found to be crucial in initiation and progression of breast cancer [23, 24] and in inducing apoptotic cell death [25]. Meanwhile, *TGF*β1 knockout in the MCF-7 breast cancer cells is also resulted in nearly 60% reduction in the cell count. Although, *TGF*β1 exerts tumor-suppressive effects that cancer cells must elude for malignant evolution, it also modulates processes such as cell invasion, immune regulation, and microenvironment modification that cancer cells may exploit to their advantage [26]. *TGF*β1 switches its role to a tumor-promoting pathway by inducing endothelial to mesenchymal transition [27, 28]. However, the mechanism underlying such spatiotemporal regulation of *TGF*β1 signaling needs in-depth investigation [29].

Activation of *p107* might suppress tumorigenesis [30], and this gene plays a fundamental role in growth control [31]. Several researches indicated that *p107* cooperates with the RB to suppress lung cancer [32, 33].

Knocking out of *CDK*4 resulted in 41.6% reduction in the cells count as preclinical studies have shown that *CDK*4 inhibition can produce rapid tumor regression and decrease in tumor burden in multiple human tumor xenograft models at high doses [34, 35]. It is also well known that overexpression of *CDK*4 is a potential unfavorable prognostic factor and mediates cell cycle progression by regulating the expression of *p21* in lung cancer [32, 35]. Knocking out *TGF*β1 also was critical for inhibiting the lung cancer cells growth, as it is a ubiquitous and essential regulator of cellular and physiologic processes including proliferation and differentiation [36]. It has a bi-functional tumor suppressor/oncogene role, and this might explain the 45.1% reduction in the lung cells count obtained in the present study. It has a bi-functional tumor suppressor/oncogene role [37], and this might explain the 45.1% reduction in the lung cells count obtained in the present study.

Amplification of the edited genes

Knocking out both *CDK*4 affected the overall cell viability as it suppresses the progression to S phase when downregulated [38]. It is amplified in approximately 20% of breast cancer cases, and the protein is over-expressed in 50% of cases [39]. *TGF*β1 knocking out decreased the cells count in both cell lines as aberrant *TGF*β1 signaling can lead to loss of growth inhibition [40]. Therapeutic targeting of the pro-oncogenic *TGF*β1 responses is currently being explored as a potential therapy against certain invasive and metastatic cancer types [35]. Meanwhile, the activation of *p107* caused the cells to commit suicide as it is involved in the negative regulation of cell cycle by interacting with E2F family members although in different combinations with respect to *RB1*[31].

*TGF*β1 is overexpressed in various tumor tissues, promotes migration and invasion of cancer cells and known to induce EMT in a number of cancer cell types and promote lung adenocarcinoma migration and invasion [41]. Several studies have been conducted to study the effect of down regulation of *TGF*β1 on lung cancer progression. Most of them used chemical substances such as sanguiin [42] and resveratrol

[43]. Inhibition of *CDK*4 activity has turned out to be the most productive strategy for the discovery and design of novel anticancer agents specifically targeting the cell cycle.

Since *CDK*4 inhibitors were demonstrated to target proliferating cells, the induction of cell death, the partial knockout of it have been proven as an effective approach in controlling the disease [38]. Meanwhile, a statistically significant inverse relationship between the histological grading (degree of malignant potential) and the expression of *p107* in squamous cell carcinomas has been indicated, meaning that an increase in grading resulted in a significant decrease in protein expression, and this protein might compensate for chronic *RB* loss [44].

Specific protein detection

For *p107* gene, CRISPR/Cas9-based activation has been performed, as this protein is important mediators of various cell processes including cell cycle progression, apoptosis and differentiation [32]. The downregulation of this gene has been widely correlated with different types of human cancers [31], and in regulating cell cycle. Meanwhile, *CDK*4, as an important regulator of the cell cycle, was knocked out from the malignant cells under study. Several researches indicated that misregulation of *CDK*4 activity can lead to cancer, as it regulates the cell cycle progression by regulating the G1-S checkpoint [34]. For that, eliminating the expression of this protein induce malignant cells to commit apoptosis by dysregulating key cellular pathways [35].

*TGF*β1 is known for its multifaceted roles in breast cancer progression [45]. Treated cells exhibited small amounts of the resemble protein, although, the effect of *TGF*β1 on cancer progression is variable: at early stages, *TGF*β1 inhibits cancer progression, while at later stages it stimulates migration, invasion and metastasis [46]. However, the molecular basis of this "*TGF*β1 paradox" is not entirely understood [47].

CONCLUSION

In the present study, the genomes of two cancer cell lines (MCF-7 and A549) were edited using CRISPR/Cas9 approach. *TGF*β1 and *CDK4* genes were knocked out while; *p107* was activated to study their role in the process of cancer progression. Data obtained indicated a significant reduction in the cell count of both cell lines after being challenged with CRISPR cassettes. Meanwhile, the semi-quantitative PCR data revealed a partial downregulation of both *CDK4* and *TGF*β1, and upregulation of *p107*. Data obtained from 2D-PAGE indicated also the presence of *de novo* protein for *p107* treatment while *TGF*β1and *CKD4* proteins were absent. Generally, these data might indicate the efficacy of CRISPR/Cas9 in editing the genome of the specified cells.

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Conflict of Interest

The authors declare that they have no competing interests.

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Authors' contributions

HS*** designed the experiments and wrote the manuscript. DA**, NJ*, ST*, MF*, BM*, and AP* performed the experiments. SEA*, OAMS*, and AS* analyzed the data, EC** draw the illustrations, HT* revised the manuscript, AFA* revised charts, and ME*** critically revised the final manuscript. All authors read and approved the final manuscript.

*Participated equally

**Participated equally

***Participated equally

Ethics approval and consent to participate

Not applicable.

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